Evaluating the role and therapeutic potential of Neuregulin-1 for remyelination in chronic multiple sclerosis

By

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Abstract:

In in chronic lesions of progressive multiple sclerosis (MS), failure in myelin regeneration and prolonged demyelination lead to permanent neurodegeneration and increased disability. Currently, minimal regenerative treatments are available to promote myelin repair (remyelination) for progressive MS. In acute demyelinating lesions, microglia have remarkable capacity to facilitate remyelination by clearance of cholesterol-rich myelin debris and supporting new oligodendrocytes to remyelinate white matter lesions. However, in chronic demyelinated lesions, microglial cholesterol metabolism and recycling process is dysregulated, resulting in a dysfunctional myelin-derived lipid-accumulating phenotype that eventually leads to microglial cell death. Currently, the underlying molecular mechanisms that drive microglia dysfunction in progressive MS are not fully understood. We previously reported downregulation of neuregulin-1 (Nrg-1), a key myelination factor, in human secondary progressive MS lesions and lesions of the MS mouse model, experimental auto-immune encephalomyelitis (EAE). Restoring Nrg-1 levels in EAE mice showed positive immunomodulatory effects and promoted a pro-regenerative phenotype in microglia in acute demyelinating lesions.

In this study, we demonstrate that persistent dysregulation of Nrg-1 levels contribute to impaired remyelination in chronically demyelinated brain lesions using a cuprizone mouse model of chronic demyelination that models progressive MS and in Nrg-1 conditional knockout mice. Using parallel *in vitro* and *in vivo* experiments, we show that therapeutic restoration of Nrg-1 promotes remyelination in chronic demyelinated lesions by enhancing oligodendrogenesis and oligodendrocyte maturation in a microglia-dependent manner. Mechanistically, Nrg-1 treatment revitalizes the innate capacity of microglia for phagocytosis of myelin debris, cholesterol recycling and efflux through activation of LXR and PPARγ signaling and upregulation of cholesterol

transporters. These changes in microglial function improve their survival and provide paracrine support for oligodendrocyte remyelination in chronic demyelinated lesions. Altogether, this study, for the first time, links dysregulation of Nrg-1 to remyelination failure in chronic demyelinated lesions, and demonstrates the therapeutic potential of Nrg-1 to augment the ability of microglia for remyelination in progressive MS.

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Dedication:

To my beloved wife, Rezvan

For her endless love, support, and patience

To my parents

Whose sacrifices and encouragement have been my guiding light

And to Mahsa, Nika, Khodanoor, Hamidreza, and all the brave young heroes who lost their lives for the want of freedom and the simple right to a normal life

Your resilience, courage, and hope inspire me every day

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Contributions:

Chapter 1 (Introduction) was independently prepared for this dissertation, with excerpts from a previously published review article (Ziaee and Karimi-Abdolrezaee, Neural Regeneration Research, 2023). Chapter 2 is a manuscript that is prepared and ready for submission to a peer-reviewed journal for publication. Chapter 3 is the discussion of the findings. The manuscripts and the contribution of authors are listed below:

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My contributions: I wrote the first draft of the manuscript that was edited by my supervisor, Dr. Soheila Karimi-Abdolrezaee.

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My contributions: I performed most experiments outlined in this manuscript. I performed all *in vitro* experiments. For *in vivo* experiments, I contributed to induction and care of cuprizone mouse model in collaboration with H.K and S.N. I managed animal treatments, inventories, animal euthanization, tissue processing, and cryo-sectioning (along with H.K.). I performed electron microscopy analyses of myelin in cuprizone mice, and majority of immunohistochemical analyses of chronic cuprizone mouse model and human MS tissue sections, interpretation of the RNA-seq

data, and data related to the Nrg-1 cKO experiments. I also analyzed the results, prepared the figures, and wrote the first draft of the manuscript. All the steps were done under the supervision and with collaboration with my supervisor Dr. Soheila Karimi-Abdolrezaee.

H.K. conceived the study and designed the project under the supervision of Dr. Soheila Karimi-Abdolrezaee. H.K performed immunohistochemical analysis of myelin in Figures 1 and 2 for chronic CPZ mice, and all oligodendrocyte analysis in Figure 3 (representative images were done by myself)..

S.N. contributed to inducing cuprizone animal model and animal care, performed all the immunohistochemical experiments from short-term cuprizone mice in Figure 1, and participated in tissue dissection and dissociation for MACS (magnetic cell sorting).

S.M.H. performed MACS and RNA extraction for RNA sequencing, and initial analysis of raw data and acquiring differentially expressed genes and modified pathways and components through Galaxy software displayed in Figure 5.

E.J. performed immunohistochemical analysis of cholesterol transporter *in vivo* displayed in Figure 6L-M.

P.G. and A.T. designed and prepared nanofibers used for myelination experiments displayed in Figure 8D.

G.M. performed electron microscopy analysis of myelin in EAE displayed in Figure 2 L-O.

V.W.Y. contributed to MS brain samples and manuscript editing.

S.K-A supervised the study, analyzed and interpreted the results, provided operational support, wrote, edited and finalized the manuscript.

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List of abbreviations:

ABCA-1	ATP-binding cassette transporter A1
ABCG-1	ATP-binding cassette transporter G1
aCSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
ApoE	apolipoprotein E
BAM	border associated macrophages
BBB	blood-brain barrier
BGII	Black Gold II
BSA	bovine serum albumin
CC	Corpus callosum
CFA	complete freund's adjuvant
CIS	clinically isolated syndrome
CM	conditioned media
CNS	central nervous system
CPZ	cuprizone
CR3	complement receptor 3
CSF1	colony-stimulating factor 1
CSF1R	colony-stimulating factor receptor 1
CSPG	chondroitin sulfate proteoglycans
DAM	disease-associated microglia
DAMP	damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
DMT	disease modifying therapy
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECM	extracellular matrix
EdU	5-ethynyl 2′-deoxyuridine
EGF	epidermal growth factor
EYFP	enhanced yellow fluorescent protein
FDA	U.S. Food and Drug Administration
FDR	False Discovery Rate
FIII	Filipin III
GFP	green fluorescent protein
GGF2	glial growth factor 2
H&E	hematoxylin and eosin
HDL	high density lipoprotein

I.P.	intraperitoneal
ICC	immunocytochemistry
IFNγ	interferon gamma
IGF1	insulin growth factor 1
IHC	immunohistochemistry
IL	interleukin
LDAM	lipid-droplet accumulating microglia
LFB	luxol fast blue
LPC	lysolecithin (lysophosphatidylcholine)
LPS	lipopolysaccharide
LXR	Liver-X Receptor
MACS	Magnetic-Activated Cell Sorting
MBP	myelin basic protein
MCM	microglia conditioned media
MDM	monocyte derived macrophage
MIMS	Microglia inflamed in MS
MLKL	mixed lineage kinase domain-like protein
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MUFA	monounsaturated fatty acid
NAWM	normal appearing white matter
NPC	neural precursor cell
Nrg-1	Neuregulin 1
Nrg-1 cKO	conditional Nrg-1 knockout
Nrg-1β1	Neuregulin 1 beta 1
Nrp1	Neuropilin-1
OL	oligodendrocyte
OPC	oligodendrocyte progenitor cell
ORO	Oil Red-O
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCL	polycaprolactone
PD	Parkinson's disease
PDL	poly-D-lysine
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PLP	proteolipid protein
PNS	peripheral nervous system
PPARα	Peroxisome proliferator–activated receptor alpha

PPARγ	Peroxisome proliferator–activated receptor gamma
PPMS	primary progressive MS (PPMS)
PSN	penicillin–streptomycin–neomycin
qPCR	quantitative polymerase chain reaction
rhGGF2	recombinant human glial growth factor 2
RIPK1	receptor-interacting protein kinase 1
RIPK3	receptor-interacting protein kinase 3
RIS	radiologically isolated syndrome
RNAseq	RNA sequencing
ROS	reactive oxygen species
RRMS	relapsing-remitting MS
RT	room temperature
S.C.	subcutaneous
SCI	spinal cord injury
SEM	standard error of the mean
SPMS	secondary progressive MS
SR-AI/II	scavenger receptor A I/II
STING	stimulator of interferon genes
TEM	Transmission electron microscopy
TFE	trifluoroethanol
TG2	transglutaminase 2
TGF-β	transforming growth factor β
Th1	helper T cell type 1
Th17	helper T cell type 17
TMEV	Theiler's murine encephalomyelitis virus
TNF-α	tumor necrosis factor alpha
TREM2	triggering receptor expressed by myeloid cells 2
WT	wild type
y/o	years old

Chapter I: Introduction

1.1 Overview of Multiple Sclerosis (MS)

1.1.1 Epidemiology and impact of MS

Multiple sclerosis (MS) is an immune-mediated condition characterized by focal inflammatory demyelination of the central nervous system (CNS), eventually leading to a neurodegenerative condition and a range of neurological disabilities [1]. MS affects 2.8 million individuals globally, with a higher incidence rate observed in Europe and North America [1,2]. Genetic predisposition plays an important role in MS susceptibility as highlighted by twin studies, and genome wide association studies have revealed more than 200 genetic variants associated with MS [3,4]. Additionally, women develop MS at a ratio of 3:1 compared to men [2]. The female to male ratio also changes with age since in children with MS the ratio is nearly equal, while it rises significantly in adolescent-onset MS and remains elevated in adults with MS. However, in case of late-onset MS, the female predominance rate decreases [5]. This elevated prevalence in females from puberty through middle age suggests crucial regulatory roles for gonadal hormones in the sex bias in MS incidence. Supporting this, active inflammatory lesions are shown to be most prevalent in people with MS with low testosterone levels or high estrogen to progesterone ratio [6,7]. Additionally, the sex differences in CNS autoimmunity may stem from differential expression of sex chromosomes genes, potentially influenced by parent-of-origin effects, such as variations in DNA methylation of the X chromosome in autoreactive T lymphocytes [8].

Environmental factors also have a major effect on MS incidence and prevalence rates. For instance, the prevalence of MS increases with latitude, hypothesized to be linked to reduced sunlight exposure and associated vitamin D deficiencies [1,9]. Moreover, viral infections, particularly with Epstein-Barr virus (EBV), have been linked more recently with triggering MS pathogenesis [10,11].

The impact of other environmental risk factors including smoking, obesity, and nutrition have also been under investigation in recent years [1].

In recent years, the incidence rate of MS has been significantly rising, although this trend has also been partially attributed to improved diagnostic tools available and elevated awareness of the disease [1]. Typically, MS is considered a condition of younger populations, with a mean age of onset around 32 years, although recent studies reveal a shift in the age of onset to older ages [1,12]. This further complicates the social and economic burden MS imposes, since due to the progressive nature of the disease, it often leads to long-term disability and reduces the quality of life and workforce participation. The costs for managing MS, alongside the need for ongoing support services, place substantial financial strain on both affected individuals and healthcare systems globally [13].

1.1.2 Courses of the disease in MS

MS presents with diverse courses of the disease. The most common course that accounts for approximately 85% of newly diagnosed cases is known as relapsing-remitting MS (RRMS) [14,15]. The typical RRMS characteristics consist of recurrent attacks (relapses) where neurological symptoms develop rapidly, followed by a complete or partial recovery (remission). The majority of individuals with RRMS will eventually progress to a secondary progressive MS (SPMS) form of the disease [14,15]. In SPMS, disabilities progressively accumulate over time and with no clear remissions. This represents a more advanced and neurodegenerative stage of the disease. On the other hand, 10-15% of the cases initially present with a progressive course at the time of diagnosis.

This primary progressive MS (PPMS) course typically occurs at more advanced ages and is associated with a rapid decline in function and a worse outcome [14,15].

There are other phenotypes of the disease that do not completely match the criteria for MS diagnosis but could be an initial stage of the disease. These include a prodromal phase with non-specific symptoms [16], a radiologically isolated syndrome (RIS) where an inflammatory demyelinated lesion is incidentally found in magnetic resonance imaging (MRI) scans of the CNS without any specific symptoms [17], and a clinically isolated syndrome (CIS) which represents a clinical inflammatory demyelination attack characteristic of MS for the first time and thereby does not fulfill the MS criteria of dissemination in time [18]. Understanding the different courses of the disease is important in MS due to the differences in pathophysiological properties and the different therapeutic approaches required for each stage of the disease.

1.1.3 Pathophysiology of MS: Inflammatory and neurodegenerative components

MS pathophysiology involves a typical inflammatory demyelinating phase, followed by a neurodegenerative course. The initial inflammatory response is mainly mediated by an adaptive immune response. During this phase, the infiltrating auto-reactive effector CD4+ helper and CD8+ cytotoxic T cells primed against myelin antigens, as well as infiltrating B cells play a major role [19]. These cells release inflammatory cytokines and recruit the innate immune cells of the CNS. This in turn results in targeting and degradation of the myelin sheath of nerve fibers [19]. Myelin plays a critical role in facilitating rapid signal transduction along the axons through salutatory conduction, as well as maintaining axonal integrity [20–22]. Therefore, if the immune mediated removal of myelin sheath is prolonged and not reversed by repair mechanisms, axonal injury

ensues which eventually leads to neuronal damage and a resulting neurodegenerative condition [21]. The inflammatory phase gives rise to the characteristic MS lesions that can be detected as demyelinating plaques using diagnostic tools such as MRI [23].

Initially during the course of MS, the focal inflammatory demyelination is followed by remyelination through repair mechanisms [21]. Therefore, the early stages of the disease are characterized by inflammatory attacks followed by complete or partial recovery. However, with disease progression and as a result of a gradual failure in these repair processes, the neurodegenerative component of the disease becomes more prominent as demyelination causes an irreversible damage to axons and neurons [21,24]. This leads to a permanent damage to the neural tissue, resulting in an accumulation of disabilities over time. Thus, there is an unmet need to unravel the cellular and molecular mechanisms that regulate demyelination, repair and neurodegeneration in MS to allow developing effective regenerative therapies for progressive MS.

1.1.4 Knowledge gap in developing therapeutics for progressive MS

Despite all the recent advances in understanding the pathophysiology of MS, there is still a considerable knowledge gap in our understanding of the pathologic mechanisms that lead to the progressive neurodegenerative nature of the disease. As a result, although the disease modifying therapies (DMTs) currently available are shown to be very effective in reducing the frequency and severity of relapses in RRMS, they have limited effectiveness in slowing the disease progression in SPMS and PPMS [24]. This is mainly due to the fact that the majority of these DMTs focus on mitigating the inflammatory response, while in progressive forms of the disease inflammation is less pronounced [24]. In recent years, the monoclonal anti-CD20 antibody ocrelizumab has shown

some promise in reducing disability accumulation in PPMS [25]. By depleting B cells, ocrelizumab exerts potent anti-inflammatory effects, resulting in near-complete suppression of relapses [26,27]. However, ocrelizumab was not shown to directly promote remyelination, alleviate oxidative stress, or modulate mitochondrial function, all of which are crucial for an effective therapeutic strategy for progressive MS [24]. Another recent advancement is the FDA approval of the sphingosine 1-phosphate receptor (S1PR) modulator, siponimod, for active SPMS [24]. Preclinical studies demonstrate that siponimod reduces inflammation, demyelination, and oxidative stress, while promoting remyelination [24]. Furthermore, phase III clinical trials show that siponimod reduces disability progression and suppresses relapses [28,29]. Despite these advancements, there is still an unmet need for more regenerative treatment strategies with the aim of achieving a more profound and effective reduction of progressive activity in MS.

A major challenge in developing effective regenerative and neuroprotective interventions for the progressive forms of MS is the complex nature of the pathologic processes that lead to failure of repair mechanisms and neurodegeneration. While recent research has identified potential processes involved in neurodegeneration, such as oxidative stress, mitochondrial dysfunction and iron toxicity, there are still major gaps in understanding the underlying mechanisms that specifically impair remyelination in chronic MS[21,24,30,31]. Various experimental models of MS have been developed to fill these knowledge gaps that will be discussed in the following sections.

1.2 Animal Models of MS Used in Research

1.2.1 The experimental autoimmune encephalomyelitis (EAE) model

The experimental autoimmune encephalomyelitis (EAE) model is a longstanding and widely used animal model of MS that more closely mimics the disease immune mediated pathology. EAE is

most commonly induced in C57BL/6 mice by immunization with myelin specific antigens such as myelin oligodendrocyte glycoprotein (MOG), in combination with an adjuvant (Freund's adjuvant in combination with Mycobacterium Tuberculosis extract) to enhance the immune response and pertussis toxin to allow permeation of the blood-CNS-barrier [32]. This immunization triggers an autoimmune attack against the myelin within the CNS and is followed by multifocal inflammation and demyelination that leads to subsequent impairment in neurological functions [32]. C57BL/6 mouse has been commonly used since it provides a reproducible and consistent model, but other strains of mice, larger rodents and non-human primates have been also used. Other myelin peptides such as myelin basic protein (MBP) have also been used for induction of EAE [32–34]. Alternatively, EAE can be induced via adoptive transfer of activated, myelin-specific auto-reactive T-cells, which directly target myelin antigens in the recipient animal [35,36]. EAE mirrors many aspects of MS pathophysiology, including a T-cell mediated immune response and formation of multifocal inflammatory demyelinating lesions [32]. Therefore, it has been instrumental in unraveling the role of adaptive immune response, particularly via helper CD4+ T-cells, in MS pathogenesis and has provided a platform for testing novel therapeutic strategies [32].

1.2.2 Toxin mediated demyelination models of MS

An important category of MS models is toxin induced demyelination. The most widely used models used in this category are cuprizone (CPZ) and lysolecithin (lysophosphatidylcholine - LPC) induced demyelination [32]. In LPC model, an injection of this toxin into white matter spinal cord or brain corpus callosum leads to focal demyelination in few days followed by an almost complete remyelination within 2-4 weeks [37–40]. This model provides a good platform to study the isolated phases of acute demyelination or remyelination, and has potential for evaluation of

remyelinating therapies, although the therapeutic time window is short [37–40]. LPC model would not allow modeling chronic demyelination.

The CPZ model of demyelination is another toxin-mediated demyelination model has been widely used for decades to study demyelination and remyelination processes. CPZ (bis-cyclo-hexanone oxaldihydrazone) is a copper chelating agent that is added to the animal diet to induce cell death in oligodendrocytes and subsequently, results in demyelination of the CNS [41,42]. The reason for this specific effect of CPZ on oligodendrocytes is yet to be uncovered; however, it is known that by chelating copper, CPZ inhibits mitochondrial enzymes such as cytochrome oxidase and monoamine oxidase, which leads to apoptosis due to oxidative stress [41,42]. Notably, oligodendrocytes have substantial energy requirements to accommodate the high level of protein and lipid synthesis needed for myelin production and maintenance [43]. These cells also simultaneously possess high intracellular iron loads and low glutathione levels, which is crucial for reducing reactive oxygen species (ROS) [44]. This composition renders oligodendrocytes particularly sensitive to fluctuations in metabolic activity and ROS levels compared to other CNS cells [45]. Consequently, disruptions in oligodendrocyte mitochondria can lead to energy deficits, ROS buildup, and interference in lipid and protein synthesis, a mechanism believed to contribute to cuprizone-induced oligodendrocyte-specific toxicity [45]. The advantage of the CPZ model is that the demyelinated lesions begin to remyelinate after withdrawal of CPZ from the diet, allowing for the study of the cellular and molecular processes during the remyelination phase and the evaluation of potential treatments [46]. A major advantage of the cuprizone model is its suitability to study both acute and chronic demyelination by the duration of CPZ in the animal's diet. Mice receive CPZ for 3-5 weeks for acute demyelination and 10 weeks or more for chronic demyelination. Considerable remyelination follows acute demyelination by CPZ withdrawal, while chronic

demyelination results in limited, if any, remyelination [47]. This chronic phase mimics the progressive neurodegenerative features of MS, providing a model to study sustained demyelination, axonal damage, and remyelination failure [21,46].

1.2.3 Other viral and genetic animal models of MS

Since viruses have been associated with MS, viral animal models of MS have been used to study this aspect of MS. Among the viral models, the most studied is the Theiler's murine encephalomyelitis virus (TMEV) model, which involves infection of mice with the picornavirus TMEV [48,49]. This leads to an acute encephalomyelitis-like disease followed by a chronic demyelination in genetically susceptible strains [48,49]. Therefore, this model is useful for studying the viral triggers and chronic progression of MS; however, the pathogenetic mechanisms in this model may differ from that of MS since it is based on an inflammatory response to a viral infection [32].

Genetic models of demyelination are also useful in the study of MS pathogenesis. One such models is shiverer mutant mouse that lacks myelin basic protein (MBP). As a result these mice develop severe hypomyelination [50]. Despite being valuable for studying the fundamental biology of myelination, this model has limited use in MS studies due to a lack of immune response [50]. Additionally, humanized mouse models in which human genes are introduced into specific immune cells of transgenic mice, offer a platform to explore human-specific immune responses in the context of MS [51]. Each of these models offers unique insights into different aspects of MS pathology, from viral triggers and immune responses to demyelination and remyelination, thus contributing to a more comprehensive understanding of the disease.

1.2.4 Limitations and strengths of MS models for studying remyelination

Despite their valuable contributions to MS research, each animal model has specific limitations that must be considered when designing a study. The EAE model captures the adaptive immune-mediated nature of MS in C57BL/6 mice that provides a reproducible model of inflammatory multifocal demyelination that can be combined with transgenic modifications [32]. However, EAE model shows several limitations for studying remyelination including its monophasic nature, without relapses or chronic progression; distribution of demyelinated lesions mainly in the spinal cord as opposed to MS where brain lesions are more common; stochastic distribution of lesions in terms of both timing and location that complicate the study of remyelination [32].

The cuprizone (CPZ) model is currently the most effective model for inducing both acute and chronic demyelination, thus providing suitable platform for studying remyelination mimicking both episodic and progressive forms of MS [32,46,47]. However, while CPZ model has a well-developed CNS innate immune response, it lacks the adaptive immune response component of MS. Therefore, it is not suitable for investigating the acute inflammatory response or auto-immune aspects of the disease [32].

The Theiler's murine encephalomyelitis virus (TMEV) model, while useful for studying acute and chronic demyelination, involves viral infection and as a result, does not directly represent the pathogenic processes MS. Moreover, its relevance to human MS is limited to certain genetically susceptible mouse strains such as SJL, restricting its broader applicability [32,48,49]. The shiverer mouse model lacking MBP provides insights into myelination process but does not incorporate the pathophysiologic components of MS [50].

In conclusion, selecting an appropriate model is highly dependent on the specific aims of the research project. While we have used EAE model in other projects, our goal in this project is to investigate the mechanisms involved in remyelination of the chronically demyelinated lesions of progressive MS, with a focus on the role of the CNS innate immunity in this process. Thereby, we employed chronic CPZ mouse model.

1.3 Demyelination and Remyelination in the CNS

1.3.1 Myelination and myelin maintenance in developing and adult CNS

Myelination is a crucial process in CNS development and homeostasis. Oligodendrocytes are the cells responsible for myelinating axons. Unlike the peripheral nervous system (PNS) where myelinating Schwan cells are only able to myelinate a single axon, oligodendrocytes in the CNS can simultaneously wrap several axons in their vicinity [52]. Oligodendrocytes produce myelin sheath in segments that cover a length of axon between two nodes of Ranvier. Myelin is extensions of oligodendrocyte plasma membrane [53]; however, its composition is specialized and distinct from normal plasma membrane. Myelin is rich in cholesterol and contains specific peptides and proteins, such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and proteolipid protein (PLP) [54]. Functionally, myelin acts as electrical insulation for axons and as result of its specific segmented structure, facilitates rapid transduction of action potential along an axon through saltatory conduction since voltage gated sodium channels are accumulated only at the nodes of Ranvier [53]. The speed of signal transduction is affected by axonal properties such as diameter, and myelin characteristics, such as internode length and relative thickness or g-ratio (the ratio of inner to outer axonal diameter) [53]. Myelin also plays a critical role in providing metabolic support to axons and thereby, maintaining axonal integrity [55,56].

CNS myelination in rodents begins with differentiation of oligodendrocytes from oligodendrocyte progenitor cells (OPCs), which themselves develop from neural stem and progenitor cells during late embryonic development [57]. Myelination continues during early neonatal phase and lasts for several weeks postnatally [57,58]. This process is tightly regulated by interactions between oligodendrocytes, neurons, and other glial cells [53]. Proper myelination is crucial for the establishment of neural circuits and affects functions such as motor coordination, cognition, and sensory processing. Any disruption in the early stages of myelination potentially leads to developmental disorders and long-term neurological deficits [53,59].

While most myelination occurs early in life, myelin maintenance via turnover and remodeling continues throughout adulthood. OPCs persist in the adult CNS and continue to differentiate into oligodendrocytes to sustain myelination [53,60]. OPCs maintain their population in the adult CNS through self-proliferation or differentiation from neural precursor cells (NPCs) [61,62]. Oligodendrocytes in the adult CNS are responsible for maintaining the integrity of the myelin sheaths and participating in remyelination following injury or disease [53]. However, myelin maintenance becomes less efficient with aging and in neurodegenerative conditions causing demyelination of the CNS [53].

1.3.2 Mechanisms of demyelination in MS

Demyelination in MS is known to be the result of an aberrant autoimmune response to specific myelin proteins within the CNS. The etiology of this response is still under investigation, but the mechanisms that lead to the removal of myelin involve both adaptive and innate immunity [63]. Initially in this process, T lymphocytes, specifically CD4+ helper T cells, Th1 and Th17, invade

the CNS, cross the blood-brain barrier (BBB) and become reactivated through interaction with antigen presenting cells of the CNS (most important among them dendritic cells at the BBB and microglia in the parenchyma) [63]. These reactivated helper T cells then start releasing pro-inflammatory and myelin toxic cytokines including interferon gamma (IFNγ) and tumor necrosis factor alpha (TNF-α) [63]. CD8+ cytotoxic T cells have been also implicated in demyelinating process as they are found in the vicinity of myelin in the lesion with autoreactive properties [64]. This demyelination initiating role of T cells have also been confirmed in EAE animal model of MS, where adoptive transfer of autoreactive T cells from an EAE animal and to a healthy animal in a process is enough to cause demyelinating EAE in the new animal [65,66].

B lymphocytes are also critically involved in MS pathogenesis as therapeutic interventions using anti B cell monoclonal antibodies show significant reduction of the disease activity and lesion formation [67,68]. The effectiveness of anti B cell therapies are shown to be independent of the antibody levels within the body, and therefore is attributed to antigen presenting and regulatory function of B cells [69].

The role of innate immunity in demyelination is also of critical importance. Microglia are the resident immune cells of the CNS that serve as the primary mediators of innate immunity within brain and spinal cord parenchyma, responding rapidly to infection, injury, and other pathological stimuli. Microglia along with astrocytes, infiltrating monocyte derived macrophages, and CNS border associated macrophages (BAMs) orchestrate a series of detrimental mechanisms that lead to inflammatory myelin damage [70]. Microglia contribute to demyelination through either antigen presentation to cells of the adaptive immunity, or production of pro-inflammatory cytokines that reactivate astrocytes to become neurotoxic, stimulate endothelial cells to facilitate infiltration of other inflammatory cells, and even modulate oligodendrocyte maturation and neuronal activity [70].

Microglia can also participate in demyelination through other mechanisms such as increasing the expression and as a result, activity of the complement system, and inducing oxidative stress through production of free radicals such as reactive oxygen species (ROS) or nitrogen species [71–73]. Despite all these destructive functions, microglia are highly involved in the remyelination process that follows demyelination [70].

1.3.3 Remyelination in MS

Remyelination is a critical repair process for recovery from demyelination in MS. Successful remyelination restores the saltatory conduction and the disrupted axonal functions due to demyelination [21]. Remyelination is enabled by activation of OPCs and their differentiation to myelinating oligodendrocytes. OPCs are activated by reactive astrocytes and microglia in response to their inflammatory mediators within the demyelinated lesion and start proliferation, while more OPCs are recruited to the site of injury from other regions of the CNS [21]. Meanwhile, the local and recruited microglia and infiltrating macrophages start clearing the myelin debris that has been shown to be inhibitory to oligodendrocyte maturation and remyelination [74,75]. The abundant population of OPCs as a result of migration and proliferation then begin differentiating into mature myelinating oligodendrocytes that engage in myelin ensheathment of demyelinated axons [21]. Although the efficiency of this repair process is high initially during the disease process and leads to a complete or near complete recovery from the symptoms, remyelination potential is known to decline in people with MS with age or progression of the disease [76,77]. The ensuing failure in remyelination can lead to accumulation of disabilities characteristic of progressive course of MS [21].

1.3.4 Mechanisms of remyelination failure in progressive MS:

The mechanisms behind remyelination failure with age and MS chronicity are not completely understood. There are also reports of heterogeneity of remyelination potential of lesions in MS, even in the same individual [78,79]. Evidence links impaired remyelination to insufficient repopulation of the lesion with OPCs, either due to extensive depletion of these cells from the lesion, or following reduced proliferation or migration to the lesion [80–82]. However, these reports are not consistent and seem to represent a lower proportion of the lesions [83]. The most consistent evidence so far, show that this remyelination failure occurs despite an abundant presence of OPCs within the lesion, suggesting a differentiation block or arrest to be the main cause. This has been shown in multiple studies using different markers for OPCs and mature oligodendrocytes [84–88]. Several mechanisms have been suggested for this maturation arrest, such as persistent activation of Notch-jagged signaling [89,90], or accumulation of glycosaminoglycan hyaluronan in chronic MS lesions that are known to inhibit OPC differentiation [91].

Like regeneration potential in other systems of the body, remyelination also declines with advancement of age. Aging alters a variety of cells with significant implications in disease progression [21]. OPC recruitment, proliferation and differentiation is reduced by aging [92]. The potential of microglia and other phagocytes in clearance of myelin debris from the lesion microenvironment is also diminished with aging and leads to accumulation of dysfunctional foamy phagocytes with crystalized cholesterol due to impaired processing of the cholesterol content of the engulfed myelin [74,93–95]. This is important since metabolism and availability of cholesterol, as a building block of myelin, is essential and rate limiting for myelination [96].

1.3.5 Cholesterol metabolism in the CNS: implications for remyelination success

A vast majority of the cholesterol content of the CNS is within the structure of the myelin sheaths [97]. This cholesterol content is all synthesized within the CNS and by almost all cell types of the parenchyma since the BBB is impenetrable to cholesterol from the periphery [97,98]. As a result, the CNS has the highest cholesterol synthesis rates among all systems and organs of the body. This is important since cholesterol availability is a rate limiting factor for myelination [96]. During the developmental myelination in late embryonic and early postnatal life, oligodendrocytes are the main cells responsible for cholesterol biosynthesis, along with some horizontal transfer from astrocytes [96,99]. However, after CNS myelination is completed, cholesterol biosynthesis by oligodendrocytes is reduced to a maintenance level to sustain myelin structure [100]. During homeostasis in adulthood, cholesterol synthesis is mainly performed by astrocytes, which then transfer it to neurons and oligodendrocytes via CNS-specific apolipoprotein E (ApoE) for their metabolic needs [99,100]. With aging, the cholesterol biosynthesis rates decline within the CNS, and this along with other aging related processes, elevate the risk of developing neurodegenerative diseases [101–103].

The cholesterol metabolism in demyelinating lesions such as MS, however, follows a different set of events. The degraded myelin debris that is released into the inflammatory microenvironment needs to be cleared away to allow formation of new myelin by oligodendrocytes [95]. The clearance of myelin debris is mainly conducted by microglia and macrophages, although astrocytes also play a smaller part in this process [104,105]. With phagocytosis of myelin debris, cholesterol and other lipids are accumulated within these phagocytes. Since mammalian cells are unable to degrade cholesterol, the excess cholesterol either has to be transferred away or recycled and used again [95]. Within acute demyelinating lesions, lipid and cholesterol laden phagocytes are rarely observed,

suggesting their ability to process the cholesterol content of the myelin debris and its recycling and efflux to be used by other CNS cells, particularly oligodendrocytes for myelin synthesis [95,106]. Upon myelin debris phagocytosis, microglia in acute lesions remarkably upregulate cholesterol biosynthesis machinery, which activates Liver-X Receptor (LXR) pathway that leads to recycling and efflux of cholesterol [107,108]. However, in chronic demyelinating lesions, an abundance of dysfunctional foamy microglia containing myelin debris is observed at the active rim of the lesion, suggesting their inability to process and release cholesterol [95]. With disruption in cholesterol recycling by microglia in chronic lesions, the only source of cholesterol for remyelination is the minor and ineffective upregulation of sterol biosynthesis by oligodendrocytes [108,109]. Thus, impaired ability of cholesterol recycling and release underlies remyelination failure [95]. Accordingly, these dysfunctional foamy microglia within chronic lesions present as a target to therapeutically revitalize their potential for remyelination by creating a more permissive microenvironment [95].

1.4 Role of Microglia in CNS Health and Disease

1.4.1 Microglia origins and functions in the healthy CNS

Microglia are multifaceted cells involved in a host of different functions during development, homeostasis, and pathologies throughout life (Figure I) [110]. These cells appear in the CNS at roughly the same time as neurons [111]. Microglia originate from erythromyeloid progenitors that migrate from the yolk sac to the neuroepithelium during early gestation [112]. Upon arrival, these cells proliferate and populate the CNS and their maturation is regulated through certain transcription factors such as PU.1 and the runt-related transcription factor 1 (Runx1), and external signaling cues including transforming growth factor β (TGF-β), and the interaction between

colony-stimulating factor receptor 1 (CSF1R) and its ligands CSF1 and interleukin (IL)-34 [112–117]. Mature microglia have low turn-over rates and maintain their own populations via self-renewal throughout adulthood without any contributions from the peripheral bone marrow derived progenitors [118–121]. These cells express a wide variety of receptors that allows them to interact with their microenvironment, detect surrounding cells and extracellular matrix, signaling ligands, and pathology-related cues called damaged- associated and pathogen-associated molecular patterns (DAMPs and PAMPs). This collection of receptors, also called the "sensome", allows microglia to perform their diverse functions within the CNS [122].

Microglia play important roles in shaping the developing CNS through phagocytosis of apoptotic or dead neuronal precursor cells (NPCs) and neurons, supporting neurogenesis, and removing excess or dysfunctional synapses, and regulating neural activity (Figure I) [123]. Microglia are shown to accumulate in the proximity of apoptotic neurons, allowing for developmental turnover of neurons, as well as regulating the number of NPCs within proliferative areas via phagocytosis during the CNS development [124–126]. Microglia also support neurogenesis and oligogenesis during development and early postnatal period through secretion of growth factors such as insulin growth factor 1 (IGF-1) and various cytokines [127–130]. Pro-inflammatory mediators such as IL-1β, IL-6, and TNFα are known to potentially contribute to inhibition of neurogenesis in pathological conditions[131,132]. However, the effects of these cytokines on neurogenesis seems to be concentration-dependent [133–135]. A more recent study demonstrates that microglia enhance neurogenesis and oligodendrogenesis by producing the combination of IL-1β, IL-6, TNFα, and IFNγ cytokines [129]. Additionally, microglia are involved in prenatal and postnatal wiring of the neural circuits and synaptic plasticity through phagocytosis of dendritic spines and synaptic pruning within diverse regions of the brain [136–139]. Finally, microglia regulate neuronal activity and

contribute to synaptic and functional plasticity through production and release of various signalling molecules, cytokines and extracellular matrix components, [140,141].

Microglia also regulate developmental myelination (Figure I). Through phagocytosis of viable OPCs, microglia modulate the ratio of OPC to oligodendrocytes within the developing CNS, and promote the maturation of myelinating oligodendrocytes, which subsequently leads to myelination [142]. Moreover, microglia phagocytose the newly formed aberrant myelin sheaths and in doing so, modify and refine the patterns of developmental myelination [143,144].

Many of the developmental functions of microglia will persist through adult CNS homeostasis, including roles in clearing apoptotic and surplus cells, synaptic pruning, modulating neuronal activity and supporting neurogenesis to maintain healthy neuronal network, specifically in neurogenic niches such as hippocampus [110,145]. During adulthood, immunologic functions of microglia become more prominent as the resident phagocytes and primary respondents within the CNS, where they constantly monitor the microenvironment for signs of infection or injury (Figure I) [123,145]. Upon detection of an insult or pathology, these cells transform and change their gene expression patterns, resulting in changes in their sensome and functional phenotype, a process traditionally termed microglial activation or reactivation [123].

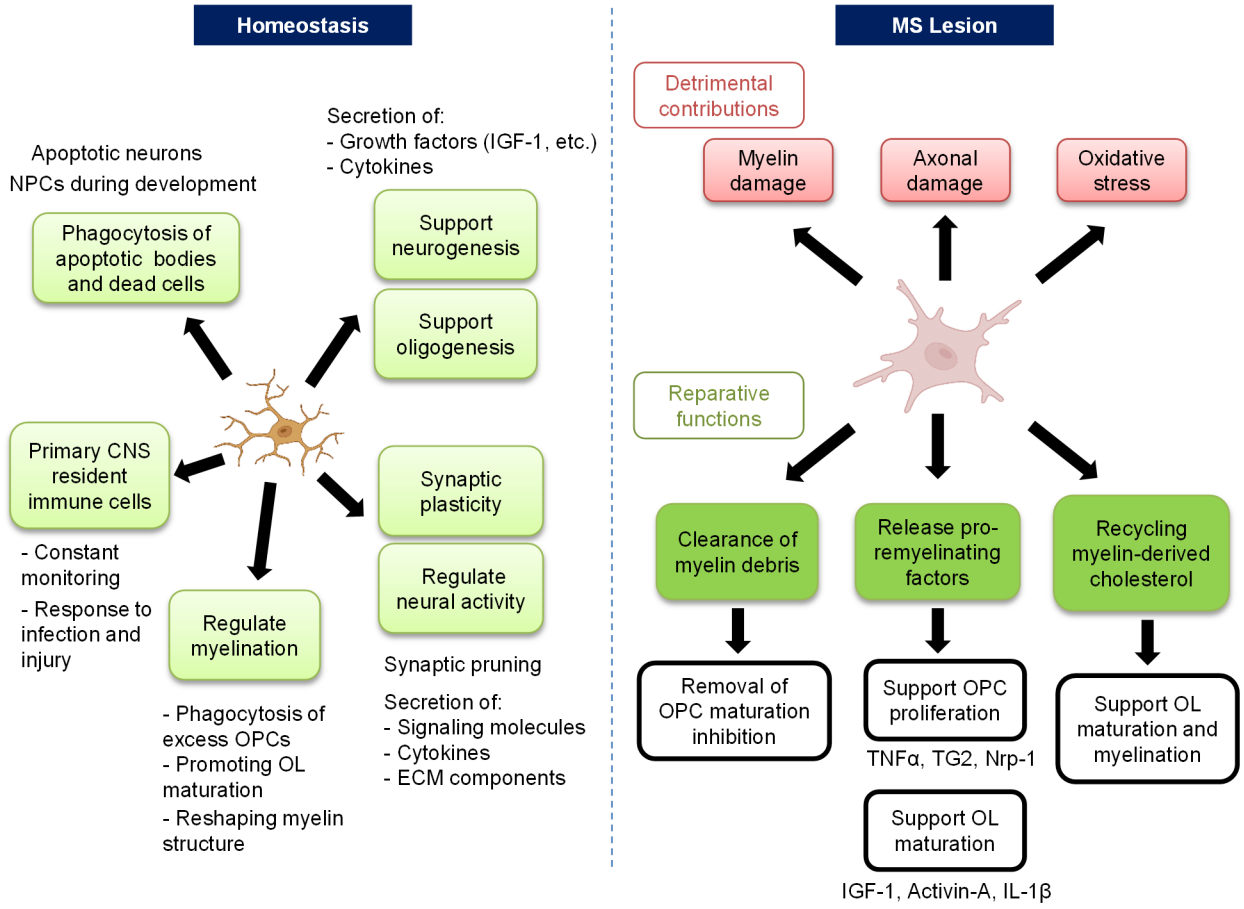


Figure I: Functions of microglia during CNS homeostasis and in MS lesion. During homeostasis microglia are involved in a plethora of functions that shape and support the CNS. These functions include phagocytosis of apoptotic bodies and dead or excessive cells, supporting neurogenesis and oligodendrogenesis through release of factors, mediating synaptic plasticity, regulating neuronal activity, regulating myelination, as well as responding to pathological conditions as primary immune cells within the CNS. Within MS lesion, microglia contribute to demyelination and axon damage and mediate oxidative stress through excessive production of reactive oxygen and nitrogen species. However, microglia also participate in the repair processes through clearing myelin debris, secreting pro-remyelination factors, and metabolising and recycling the cholesterol content of myelin debris that facilitate remyelination. CNS: central nervous system; ECM: extracellular matrix; IFNγ: interferon gamma; IGF-1: insulin-like growth

factor 1; IL-1β: interleukin 1 beta; IL-6: interleukin 6; MS: multiple sclerosis; NPC: neural precursor cell; Nrp-1: Neuropilin-1; OL: oligodendrocytes; OPC: oligodendrocyte progenitor cell; TG-2: transglutaminase 2; TNFα: tumor necrosis factor alpha. Figure generated using items from Biorender.

1.4.2 Microglial reactivity in CNS pathologies: From neurodegeneration to neuroprotection

Microglia are extremely dynamic cells known to shift their transcriptional profile, sensome composition, functional phenotypes and structural and morphological properties in reaction to injury, infection or other pathological conditions [123]. In neurodegenerative disease such as Alzheimer's disease, Parkinson's disease and MS, microglia respond to various stimulating agents in the microenvironment such as accumulation of pathological peptides, apoptotic or necrotic cells, and cytokines and chemokines released by infiltrating immune cells. This reactivity leads to a shift in microglia phenotype to a disease associated state and their increased recruitment to the site of pathology [146]. As a result, these reactive microglia engage in pro-inflammatory processes which may exacerbate the damage and progression of the pathologic condition [146]. In MS specifically, microglia engage in damaging myelin sheaths as evidenced by intracellular accumulation of myelin degradation products in these cells within MS lesions (Figure I) [147,148]. Disease associated microglia seem to mediate axonal injury as they are often found in high numbers in close proximity of damaged axons [149,150]. These cells are also shown to be heavily involved in oxidative stress within MS lesions, a process known to contribute to oligodendrocyte and axon damage [73,151]. In support of these observations, studies on EAE mice have shown that prevention of microglial production of inflammatory mediators or neutralizing microglial production of reactive oxygen and nitrogen species protects axons from damage [152–154].

Microglia have also been shown to acquire neuroprotective phenotypes in MS and contribute to repair mechanisms (Figure I) [155–157]. In these neuro-regenerative states, microglia are known to promote remyelination through clearing myelin debris and releasing neurotrophic and pro-remyelinating factors [104,157–159]. Clearance of myelin debris by phagocytes (microglia and macrophages) is an essential step in the repair process following demyelination since myelin debris is known to be inhibitory to oligodendrocyte maturation and remyelination [74,160–162]. Microglial phagocytosis of myelin debris is receptor mediated and the main receptors involved in this process include Mer tyrosine kinase (MerTK) [163], triggering receptor expressed by myeloid cells 2 (TREM2) [164,165], CD36 [166], scavenger receptor A I/II (SR-AI/II) [167,168], complement receptor 3 (CR3) [168,169], and FCγ receptor [170]. Microglia can also support remyelination through releasing a host of factors, such as cytokines, chemokines and growth factors. These factors promote OPC proliferation, as seen with secretion of TNFα [171], transglutaminase 2 (TG2) [172] and Neuropilin-1 (Nrp1) [155], and OPC differentiation to mature myelinating oligodendrocytes as observed by production and release of insulin-like growth factor 1 (IGF-1) [173], activin-A [157], and interleukin-1β (IL-1β) [174]. It is also recently proposed that by recycling the cholesterol content of myelin debris, microglia promote remyelination through facilitating OPC differentiation into mature oligodendrocytes, and providing sufficient cholesterol to these oligodendrocytes for myelin production [95,108].

This dual contribution of microglia in damage and repair associated mechanisms in MS is dependent on the transcriptional state and functional phenotype that these cells acquire upon responding to CNS insults [175]. Understanding the different states of reactive microglia has been at forefront of MS research.

1.4.3 Microglial phenotypes: a rapidly developing concept

Microglia were traditionally categorized into "resting" phenotype during homeostasis versus "activated" when challenged by a pathology [176]. Activated microglia were further divided into the pro-inflammatory neurodegenerative M1 versus pro-regenerative and neuro-protective M2 phenotypes, a categorization that was generally accepted until a few years ago [157,177]. However, due to recent advances in omics studies, specifically single-cell transcriptomics, it is now known that microglia are more dynamic than previously believed and can present multiple phenotypical states, suggesting much further complexity in their functional capacity and response to insult and injury [178]. For example, single-cell transcriptomic studies have shown more than one transcriptomic states for the homeostatic microglia, where cells in the developing, adult, and aging brain display distinct signatures [178–181]. As an example of microglial states in response to an insult, disease-associated microglia (DAMs), originally detected in models of Alzheimer's disease (AD), are shown to be present across a number of pathologies. Microglia inflamed in MS (MIMS) are considered to be a specific signature displayed in MS [71,182]. In animal models of demyelination and remyelination, several microglial states are shown to be present simultaneously within the lesions [178,183]. Thus, when interpreting these transcriptomic states, one should keep in mind the specific context of pathology, including species, sex, region and time [178]. It should also be noted that transcriptomics does not exactly reflect functional changes, and complementary multi-omics studies on translation, proteins, post-translational changes and metabolic properties are required to elucidate the functional phenotype of microglia.

1.5 Dysfunctional Microglia in Progressive MS

1.5.1 Phenotypic changes in microglia with aging and MS chronicity: formation of dysfunctional foamy microglia

The function of microglia deteriorates over time with aging and leads to formation of a dysfunctional inflammatory phenotype. These profound functional changes are a result of prolonged and chronic low-intensity inflammatory signals and result in impaired phagocytosis of cellular debris, heightened production of reactive oxygen species (ROS), an elevated secretion profile of pro-inflammatory cytokines such as Il-1β, IL-6 and TNFα, and a tendency for an exaggerated response upon immune challenge [184–186]. Recently, it was shown that aging also leads to the formation of a distinct lipid-droplet accumulating microglia (LDAM) transcriptomic signature within the CNS of mice. This microglia phenotype was shown to have specific dysfunctional properties, for instance in their impaired phagocytic capacity and elevated production of ROS, and that it was the main detrimental phenotype of microglia in the aged brain [187]. This finding is also corroborated by the earlier reports in peripheral innate immunity that show dysfunctional and detrimental phenotypes in lipid accumulating macrophages called “foam cells” in the atherosclerotic lesions [188]. In recent literature, cholesterol accumulation has also been reported in the microglia within focal LPC-demyelinated lesions in aged mice. The accumulated cholesterol disrupts phagolysosomal membranes through formation of crystals, which consequently lead to stimulation of inflammasome and a dysfunctional inflammatory microglia phenotype that diminishes remyelination [189].

Initially during the course of MS, microglia are present throughout the acute active lesions and contribute to repair by clearing myelin debris through phagocytosis (Figure II) [190]. These myelin debris engulfing cells appear foamy due to transient accumulation of myelin derived lipids, but

have a pro-regenerative profile as evident by increased levels of IL-1ra, CCL18, IL-10, TGF-β and IL-4 expression [191], reduced nitric oxide production [192], and activation of PPARβ/δ [192] and LXR [193] pathways. Therefore, these cells are capable of processing and exporting their lipid and specifically, cholesterol contents [95,190]. However, with chronicity of the disease, overloading of myelin debris elevates the burden of myelin processing and eventually leads to microglial dysfunction [108,190], possibly due to senescence [194]. These dysfunctional foamy microglia are frequently reported to be abundant in the rim of the mixed active-inactive (chronic active) lesions present in the progressive phase of MS [195,196]. Notably, progressive forms of MS (both primary and secondary) typically occur later in life compared to relapsing-remitting forms, and therefore both chronicity related progression of the disease process and aging might play a role in formation of these dysfunctional foamy phagocytes within lesions [95].

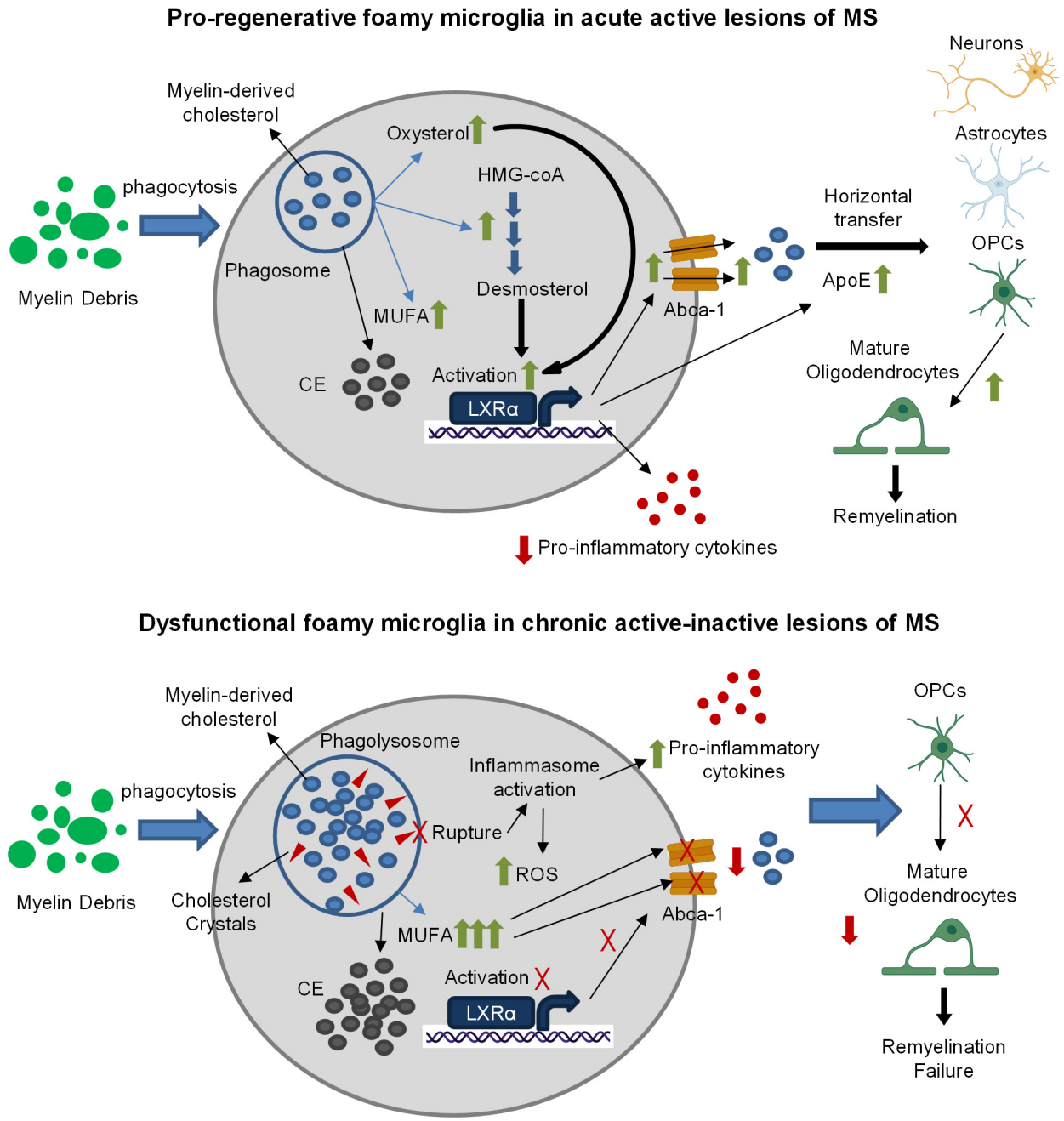


Figure II: Microglial cholesterol metabolism in acute versus chronic MS lesions and its implications in remyelination. In acute active lesions of MS, microglia clear myelin debris and process its cholesterol content. This leads to activation of cholesterol synthesis pathway and production of desmosterol. Desmosterol along with oxysterols (generated from myelin debris cholesterol) activate LXRα pathway, which then upregulates cholesterol efflux transporters (Abca-

1) that excrete intracellular accumulated cholesterols, and ApoE that transfers extracellular cholesterol. LXRα activation also reduces pro-inflammatory cytokine secretion. Additionally, myelin-derived lipids give rise to MUFAs that assist in esterification of toxic free cholesterols. All these events lead to "horizontal transfer" of cholesterols to other CNS cells and support maturation of OPCs to myelinating OLs and consequently, remyelination. In chronic mixed active-inactive lesions, microglial processing of cholesterols becomes ineffective, therefore LXRα is not activated, leading to reduced Abca-1 production. Excessive production of MUFAs also disrupts existing cholesterol efflux channels. This leads to ineffective cholesterol efflux, and as a result, cholesterol accumulation. In long term, cholesterol becomes crystalized, rupturing the phagolysosome membrane, which in turn activates inflammasome that generate pro-inflammatory cytokines. This inflammatory profile also leads to excessive ROS production. The release of these cytokines along with lack of cholesterol efflux creates a non-permissive microenvironment for OPC maturation that leads to remyelination failure. Abca-1: ATP-binding cassette A1; ApoE: apolipoprotein E; CE: cholesteryl esters; HMG-coA: 3-hydroxy-3-methylglutaryl coenzyme A; LXRα: Liver-X receptor alpha; MS: multiple sclerosis; MUFA: mono-unsaturated fatty acids; OPC: oligodendrocyte progenitor cell; ROS: reactive oxygen species. Figure generated by items from Biorender.

1.5.2 Role of microglia in impaired cholesterol metabolism in progressive MS

As shown in experimental animal models of demyelination, microglia uptake of myelin debris from the lesion leads to accumulation of cholesterol within these cells (Figure II). It is well established that cellular cholesterol accumulation suppresses cholesterol synthesis pathways at transcriptional, translational and post-translational levels, as well as production of oxysterols [197–

[199]. However, it was recently shown that in active demyelinating lesions, myelin derived cholesterol accumulation conversely leads to an upregulation of cholesterol synthesis pathways within microglia and an excessive production of immediate cholesterol precursor, desmosterol (Figure II) [108]. As both these sterols are potent LXR activators, the higher levels of desmosterol and probably oxysterols within these phagocytes induces an upregulation of cholesterol efflux transporters such as ABCA1 and ABCG1 and limits the inflammatory profile as a result of LXR pathway activation [107,108,199,200]. This leads to an enhanced myelin derived cholesterol recycling, which likely supports repair mechanisms by providing cholesterol to oligodendrocytes, astrocytes and neurons, presumably via ApoE mediated horizontal cholesterol transfer [100,108,109,201,202]. ApoE is another gene regulated by LXR pathway besides cholesterol efflux transporters, and is shown to be necessary for clearance and recycling of cholesterol in demyelinating lesions [189,203].This crucial role of LXR pathway in MS lesion repair and remyelination is further supported by the observed increased risk of familial MS associated with mutations in the LXR gene [204].

With aging and chronicity of the disease, these compensatory mechanisms in myelin-derived lipid recycling by microglia exhaust and become inefficient in progressive MS (Figure II) [95]. Prolonged myelin exposure has been shown to result in high concentrations of monounsaturated fatty acids (MUFAs) within phagocytic microglia [190]. MUFAs are beneficial in acute lesions since they help esterification of free cholesterols that can have toxic effects [190,205]. However, accumulation of MUFAs in a chronic lesions leads to destabilization of ABCA1 and a resulting interruption in cholesterol efflux, and consequently formation of dysfunctional foamy phagocytes and a disrupted remyelination [190,206–209]. These cholesterol accumulating microglia are shown to activate inflammasome response and become detrimental to the repair processes as a result of formation of cholesterol crystals which rupture the membrane of phagolysosomes (Figure II) [189]. Despite the

crucial roles of LXR in appearance of these dysfunctional foamy phagocytes, it is not the only disrupted pathway involved in cholesterol trafficking and efflux in these cells. Other pathways such as PPARα and γ have also been shown to be compromised in foamy microglia [210,211]. The extensive intracellular accumulation of lipids and cholesterol is consequently followed by activation of pathways involving inflammasomes, which ultimately leads to microglia death [108,189,212]. A recent study has shown that dysfunctional and impaired microglial cell death within remyelinating lesions is driven by necroptosis [213]. Necroptosis is a non-apoptotic form of programmed cell death, which exerts an inflammatory response due to leakage of intracellular contents and is implicated in a variety of pathologies including neurodegenerative conditions [214].

In recent decades, promoting remyelination has been one of the main targets in developing therapeutic strategies for progressive MS [83]. Modulation of cholesterol metabolism in chronic lesions have been one of the proposed strategies. LXR pathway specifically has received special attention due to its double role in both facilitating recycling of cholesterol for remyelination and suppressing the inflammatory response of phagocytes [95]. It was demonstrated recently that LXR agonists could promote repair and remyelination in animal models of demyelination [108,215]. Additionally, mimetics of LXR activating molecules such as desmosterol, and LXR activation products such as ApoE have separately exhibited repair promoting properties [108,216,217]. Excitingly, fingolimod, the first oral DMT for MS, has been shown to affect intracellular cholesterol transport through Niemann-Pick type C protein and elevate high-density lipoprotein (HDL) levels in plasma [218–220]. All these findings suggest a potential in modulation of intracellular cholesterol transport as a viable and promising therapeutic strategy for progressive MS. Therefore, unraveling endogenous mechanisms that influence and harness the potential beneficial effects of the otherwise dysfunctional lipid laden microglia within chronic demyelinating lesions may facilitate

development of new therapies for progressive MS. Our group has shown, for the first time, that Neuregulin-1 (Nrg-1) has the potential for enhancing remyelination in acute demyelinating lesions, and simultaneously, promotes a shift in the phenotype of microglia from a pro-inflammatory to a pro-regenerative state [221–223]. In the next sections, I will discuss Nrg-1 as a promising potential target for treatment of chronic demyelinating lesions.

1.6 Neuregulin 1 (Nrg-1): Structure, Function, and Signaling Pathways

1.6.1 Overview of Nrg-1 and its isoforms

Neuregulin-1 (Nrg-1) is a ligand and a growth factor with crucial roles in development and homeostasis of the central and peripheral nervous systems [224]. In the peripheral nervous system (PNS), Nrg-1 is vital for Schwann cell differentiation, survival and myelinating function, while in the CNS, it is implicated in a wide range of developmental processes such as radial glial cell formation, developmental neuronal migration, synapse formation, and regulation of myelination [224]. Nrg-1 has also been shown to have allelic and haplotypic association with psychiatric disorders and is known to be a susceptibility gene for schizophrenia and bipolar disorder, highlighting the importance of this molecule and its signaling pathway in CNS development and physiology [225–227].

Nrg-1 is a member of the Neuregulin family of growth factors (Nrg-1 to 4) and is known to have 6 types (Type I-VI) and almost 30 isoforms, all containing the same active site on their extracellular domain called the epidermal growth factor (EGF)-like domain [224,228]. Except for Nrg-1 type III that is membrane bound, other types and isoforms are released through an enzymatic cleavage as a soluble ligand. Nrg-1 type III is mainly expressed by neurons and is located on axons

[224,229]. These different types and isoforms of Nrg-1, as well as distinct enzymatic processing of those isoforms have been shown to exert different functions [230–233]. For example, Nrg-1 type 1 has major roles in embryogenesis, Nrg-1 type II is mainly involved in late developmental stages of nervous system, and Nrg-1 type III is mainly involved in regulating myelination and behaviour of neurons [224,229,232,234]. Regardless of type and isoform, Nrg-1 has been shown to be mainly expressed by neurons (with an axonal bias) and oligodendrocyte lineage cells in the adult CNS [224].

1.6.2 Nrg-1 signaling pathways in the CNS

Nrg-1 exerts its functions through interaction with its specific receptors from the ErbB tyrosine kinase receptor family. Nrg-1 receptors are homodimers or heterodimers of ErbB2, 3 and 4 which interact with the active EGF-like domain of Nrg-1 isoforms [235]. Notably, ErbB1 is not involved in Nrg-1 signaling and is a specific receptor for EGF [236,237]. ErbB2 does not show a high affinity ligand binding to the EGF-like domain of Nrg-1, and ErbB3 does not possess the required kinase domain to activate the downstream signaling pathways intracellularly [238–240]. Therefore, homodimers of either ErbB2 or ErbB3 do not result in effective Nrg-1 signaling; however, when in a heterodimer structure with other ErbB proteins, they can form functional receptors [240–242]. ErbB4 on the other hand has both high ligand affinity and effective kinase activity, resulting in its ability to form functional Nrg-1 receptors either with homodimerization, or heterodimerization with other ErbB proteins [243]. ErbB receptors of Nrg-1 are expressed by all CNS cells, including neurons, OPCs, oligodendrocytes, astrocytes and microglia [224].

Upon activation of ErbB receptors following interaction with Nrg-1, intracellular phosphorylation of the ErbB partner recruits effector molecules that facilitate downstream signal transduction. Of

the major downstream pathways of Nrg-1 signaling are phosphatidylinositol 3-kinase (PI3K) dependent activation of JAK/STAT3 and AKT/mTOR/S6K, Raf/MEK/ERK pathway, and JNK/c-JUN pathway [224,244–246]. Moreover, it has been shown that ErbB4 intracellular domain can be cleaved upon interaction with Nrg-1, translocate to the nucleus, and modify gene expression [247,248]. The Nrg-1/ErbB interaction can also activate signaling pathways traditionally linked with EGF receptors [245]. These pathways and other modes of signalling implicated in Nrg-1/ErbB interactions such as backwards signaling by released ErbB4 on membrane bound forms of Nrg-1 [234], exert a complex set of functions in different cells in a context dependent manner [224].

1.6.3 Role of Nrg-1 in oligodendrocyte development and myelination

Nrg-1 signaling is implicated in oligodendrocyte maturation and physiology. Unlike PNS myelination that is heavily dependent of Nrg-1 signaling due to its roles in Schwann cell survival and function [249,250], the role of Nrg-1 in CNS myelination remains elusive. Expression of all ErbB receptors by OPCs and other cells of the oligodendrocyte lineage implies that Nrg-1 signaling is important in these cells [251,252]. Early studies showed that knockout of Nrg-1 results in impaired oligogenesis and supplementation with exogenous Nrg-1 reversed this absence of oligodendrocytes [253]. *In vitro* studies on rat cortical OPC cultures also showed that Nrg-1 treatment enhances OPC proliferation and oligodendrocyte survival [252]. Furthermore, Nrg-1/ErbB signaling is necessary for OPC differentiation into oligodendrocytes as its disruption blocks this process [254]. Both ErbB2 and ErbB3 are implicated in Nrg-1 signaling dependent oligodendrocyte differentiation and maturation [254,255]. Notably, ErbB3 is shown to be dispensable for this process [256]. Previous studies from our group has also shown that Nrg-1 treatment enhances oligogenesis of mouse neural precursor cells (NPCs) [257]. Maturation of both OPC and NPC derived

oligodendrocytes is enhanced by exogenous Nrg-1 and promotes myelination in an *in vitro* setting [222]. These effects were shown to be through ErbB2 and ErbB4 receptors [222].

Although Nrg-1 signaling is critically involved in oligodendrocyte physiology, the data regarding the impact of this pathway on myelination is rather controversial. This is partly due to the fact that Nrg-1 is essential in cardiac development during embryogenesis, thus mice with global embryonic knockout of Nrg-1 and ErbB receptors do not survive past the late stages of embryonic life when myelination occurs [230,231,258–261]. As a result, most of the transgenic studies are limited to the use of heterozygous or conditional knockout mouse lines or overexpression models. Heterozygous Nrg-1 +/- mice shows thinner myelin sheaths around the axons of corpus callosum [262]. However, a later study using tamoxifen inducible conditional knockout mice where Nrg-1 was ablated postnatally, reported oligodendrocyte or axon specific knockout of Nrg-1 does not results in noticeable changes in CNS myelination [263]. Overexpression of Nrg-1, however, was resulted in hyper myelination of the developing CNS. Altogether, despite the advancement of our knowledge on the roles of Nrg-1 in oligodendrocyte physiology, further investigation is required to better understand how this signalling pathway affects myelin maintenance and repair in healthy and pathologic conditions.

1.6.4 Nrg-1 as a therapeutic target in neurodegenerative diseases

In addition to its roles in neurodevelopment and adult CNS homeostasis, Nrg-1 has emerged as a regulator PNS and CNS injury and repair. Nrg-1 has been long studied in the context of PNS injury with beneficial effects on the repair processes. Elevation of Nrg-1 expression has been correlated with axonal regeneration and repair after PNS injury in animal models [264–266]. In sciatic nerve

crush injury, Nrg-1 overexpression promotes remyelination [266]. In recent years, emerging evidence also implicates Nrg-1 in various CNS pathologies including neurotrauma, ischemia and neurodegenerative disease including MS [221,224,257]. In spinal cord injury (SCI), our group has shown a persistent downregulation of Nrg-1 within the lesion and its functional ramifications on the injury mechanisms as its therapeutic restoration exerts neuroprotective effects and improve recovery [223,257,267]. In brain ischemia and SCI, Nrg-1 treatment is neuroprotective through reducing neuronal apoptosis and inflammatory cytokine production and secretion within the lesion [223,268–271].

Nrg-1 has also been associated with neurodegenerative diseases. A recent study on SOD1-linked mouse model of amyotrophic lateral sclerosis (ALS) reported downregulation of Nrg-1 and showed neuroprotective effects of exogenous Nrg-1 treatment through promoting motor neuron survival [272]. Interestingly, a study on Parkinson's disease (PD) patients has reported reduced plasma levels of Nrg-1 [273]. Studies on mouse models of PD have also shown protective roles for Nrg-1 treatment in nigral dopaminergic neurons with an indispensable role for ErbB4 [274,275]. In transgenic mice and *in vitro* models of Alzheimer's disease (AD), Nrg-1 treatment is neuroprotective and attenuates cognition impairments [276,277].

1.7 Therapeutic Potential of Nrg-1 in MS

1.7.1 Previous studies on the role of Nrg-1 in MS

Our group previously discovered that Nrg-1 is dysregulated within demyelinated lesions of people with MS [221] (Figure III). This was observed in samples from both RRMS and the chronic lesions of SPMS. Interestingly, these reduced Nrg-1 levels were also reflected in the peripheral immune

tissues such as spleen and even in the serum of individuals with clinically isolated syndrome (CIS) who are at the early stage of developing MS. In EAE mice, Nrg-1 is also significantly dysregulated within demyelinated lesions [221] (Figure III). Therapeutic restoration of Nrg-1 through systemic administration of an exogenous Nrg-1 peptide was sufficient to alleviate the severity of EAE symptoms. This improvement in the outcome of the disease was observed when the treatment was given pre-symptomatically, at onset, at the peak of the disease or after the EAE peak. The importance of these findings was accentuated by the fact that EAE pathogenesis is the most relevant to that of RRMS among the MS animal models [32]. Additionally, earlier studies by our group in LPC model of acute demyelination showed that Nrg-1 treatment promotes remyelination in these lesions [222] (Figure III). In an early study on a chronic relapsing model of EAE, administration of another isoform of Nrg-1 named glial growth factor 2 (GGF2) enhanced remyelination [278]. Pro-remyelination effects of Nrg-1 treatment has also been demonstrated in SCI [279]. There have been efforts in understanding how these beneficial effects of Nrg-1 occur and what mechanisms are involved in promotion of remyelination.

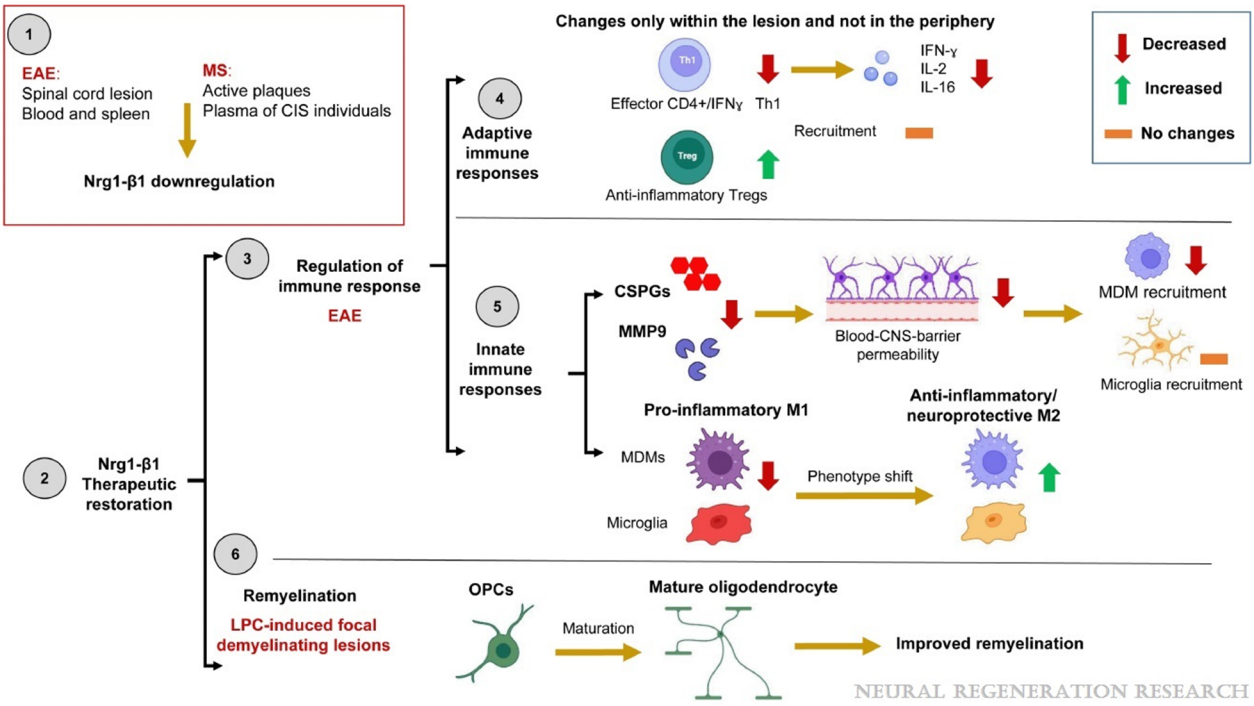


Figure III: The current proposed role and mechanisms of Nrg-1β1 in pathophysiology of MS and EAE. (1) We have demonstrated that expression level of Nrg-1β1 protein is dysregulated in the spinal cord lesions, peripheral blood, and spleen of EAE mice, as well as active plaques of individuals with MS and plasma of CIS individuals. (2) Restoration of Nrg-1β1 levels using recombinant human Nrg-1β1 peptide therapy has been beneficial for recovery in animal models of MS through various mechanisms: (3) Modulation of the immune responses through the regulation of both adaptive and innate components of neuroinflammation. (4) In adaptive immune response, while Nrg-1β1 treatment does not affect the total number of T helper cells in the blood or to the spinal cord of EAE, it significantly decreases the population of CD4+/IFNγ+ Th1 cells that leads to reduced levels of inflammatory cytokines such as IFNγ, IL-2 and IL-16. Nrg-1β1 treatment increases anti-inflammatory Tregs within the EAE lesions in the spinal cord. (5) In innate immune response, restoring Nrg-1β1 levels reduces monocytes in the blood and their infiltration into the CNS and consequently a decrease in the number of MDMs, while the recruitment of microglia

remains unaffected. This was correlated with a reduction in the production of CSPGs and MMPs that are known mediators involved in the blood-CNS permeability in EAE and MS. Availability of Nrg-1β1 also supports a phenotype shift from pro-inflammatory M1 to neuroprotective M2 in both microglia and MDM populations. (6) Using LPC model, we have demonstrated that Nrg-1β1 treatment promotes differentiation of OPCs to mature myelinating oligodendrocytes that results in accelerated and improved remyelination of demyelinating lesions of the spinal cord. CIS: Clinically isolated syndrome; CNS: central nervous system; CSPGs: chondroitin sulfate proteoglycans; EAE: experimental autoimmune encephalomyelitis; IFNγ: interferon γ; IL: interleukin; LPC: lysolecithin induced focal demyelination; MDM: monocyte-derived macrophage; MMP9: matrix metalloproteinase-9; MS: multiple sclerosis; Nrg-1β1: neuregulin-1beta1; OPC: oligodendrocyte progenitor cell; Th1: helper T cell type 1; Tregs: regulatory T cells. This figure is created with BioRender.com. Figure reprinted and figure legend quoted from *Ziaee, S. M. & Karimi-Abdolrezaee, S. Emerging role of neuregulin-1beta1 in pathogenesis and progression of multiple sclerosis. Neural Regen. Res.* ***18****, 133–134 (2023).*

1.7.2 Role and mechanisms of Nrg-1 in remyelination

Recent studies have provided an insight on the involvement of Nrg-1 in regulation of remyelination. Our group has previously shown that therapeutic restoration of Nrg-1 promotes oligogenesis and has protective effects on oligodendrocytes and axons in SCI and acute LPC-induced demyelinated lesions [222,257]. Of note, Nrg-1 treatment was shown to promote an oligogenic fate in NPCs, facilitate differentiation of adult OPCs, enhance oligodendrocyte morphological complexity, and support oligodendrocytes in myelination of axons [222,257]. This was accompanied by relatively thicker myelin sheaths and better preservation of axons after acute

demyelination [222]. A study on SCI also showed that genetic ablation of Nrg-1 signaling resulted in impairment of spontaneous Schwann cell mediated remyelination [279]. Accumulating evidence has suggested a role for Schwann cells in remyelination of spinal cord demyelination, and it has been recently shown that these Schwann cells could be generated from endogenous OPCs within the lesion [280,281]. Notably, Nrg-1/ErbB signaling has been shown to promote this OPC derived Schwann cell generation [281].

Nrg-1 treatment also exerts its regenerative effects indirectly through modification of extracellular matrix (ECM) and modulation of immune responses [282] (Figure III). For instance, chondroitin sulfate proteoglycans (CSPGs), known for their inhibitory effects on regenerative processes in CNS pathologies, have been shown to be upregulated in demyelinated lesions of LPC and EAE, hindering OPC recruitment and maturation, and consequently, remyelination [221,222,283–285]. Our group has shown that Nrg-1 treatment reduces CSPG production by activated astrocytes *in vitro*, as well as lowering its levels within demyelinated lesions of LPC and EAE animal models [221,222,267]. Moreover, Nrg-1 reduces activity of matrix metalloproteinase 9 (MMP9) in EAE lesions that play key roles in MS pathogenesis through disruption of BBB and facilitating immune cell infiltration, [221,267] (Figure III). This effect is likely an underlying mechanism of the immunomodulatory effects of Nrg-1 in attenuating innate and adaptive immune responses within EAE lesion. These studies showed a reduction in the number of effector CD4+/IFNγ+ helper T cells, along with a simultaneous increase in the population of anti-inflammatory regulatory T cells (Tregs) within the lesions [221] (Figure III). This resulted in a reduction in the local levels of inflammatory cytokines such as IFNγ, IL-2 and IL-16. Tregs are known for their suppression of effector helper T cell responses, but are also capable of inducing pro-regenerative phenotypes in microglia and macrophages [286,287]. These immunomodulatory effects on helper T cells and Tregs

was also observed with Nrg-1 treatment in previous study by our group in SCI suggesting its common immunomodulatory mechanisms in various neurological disorders [223]. Interestingly, Nrg-1 treatment also plays an important role in modulating microglia and macrophages directly, and induce a pro-regenerative response in these cells [221,223] (Figure III). In the next section, I will discuss the specific effects on Nrg-1 treatment in microglia and how they can modify the remyelination capacity.

1.7.3 Role of Nrg-1 in microglia modulation in MS

Microglia along with the infiltrating macrophages are the main responders of innate immunity during demyelinating events [123,145]. In EAE, Nrg-1 treatment fosters a phenotype shift in microglia and macrophages from a pro-inflammatory to a neuro-regenerative state [221]. This modulation of microglia function was caharacterized by a decrease in local levels of IL-1β, IL-6 and TNFα cytokines along with a significant reduction reactive oxygen species (ROS) [221]. These cytokines are among the most important inflammatory cytokines associated with inflammatory microglia phenotypes, while these cells are known to engage in exacerbation of damage through excessive production of ROS [146,288]. Similar phenotype shift from inflammatory to pro-regenerative microglia with Nrg-1 treatment was also earlier reported by our groups in SCI [223]. Microglia in pre-regenerative states are capable of promoting OPC differentiation, oligodendrocyte maturation, and consequently, enhance remyelination in animal models of demyelination [108,157]. Therefore, there seems to be a potential for Nrg-1 treatment in promoting remyelination through modulation of microglia function. Considering this combined with the direct beneficial effects of Nrg-1 signaling on oligodendrocyte maturation and its capacity to enhance remyelination of acutely

demyelinated lesions, Nrg-1 emerges as a promising candidate for developing remyelination therapies for progressive MS in which microglia are abundantly present in these lesions.

1.8 Research Objectives and Hypothesis

1.8.1 Rationale for studying Nrg-1 in chronic demyelination

The pathologic hallmark of MS is focal inflammatory demyelination in the CNS [289]. Thus, remyelination is a critical repair process for MS recovery [290]. While remyelination occurs at early stages enabling remission, it declines with disease progression and aging [21,77,291,292]. Although mechanisms underpinning the remyelination decline in chronic MS are yet to be understood, evidence suggests this failure may reflect a decline in maturation of oligodendrocyte progenitor cells (OPCs) to myelinating oligodendrocytes, a prerequisite for remyelination [21,84,92,293]. Importantly, myelin is essential for axon integrity, and remyelination failure and prolonged demyelination is an underlying mechanism for white matter degeneration and disability accumulation [21,22,294]. Thus, it is imperative to unravel how remyelination is regulated in chronic, progressive MS. This knowledge is critical as 10-15% of people with MS start with a primary progressive form and many people with relapsing remitting MS transition to secondary progressive MS [295]. This research aims to develop remyelination strategies for chronic MS.

Our group identified, for the first time, that Nrg-1 protein is dysregulated in human MS lesions and EAE mice, with a positive association with disease progression and neurological disability [221]. Nrg-1 is a ligand produced by neurons and oligodendrocytes with defined developmental roles in oligodendrogenesis, myelination and axonal maintenance [224]. Our EAE studies revealed a novel immunoregulatory role for Nrg-1 as its restoration by systemic peptide treatment promoted a

phenotype shift in microglia from pro-inflammatory to pro-regenerative states in EAE mice [221,282]. In acute demyelinating lesions of lysolecithin (LPC), our group also showed the promise of Nrg-1 treatment in promoting remyelination that was associated with an increase in pro-regenerative microglia [222,282]. In acute MS lesions, these microglia are shown to facilitate remyelination through uptake and metabolism of damaged myelin, and recycling of its cholesterol content that supports OPC maturation [108,157,190]. Dysfunctional myelin-derived lipid-laden microglia are known to be present in chronic MS lesions [108,190]. This presents an opportunity for therapeutic interventions to promote the reparative phenotype of microglia for facilitating remyelination in chronic MS. Therefore, we investigate here whether therapeutic use of Nrg-1 leads to remyelination enhancement in chronically demyelinated lesions through induction of a phenotype shift in dysfunctional foamy microglia within these lesions. We propose this phenotype shift involves enhancement of the cholesterol recycling process by these cells. Proteomics data from a previous work in our lab supports this hypothesis since it depicts an increase in lipid metabolism under Nrg-1 treatment in EAE mice [221].

1.8.2 Hypothesis

Restoration of the deficient levels of Nrg-1 in chronic demyelinating lesions promotes OPC maturation and remyelination by harnessing microglia potential for myelin debris phagocytosis and cholesterol recycling and release.

1.8.3 Objectives

1- To investigate the impact of Nrg-1 on OPC recruitment, maturation and remyelination in a chronic cuprizone mouse model of progressive MS

2- To determine whether Nrg-1 mediates its effects on remyelination by modulating microglia response using *in vitro* and *in vivo* models

3- To determine the impact of endogenous Nrg-1 on OPC recruitment/maturation, myelin maintenance, and remyelination using a loss of function model.

These objectives were addressed in a research manuscript that is reported in Chapter 2 of this thesis.

Chapter II: Harnessing microglia capacity to facilitate myelin regeneration in chronically demyelinated brain lesions

This collaborative work is has been finalized and is written as a manuscript ready for submission

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Conflict of interest: S.K-A and H.K. are inventors on the patent application (Use of Nrg-1β1 for Detection and/or Treatment of Multiple Sclerosis - US Patent App. 18/043,199, 2024). The relevant interests are managed by the University of Manitoba. A.T. is a co-founder of InPrint Bio and 3D PenBone, neither of which however, participated in or bias the work. Other authors declare no competing financial interests.

Authors' contributions: S.K-A and H.K. conceived and designed the study. S.M.Z performed majority of experiments, analyzed the results, prepared the figures, and wrote the first draft of the manuscript. H.K. designed *in vitro* experiments and cuprizone studies. H.K. and S.M.Z. performed oligodendrocyte and myelin analysis *in vivo*. S.N. contributed to animal experiments and performed some imaging and tissue analysis. S.M.H performed MACS, RNA extraction and bioinformatics. S.M.Z. and S.M.H. performed data interpretation for RNA sequencing. P.G. and A.T. designed and prepared nanofibers. E.J. performed immunohistochemical analysis of cholesterol transporter *in vivo*. G.M. analyzed myelination in EAE. V.W.Y. contributed to MS brain samples. S. K-A. supervised the study, analyzed and interpreted the results, provided operational support, and edited and finalized the manuscript. All authors reviewed the manuscript.

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2.1 Abstract

Failure in remyelination is a characteristic of chronically demyelinated lesions in multiple sclerosis that results in progressive neurodegeneration and permanent neurological impairments. Microglia have remarkable capacity to facilitate oligodendrocyte remyelination through cholesterol metabolism and release. However, they become dysfunctional in dysregulated milieu of chronic demyelinated lesions by intracellular accumulation of lipid that eventually results in foamy microglia and cell death. Currently, the molecular mechanisms that drives microglia dysfunction in progressive multiple sclerosis are not fully understood. Here, we unravel that disruption in neuregulin-1 signaling in chronically demyelinated brain lesions underlies impaired remyelination by suppressing microglia pro-remyelination function. Therapeutic restoration of neuregulin-1 promotes remarkable myelin regeneration by revitalizing the innate capacity of microglia for clearance of myelin debris, cholesterol recycling and efflux in chronic demyelinated lesions. These findings highlight the promise of neuregulin-1 as an endogenous target to therapeutically facilitate microglia reparative functions in progressive multiple sclerosis in which there is an unmet critical need for treatment development.

2.2 Introduction

Progressive multiple sclerosis (MS) is a debilitating neurodegenerative disorder characterized by chronic demyelination that results in accumulation of permanent neurological disabilities [295–297]. Remyelination is a repair process that is essential for maintaining axonal integrity, restoring neuronal function and reversing the symptoms of demyelination [21,77,290,292]. Unlike the relapsing-remitting form of MS, remyelination is severely impaired in progressive MS causing permanent neurodegeneration [76,77]. Although the underlying molecular mechanisms are yet to be elucidated, lack of support for the maturation of oligodendrocyte progenitor cells (OPCs) to myelinating oligodendrocytes is thought to be a contributing factor to remyelination failure in chronic demyelinated lesions [84–91].

Microglia play diverse roles in CNS homeostasis and pathologies. In MS, microglia are involved in both neurodegenerative events and the repair processes, depending on the phenotype they acquire [298–301]. Growing evidence has shown a pivotal role for microglia in remyelination, partially through their ability for myelin clearance and cholesterol recycling [108,156,190,213,302]. Despite this potential, microglia often exhibit a dysfunctional phenotype in chronic active lesions of progressive MS and with aging that is characterized by impaired cholesterol metabolism following phagocytosis of myelin debris that leads to the formation of foamy phagocytes [95,108,187,189,190,196]. These foamy microglia are ineffective in cholesterol trafficking through efflux channels due to the dysregulation of cholesterol biosynthesis and export signaling pathways such as LXRs and PPARs [95,108,190,210,211]. Therefore, dysfunctional foamy microglia have been implicated in impaired remyelination that results in sustained demyelination and neurodegeneration [95,108,187,189,190,194]. Given the abundance of microglia in chronic active MS lesions [195,196,303,304], revitalizing their intrinsic capacity for remyelination offers a promising

therapeutic target. Here, we reveal novel evidence that persistent dysregulation of Nrg-1 drives microglia dysfunction that causes impaired oligodendrocyte maturation and consequently sustained demyelination in the brain. Nrg-1 is a key signaling protein in the developing and adult CNS that we recently discovered to play a role in pathogenesis and immune modulation in MS models [221,222]. To date, the role of Nrg-1 in progressive MS is unexplored. In a mouse model of chronic demyelination that models progressive MS, we report that therapeutic restoration of Nrg-1 allows remarkable remyelination in chronically demyelinated white matter. Intriguingly, we found the beneficial effects of Nrg-1 is microglia-dependent as their ablation from chronic demyelinated lesions abolishes Nrg-1 effects. Mechanistically, Nrg-1 promotes microglia integrity and function by potentiating their capacity for myelin clearance and concomitant augmentation of microglia cholesterol biosynthesis and efflux machinery through activation of LXR and PPARγ signaling. This study unravels endogenous mechanisms of remyelination failure in progressive MS, and introduces a novel targeted regenerative therapy for the neurodegenerative phase of MS.

2.3 Material and methods

2.3.1 Animal studies

All procedures involving animals and experimental protocols received approval from the University of Manitoba's Animal Ethics Care Committee, following the Canadian Council of Animal Care's guidelines for the care and use of research animals. Mice were kept in standard plastic cages at a constant temperature of 22°C, with a 12-hour light/dark cycle and access to drinking water and pelleted food *ad libitum*. PDGFRα-Cre^{ERTM} mice (Jackson Lab stock # 018280)[305] and the membrane-tethered Rosa26-mGFP(mT/mG) (Jackson Lab Stock # 007676) were purchased from the Jackson Laboratory, both on a C57/BL6 background, and cross-bred to

obtain PDGFRα-Cre^{ERTM}: Rosa26mGFP (mT/mG) (in short PDGFRα-Cre) mice. All experiments included age-, sex-, and genotype-matched mice as controls. For Nrg-1 conditional knockout mice, the CRISPR/Cas9 system was utilized to generate mutant mice harboring floxed alleles of the Nrg1 gene (Nrg1$^{fl/fl}$), similar to the previously described global knockout Nrg-1 mice [306,307]. The Nrg1$^{fl/fl}$ mice were then bred with Cre driver mice to generate Nrg1$^{fl/fl}$: UBC-Cre ^{ERTM} mice (Nrg1 Cre). The loxP sites flanked exons 6–8, which encode the EGF-like domain of Nrg-1, the biologically active domain present in all Nrg-1 isoforms [224,306]. Upon tamoxifen administration, Cre recombination results in the ablation of the EGF-like domain. It should be noted that embryonic knockout of Nrg-1 is lethal due to its essential role in heart development [230,231]. This conditional knockout line was established and maintained in our laboratory. Additionally, adult mice (12 weeks old) expressing enhanced yellow fluorescent protein (EYFP) [308] were used in this study to extract EYFP+ myelin particles from the CNS. These mice were from our transgenic colony [strain 129-Tg(ACTB-EYFP)2Nagy/J; in short: EYFP Tg] maintained at the University of Manitoba, Winnipeg, Canada. The initial colony founders were generously supplied by Dr. Andras Nagy from the Lunenfeld-Tanenbaum Research Institute in Toronto, Ontario, Canada. All animals were provided by Central Animal Facility, University of Manitoba, Canada.

2.3.2 Tamoxifen administration

Tamoxifen (Biosynth, FT27994) was prepared in corn oil (Sigma Aldrich, C8267) at 30mg/ml concentration. Two weeks before the start of cuprizone feeding, mice from the PDGFRα-CreER (100mg/kg) and Nrg1-Cre (180mg/kg) colonies were intraperitoneally (I.P.) injected with Tamoxifen daily for five days to induce recombination.

2.3.3 Cuprizone induced demyelination and Nrg-1 treatment

At 6-8 weeks of age, both male and female PDGFRα-Cre and Nrg1-Cre mice were provided with either a standard milled rodent diet (North American Lab supplies) as a control or the same diet mixed with 0.2% w/w cuprizone (CPZ) [bis(cyclohexanone) oxaldihydrazone] (Thermo Scientific, A10628.22). This CPZ diet was continued for a total of 5 or 10 weeks to induce short-term or chronic demyelination, respectively [46,47]. Following this period, the mice were switched back to a regular diet to allow for remyelination. (Fig. 1A). The animals were randomly assigned to either a vehicle or an Nrg-1 treatment group. Those in the Nrg-1 group received daily subcutaneous (S.C.) injections of Nrg-1β1 peptide (~8 kDa), containing the bioactive epidermal growth factor (EGF)-like domain (Shenandoah Biotechnology, USA, 100-46), at a dose of 600 ng/mouse/day (equivalent to 30 μg/kg). Mice in the vehicle group were given the same volume of a solution containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, A9647) in saline, which was used as the preparation medium for Nrg-1β1. To track proliferating cells, EdU (5-ethynyl 2′-deoxyuridine) (Biosynth Carbosynth Group, Cat # NE08701) was injected intraperitoneally (12.5 mg/kg body weight, in PBS) to mice according to the experimental timelines presented in the figures. EdU is a thymidine analogue incorporated into the DNA during cell cycle [309].

2.3.4 Microglia depletion

To achieve pharmacological depletion of microglia from the central nervous system (CNS), PLX5622 (Cayman Chemical, 28927), a colony stimulating factor 1 receptor (CSF-1R) inhibitor, was incorporated to the diet of the mice at a concentration of 1200 ppm [310,311]. The administration of PLX5622 was started at week 9 of CPZ diet (1 week prior to CPZ withdrawal) and was continued until the study endpoint, either 2 or 4 weeks post-CPZ withdrawal.

2.3.5 EAE induction and treatments

Eight-week-old female C57BL/6 mice were obtained from the University of Manitoba's Central Animal Facility. To induce EAE, mice were immunized with myelin oligodendrocyte glycoprotein (MOG)35-55, following previously established protocols [221]. In summary, after a 7-day acclimatization period, mice were immunized with 100 μL (50 μg) of MOG 35–55 peptide emulsified in complete Freund's adjuvant (CFA), which includes incomplete Freund's adjuvant with 5 mg/mL heat-inactivated Mycobacterium tuberculosis H37Ra (Thermo Fisher Scientific). A 50 μL emulsion was injected subcutaneously (s.c) on each side of the tail base. Additionally, 300 ng of pertussis toxin (List Biological Laboratories) was administered intraperitoneally (i.p.) on days 0 and 2 post-immunization. EAE mice were monitored daily, with clinical scores recorded as per previous methods. EAE mice were then randomly divided into vehicle and Nrg-1β1 treatment groups, receiving daily s.c. injections of either Nrg-1β1 peptide (600 ng/day) or vehicle as described above. Treatments were administered starting at the peak of the disease (clinical score of 2.5-3) for 4 weeks.

2.3.6 Tissue processing

At designated end-points, mice were deeply anesthetized with a mixture of isoflurane and propylene glycol (40:60 v/v) and perfused with cold 0.1M phosphate-buffered saline (PBS) followed by 3.5% paraformaldehyde (PFA) for immunohistochemical analysis. Brain tissue was then post-fixed overnight at 4°C in 10% sucrose within 3.5% PFA, then cryoprotected in 20% sucrose for an additional 24–48 hours, and finally embedded in Tissue-Tek®-OCT (Electron Microscopy Sciences). Brain samples were sectioned at 16 μm thickness using a cryostat (Leica Biosystems) and stored at -80°C for subsequent analysis.

2.3.7 Immunofluorescence staining

Three coronal brain sections, spaced 100 μm apart, were chosen from each animal for each immunohistochemical analysis. Frozen slides were first air-dried at room temperature (RT) and then rinsed with PBS for 5 minutes. Sections were blocked for 1 hour at RT with a solution containing 1% BSA, 5% non-fat milk, and 0.3% Triton X-100 in PBS. Next, the tissue sections were incubated overnight with the primary antibody in blocking solution at 4°C. After rinsing with PBS, the sections were incubated with secondary antibodies (Alexa 405, Alexa 488, Alexa 568, or Alexa 647 conjugated; 1:500; Invitrogen) suitable for co-labeling with anti-mouse, anti-rabbit, anti-rat, anti-chicken, or anti-goat targets. Nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI), and the sections were cover-slipped with Mowiol mounting medium. Antibody specificity was confirmed with both a negative control, excluding the primary antibody in the immunostaining protocol, and a positive control using tissues known to express the target antigen. All samples were processed in parallel under the same condition and imaged using a Zeiss LSM700 Spectral Confocal Microscope or a Zeiss AxioImager M2 fluorescence microscope (Zeiss) under consistent settings.

Supplementary table 1: Antibodies used for the study. (IHC: immunohistochemistry, ICC: immunocytochemistry).

Antibody	Host species	Application	Dilution rate	Source (CAT#)
Abca-1	rabbit	IHC	1:200	Sigma (SAB4300712)
APC	mouse	IHC	1:50	Millipore (OP80)
CD11b	rat	IHC, ICC	1:25	DSHB (M1/70.15.11.5.2-s)

EGF domain-specific Nrg-1β1	goat	IHC	1:200	R&D (AF396NA)
GFP	mouse	IHC	1:500	Millipore (MAB3580)
Iba-1	rabbit	IHC, ICC	1:500	Wako (019-19741)
MBP	mouse	IHC	1:1000	Biolegend (836504)
MBP	rat	ICC	1:200	Millipore (MAB386)
O4	mouse	ICC	1:200	R&D (MAB1326)
Olig2	rabbit	IHC, ICC	1:500	Chemicon (AB9610)
P2y12	rabbit	IHC	1:200	Anaspec (55043A)
RIP3	rabbit	IHC	1:50	Pro Sci (2283)
Tmem-119	mouse	IHC, ICC	1:100	Abcam (ab209064)

2.3.8 Black Gold II myelin staining

Black Gold II staining (Black Gold II myelin stain [Histo-Chem Inc., 1BGII]) was carried out following the manufacturer's protocol. Briefly, tissue sections were incubated in 0.3% Black Gold II at 60°C for 12–20 min until the finest myelin fibers were visibly stained. The slides were then fixed in 1% sodium thiosulfate for 3 min, rinsed in deionized water, dehydrated via a series of gradated ethyl alcohols, cleared in xylene for 30 minutes, and cover-slipped with Permount permanent mounting media (Fisher Chemical, SP15500). A total of 3 sections were examined per mouse, and 3-5 mice (both male and female) were analyzed per group.

2.3.9 ORO and Filipin III staining in vitro and in vivo

ORO staining was performed on PFA fixed cultured cells and tissue sections to visualise esterified lipids according to the manufacturer’s instructions. Briefly, cells and tissue sections were washed

in 60% isopropanol for 3 minutes, followed by staining with 0.3% ORO (Thermo Scientific Chemicals, AAA1298914) for 15 minutes. To determine the number of cultured cells per imaging field, hematoxylin incubation was used to counterstain for nuclei. Filipin III staining was utilized to detect free cholesterols in PFA fixed cells. The cells were incubated with 50 µg/ml of Filipin III (Sigma-Aldrich, F-9765) for 2 hours. To determine the number of cells per imaging field, Propidium Iodide (Polysciences, 03748-100) (1mg/mL) counterstaining for nuclei was used. Imaging was done using a Zeiss AxioImager M2 fluorescence microscope (Zeiss). Images were analyzed by measurement of the integrated density of the ORO and Filipin III signal using the ImageJ software.

2.3.10 Transmission electron microscopy

To process the tissues for electron microscopy, the mice were perfused with ice-cold Sorensen's Phosphate buffer, followed by perfusion with a solution of 2% PFA/2% glutaraldehyde in Sorensen's Phosphate buffer as we described before [222]. The corpus callosum within the region of interest was dissected and fixed again in 2% PFA/2% glutaraldehyde in Sorensen's Phosphate buffer for 2 hours and stored in Sucrose/Phosphate buffer at 4°C until used. Specimens were further fixed with 1% Osmium tetroxide at room temperature for 2 h and dehydrated through a series of graded ethanol, with a final methanol wash. Tissue was then transitioned through Propylene oxide and embedded in resin. Resin was allowed to cure in at 60°C for 30 hours. Samples were flat embedded to ensure the correct sectioning orientation for visualization of axons/myelin. Blocks were trimmed on a Leica EM Trim 2 (Vienna, Austria) with a milling diamond. Sections 0.5-1µm thick were cut on a Leica ARTOS 3D (Vienna, Austria) ultramicrotome with a diamond knife and stained with toluidine blue to determine to region of interest. Thin sections were taken at 80-100nm and placed on 100 or 200 mesh Cu Grids from

EMS (Cat #G200-Cu, #G100-Cu) and stained with Uranyless (Cat # 22404) and Lead citrate (Cat # 22410 from EMS). Images were acquired using a Phillips CM10 Electron Microscope Model PW 6020/15 (Eindhoven, Netherlands) with a Tungsten filament (60 kV) at 800 – 64,000 x magnification with an Advantage HR-B Digital CCD camera and AMT software. Myelin g-ratio (the ratio of inner to outer diameter of myelinated axons) was used as a measure. Image J software was used for measurements on high-resolution digital images from non-overlapping, sequential microscope fields within the demyelinated area. For analysis, a grid was added to the images with evenly spaced tile dimensions. All axons present at the intersections of the grid lines were used for measurements. A minimum of 200 axons and 3 images were evaluated for each animal. The examiner was blinded to the experimental conditions and conducted all counts on the same computer with the same grid settings for consistency across all animals.

2.3.11 Magnetic-Activated Cell Sorting (MACS) and RNA sequencing

RNA sequencing was conducted on microglia/macrophages isolated from chronic CPZ mice using MACS sorting. Animals were euthanized under deep anesthesia induced by an isoflurane and propylene glycol (60:40 ratio) mixture, followed by cervical dislocation. Brains were quickly removed and placed in cold artificial cerebrospinal fluid (aCSF, 2 brain per each N and N=3-5 per group). The corpus callosum was dissected and enzymatically dissociated using a solution containing kynurenic acid 0.2 mg/ml, trypsin 1.33 mg/ml, and hyaluronidase 0.2 mg/ml for 20 minutes at 37°C. The enzyme solution was then replaced with 1 mg/ml trypsin inhibitor, and the cell suspension was filtered through a 40µm cell strainer. Cell labeling was performed using specific antibodies according to the MicroBead kit protocols (Miltenyi Biotec). Microglia/macrophages were labeled with an anti-CD11b antibody (1:10; Miltenyi Biotec, 130-093-636). Antibody incubation was carried out for 20 minutes at 4°C, followed by a 10-minute

wash with PBS. Cells were isolated by passing the labeled cell suspension through magnetic columns (Miltenyi Biotec, 130-042-401) attached to a magnetic separator (Miltenyi Biotec, 130-091-051). RNA was extracted from the sorted cells by homogenization in TRI Reagent (Sigma, T9424), followed by purification using the PureLink RNA Mini Kit (Thermo Fisher Scientific, 12183018A). Library preparation and RNA sequencing were performed at Genome Quebec using the Illumina NovaSeq PE 100 platform. Transcriptomics analysis was conducted using Galaxy workflows [312–314] and the iDEP platform [315]. Alignment of transcripts to the mouse genome (mm10) was performed with the HISAT2 package, and gene counts were extracted using the featureCounts package. Differential gene expression was identified using the DESeq2 package, with criteria set at a False Discovery Rate (FDR) of less than 0.05 and fold changes greater than 2. Gene Ontology pathway analysis was performed with FDR values calculated using the Benjamini–Yekutieli method, applying a threshold of 0.05.

2.3.12 Total RNA isolation and quantitative PCR

Total RNA was extracted from isolated microglia using TRIzol reagent (Thermo Fisher Scientific; catalog 15596026) according to the manufacturer's guidelines. DNase-treated RNA samples were then used for quantitative PCR (qPCR) with a QuantiFast SYBR Green RT-PCR kit (Qiagen, 204154), following the manufacturer's instructions. Reactions were conducted using a LightCycler (RocheDiagnostics) as previously described [223]. Analysis of results was performed with LightCycler software version 3.5 (Roche diagnostics). Expression values were normalized to the mean of two housekeeping genes (Rplp0, Gapdh). Quantification was achieved via the ΔΔCt method, with values normalized to experimental control samples (set to 1). All primers used (detailed in Supplementary Table 2) were designed for optimal efficiency with criteria including

primer length (18–22 bp), melting temperature (52–58 °C), GC content (40–60%), minimal repeats and amplicon length (<220 bp). All primers were intron spanning.

Supplementary table 2: list of primers used in this study.

Primer name	Sequence	Species
Abca1 F	CTG TTT CCC CCA ACT TCT G	Mouse
Abca1 R	TCT GCT CCA TCT CTG CTT TC	Mouse
Abcg1 F	TCT TTG ATG AGC CCA CCA GT	Mouse
Abcg1 R	GGG CCA GTC CTT TCA TCA	Mouse
Rplp0 F	AGA TTC GGG ATA TGC TGT TGG C	Mouse
Rplp0 R	TCG GGT CCT AGA CCA GTG TTC	Mouse
GAPDH F	CTG GAG AAA CCT GCC AAG TAT GAT	Mouse
GAPDH R	GAC AAC CTG GTC CTC AGT GTA	Mouse
UBC F	GAC GTC ACC CGT TCT GTT G	Mouse
UBC Cre R	AGG CAA ATT TTG GTG TAC GG	Mouse
Nrg-1 3' Lox-P site WPG1249	CTG CCT TCA ATC TAT GAC TAG TTT ATG	Mouse
Nrg-1 3' Lox-P site WPG1311	CCG GCC TCT CAT TTG AGT TCT	Mouse
Nrg-1 5' Lox-P site WPG1253	GTG CAT ACT CAG TGG TCC AT	Mouse
Nrg-1 5' Lox-P site WPG1254	GTC TGA CTG CCA ACA TTC T	Mouse
PDGFR Cre F	TCA GCC TTA AGC TGG GAC AT	Mouse
PDGFR Cre R	ATG TTT AGC TGG CCC AAA TG	Mouse
PDFGR ITC F	CAA ATG TTG CTT GTC TGG TG	Mouse
PDFGR ITC R	GTC AGT CGA GTG CAC AGT TT	Mouse

Rosa (mT/mG) WT F	AGG GAG CTG CAG TGG AGT AG	Mouse
Rosa (mT/mG) MU F	TAG AGC TTG CGG AAC CCT TC	Mouse
Rosa (mT/mG) Co R	CTT TAA GCC TGC CCA GAA GA	Mouse

2.3.13 Myelin preparation

Myelin was extracted from the brains of 12-week-old EYFP-Tg mice [108]. The brains were first homogenized in a 0.32 M sucrose solution and carefully layered over a 0.85 M sucrose solution in a centrifuge tube. This mixture was centrifuged at 15,000g for 2 hours. The resulting interphase, which contained partially purified myelin, was carefully collected, rinsed with water, and centrifuged again at 15,000g for 15 minutes. To induce osmotic shock, the myelin was incubated in water for 15 minutes, followed by another centrifugation step at 12,000g for 15 minutes. The pellet was then resuspended in a 0.35 M sucrose solution, layered on top of a 0.85 M sucrose solution, and centrifuged for 1 hour at 15,000g. After this step, the purified myelin was rinsed with water and subjected to a final centrifugation for 30 minutes at 15,000g. The pellet was then resuspended in PBS, homogenized thoroughly, and stored at –80 °C until further use.

2.3.14 Preparation of electrospun polycaprolactone (PCL) nanofibers

Transparent solution 10% w/v polycaprolactone (PCL) in fluorinated alcohol of 2,2,2-trifluoroethanol (TFE) was prepared by dissolving PCL polymers in TFE with sufficient stirring under room temperature. An adjustable high-voltage power supply was used to apply 10 kV DC voltage. The PCL solution was placed in a 5-mL syringe and loaded into a Harvard Apparatus PhD 2000 syringe pump (with a continuous flow rate of 0.2ml/min. Under a chemical fume hood, the positive electrode of the high-voltage power supply was clamped directly to a needle (23 G). The negative electrode was connected to a metallic rotatory collector wrapped with aluminum foil. Collecting circular 12mm diameter cover glasses were placed on the aluminum foil surface using

double-sided carbon tape. The distance between the needle and collector was set to 12cm and the speed of the collector was set on 1000 rpm toward the needle to produce relatively aligned PCL fibers. The quality of the fiber's alignment and density was assessed using a table top Hitachi scanning electron microscope (TM-1000, HITACHI). All electro spun fibrous membranes on the cover glasses were detached from the collector and placed in 24 well plates under vacuum for 24 hours to ensure the evaporation of the solvent. The samples were then washed with 70% ethanol for 5 min and then three times with DPBS for 5 min each. The samples were then exposed to UV for 1 hour to be sterilized.

2.3.15 Isolation of mouse microglia and OPC for in vitro studies

Microglia and OPCs cultures were prepared as we described earlier [221,222]. Briefly, cerebral cortices were isolated from neonatal CD1 pups (P0-P2), followed by enzymatic digestion at 37°C for 45 minutes in a solution containing papain (0.9 mg/ml; Worthington Biochemical), L-cysteine (0.2 mg/ml; Sigma), and EDTA (0.2 mg/ml; Sigma) diluted in HBSS (Invitrogen). The resulting cell suspension was then filtered through a 40µm cell nylon strainer and transferred to poly-D-lysine (PDL)-coated flasks in Dulbecco's modified Eagle medium (DMEM) (high glucose, containing L-glutamine, containing sodium pyruvate) medium supplemented with 10% FBS (Gibco, by Thermo Fisher Scientific, MD, USA) and 1% penicillin–streptomycin–neomycin (PSN, Invitrogen). Cultures were incubated at 37°C in a 5% CO_2 environment, with media refreshed every 2-3 days. After 10-12 days and upon reaching confluence, cultures were shaken for 2h at 250 rpm at 37ºC and the supernatant media containing microglia were collected. Then the cells were seeded in PDL coated dishes in complete DMEM media with 10% fetal bovine serum (FBS). For collection of OPCs, after the removal of microglia, the medium was refreshed and the cultures were shaken overnight for 16-18 hours at 250 rpm at 37ºC. The OPC-containing medium was

filtered through a 40-µm cell nylon strainer (BD Biosciences), centrifuged at 1,000 rpm for 10 min, and transferred to uncoated Petri dishes (BD Biosciences) to eliminate astrocytes and the remaining microglia. After 1 hour, the media was collected again, centrifuged for 10 min at 1,000 rpm, and the isolated OPCs were collected. These cells were then plated for use in various conditions as described below.

2.3.16 Microglia experiments

Microglia were seeded on PDL-coated 8-well multi-chamber glass slides (20,000 cells per well, LabTek) for immunofluorescence assessments, and on 6-well plates (200,000 cells per well) in complete DMEM media. 24 hours after the initial seeding, the cells were switched to serum free Dulbecco's modified Eagle media supplemented with PSN and treated with vehicle (0.1% BSA), Nrg-1β1 (200ng/ml), lipopolysaccharide (LPS) (100 ng/ml) + IFNγ (20 ng/ml) or Nrg-1β1 + LPS + IFNγ. Various assessments were conducted in these conditions as described below.

2.3.16.1 Phagocytosis experiments

EYFP+ myelin particles were added to each condition at 100µg/ml. Cells seeded on glass slides were fixed after 6 or 24 hours with 3% PFA and co-labeled with DAPI to assess myelin phagocytosis by microglia (YFP signal intensity/DAPI).

Microglia were stained with Filipin III or Oil-Red-O (ORO). Microglial expression of cholesterol biosynthesis and release pathways was done through qPCR on RNA lysates collected after 6 and 72 hours. The conditioned media was also collected from each condition after 72 hours for assessment of cholesterol content and for use in OPC maturation and myelination assays as described below. Cholesterol content of conditioned media was measured using the Amplex™ Red Cholesterol Assay Kit (Invitrogen, Cat# A12216) per manufacturer's instructions. To

investigate if the effects of Nrg-1 are through cholesterol efflux machinery, we combined microglia activation, Nrg-1, and myelin treatment with pharmacological antagonists of the upstream pathways to cholesterol efflux transporters, namely LXR (GSK2033; Tocris, 5694) and PPARγ (GW9662; Santa-Cruz, sc-202641). Multiple doses were used for each inhibitor based on previous studies to determine their efficacy and toxicity (GSK2033: 0.5, 1, 5, 10 and 20 μM, GW9662: 1, 5, 10, 15 and 20 μM). Toxicity assessment was done using a Live/Dead Viability/Cytotoxicity Kit (Invitrogen, L3224). RNA lysates and conditioned media were collected from these conditions after 72 hours, for qPCR and assessment of cholesterol content, respectively.

2.3.16.2 Cholesterol measurement

To determine the concentration of cholesterol in the conditioned media of the microglia, the Amplex™ Red Cholesterol Assay Kit (Invitrogen, A12216) was used. In summary, microglia conditioned media was diluted in the reaction buffer provided in the kit. A working solution of 300 μM Amplex® Red reagent containing 2 U/mL HRP, 2 U/mL cholesterol oxidase, and 0.2 U/mL cholesterol esterase in the reaction buffer was prepared and added to the conditioned media in each well. A no cholesterol condition (blank) as negative control and a gradient of known cholesterol concentrations (from 0 to 2 μg/ml) were used to create a standard curve. The reactions were incubated in 37° C protected from light for 30 minutes and the fluorescence signal was measured every 2 minutes in a fluorescence microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm. The cholesterol concentrations were interpolated based on the standard curve, using the GraphPad Prism software after correcting for the initial dilution rate of the samples in the reaction buffer.

2.3.17 OPC proliferation and maturation assays

For assessment of OPC maturation in the presence of microglia conditioned media (MCM), OPCs were plated on PDL-coated 8-well multi-chamber glass slides at 20,000 cells per well in complete DMEM media. After 24 hours, the cells were divided into 10 different conditions: vehicle treated, Nrg-1β1 treated (200 ng/ml), and MCM treated from the 4 microglia conditions (vehicle, Nrg-1β1, LPS + IFNγ, Nrg-1β1 + LPS + IFNγ) without and with myelin exposure. The media was changed to OPC culture media [222] (DMEM [Invitrogen], insulin [10 µg/ml], Apo-transferrin [100 µg/ml], sodium selenite [5.2 ng/ml], hydrocortisone [18 ng/ml], putrescine [16 µg/ml], progesterone [6.3 ng/ml], biotin [10 ng/ml], N-acetyl-L-cysteine [5 µg/ml], BSA (100 µg/ml), 1% B27, and PSN [Invitrogen]) along with the treatment specific to each condition. For MCM treated conditions, MCM was added to the OPC culture medium at a ratio of 1:1. The cultures were maintained at 37°C and 5% CO_2 and the media was refreshed every other day. After 14 days, the cells were fixed with 3% PFA and used for immunostaining. For assessment of OPC proliferation, 10 µM EdU was added to the OPC culture medium and the same experiments were repeated. After 72 hours, the cells were fixed with 3% PFA and used for immunostaining.

2.3.18 Myelination assay

To assess the myelination capacity of oligodendrocytes treated with MCM, OPCs were seeded on PDL-coated glass coverslips in 24-well plates in the presence of electrospun polycaprolactone (PCL) nanofibers at 20,000 cells per well in complete DMEM medium [316]. After 24 hours, the media was switched to OPC culture media with vehicle or MCM from LPS + IFNγ + myelin treated or Nrg-1β1 + LPS + IFNγ+myelin treated microglia. For MCM treated conditions, MCM was added to the OPC culture medium at a ratio of 1:1. The cultures were maintained at 37°C and

5% CO_2 and the media was refreshed every other day. After 28 days, the cells were fixed with 3% PFA and used for immunostaining.

2.3.19 Human brain MS specimens

Frozen post-mortem brain tissues were sourced from the United Kingdom Multiple Sclerosis Tissue Bank at Imperial College, London (www.ukmstissuebank.imperial.ac.uk; provided by Richard Reynolds and Djordje Gveric). All MS tissues were obtained and utilized with approval from the institutional ethics committee of the University of Calgary. Four brain tissue samples from individuals with chronic MS, each containing active lesions, were analyzed in this study. The lesions met the morphologic criteria for active inflammatory demyelinating process characteristic of MS when stained with H&E-LFB. Detailed information on individual MS samples is available in Supplementary Table 3. Before immunohistological staining, human tissue sections were fixed with 3.5% PFA.

Supplementary table 3: list of details of MS individual samples.

Sample	Age	Sex	Disease type
MS 1	42 y/o	F	SPMS
MS 2	58 y/o	F	SPMS
MS 3	50 y/o	F	SPMS
MS 4	43 y/o	M	SPMS

2.3.20 Statistical analysis

For all analyses, we ensured unbiased evaluation by employing randomization and blinding of experimental conditions. When comparing more than two groups, One-way ANOVA with Tukey's

post-hoc correction was applied. For comparisons involving two variables across more than two groups, two-way ANOVA was used, with Tukey's post-hoc test applied to compare means at each time point. For non-parametric data, the Mann-Whitney test was conducted, while a Student's t-test was used for comparisons between two groups. Data are presented as means ± standard error of the mean (SEM) unless otherwise noted, and a p-value of ≤ 0.05 was considered statistically significant in all the analyses.

2.4 Results

2.4.1 Remyelination is impaired in chronic demyelinating lesions despite the presence of OPCs and is associated with dysregulation of Nrg-1β1

We employed a cuprizone (CPZ) mouse model of demyelination that is well suited for investigating the status of spontaneous remyelination in a progressive manner. We induced CPZ demyelination in PDGFRα-Cre mice that allows tracking OPCs progenies by GFP expression to study proliferation, maturation and remyelination by OPCs and new oligodendrocytes in demyelinating lesions (Fig. 1 A). Mice were subject to 5 and 10 weeks of CPZ diet to induce short-term verses chronic demyelinated lesions, respectively. To allow remyelination, CPZ mice underwent a recovery period for 2 and 4 weeks with re-introduction of normal diet in short-term and chronic paradigms, respectively. We assessed remyelination within the corpus callosum by Black Gold II (BGII) staining that provides a high resolution and myelin-specific histochemical detection method [317]. Our analyses confirmed robust demyelination in the corpus callosum of CPZ mice after both short-term (86%) and chronic demyelination (94%) compared to their age-matched healthy control. Interestingly, mice with short-term demyelination showed significant spontaneous

remyelination after 2 weeks of recovery achieving 68% of baseline control myelination, while extent of remyelination in chronic demyelinated lesions was inadequate (32% of baseline) even 4 weeks after CPZ withdrawal (Fig. 1 B-D). Importantly, we found that impaired remyelination after prolonged demyelination occurs despite the presence of GFP+ oligodendrocyte progenies in the corpus callosum. This suggests that oligodendrocyte population is replenished; however, their maturation to myelin forming cells is inhibited in the milieu of chronic demyelinated lesions.

We previously showed dysregulation of Nrg-1β1 in human MS lesions, as well as in lesions of experimental autoimmune encephalomyelitis (EAE) mouse model of MS with a positive association with disease pathogenesis and progression [221]. Given Nrg-1β1 is well-known for its developmental role in myelination [224], we sought to investigate the expression level of Nrg-1β1 in short-term and chronic CPZ lesions and whether it is correlated with the success of remyelination. Immunohistochemical analysis revealed significant downregulation of Nrg-1β1 in the cortex (89 and 85% reduction) and corpus callosum (90 and 91% reduction) of CPZ mice after both short-term and chronic demyelination, respectively, compared to the age-matched control mice (Fig. 1 E-L). Of note, in the CNS, Nrg-1β1 is largely expressed by neurons and oligodendrocytes, and it is also axonally localized. Thus, its downregulation within white matter demyelinating lesions likely reflects oligodendrocyte loss as well as injury and loss of axons. Intriguingly, downregulation of Nrg-1β1 was restored significantly during recovery from short-term demyelination in the cortex and corpus callosum to 74% and 78% of the baseline control, respectively, while its decline persisted in chronic demyelinated lesions with no restoration even after 4 weeks of recovery (Fig. 1 I-L). These findings indicated a positive correlation between persistent dysregulation of Nrg-1β1 in chronic demyelinating lesions and impaired remyelination.

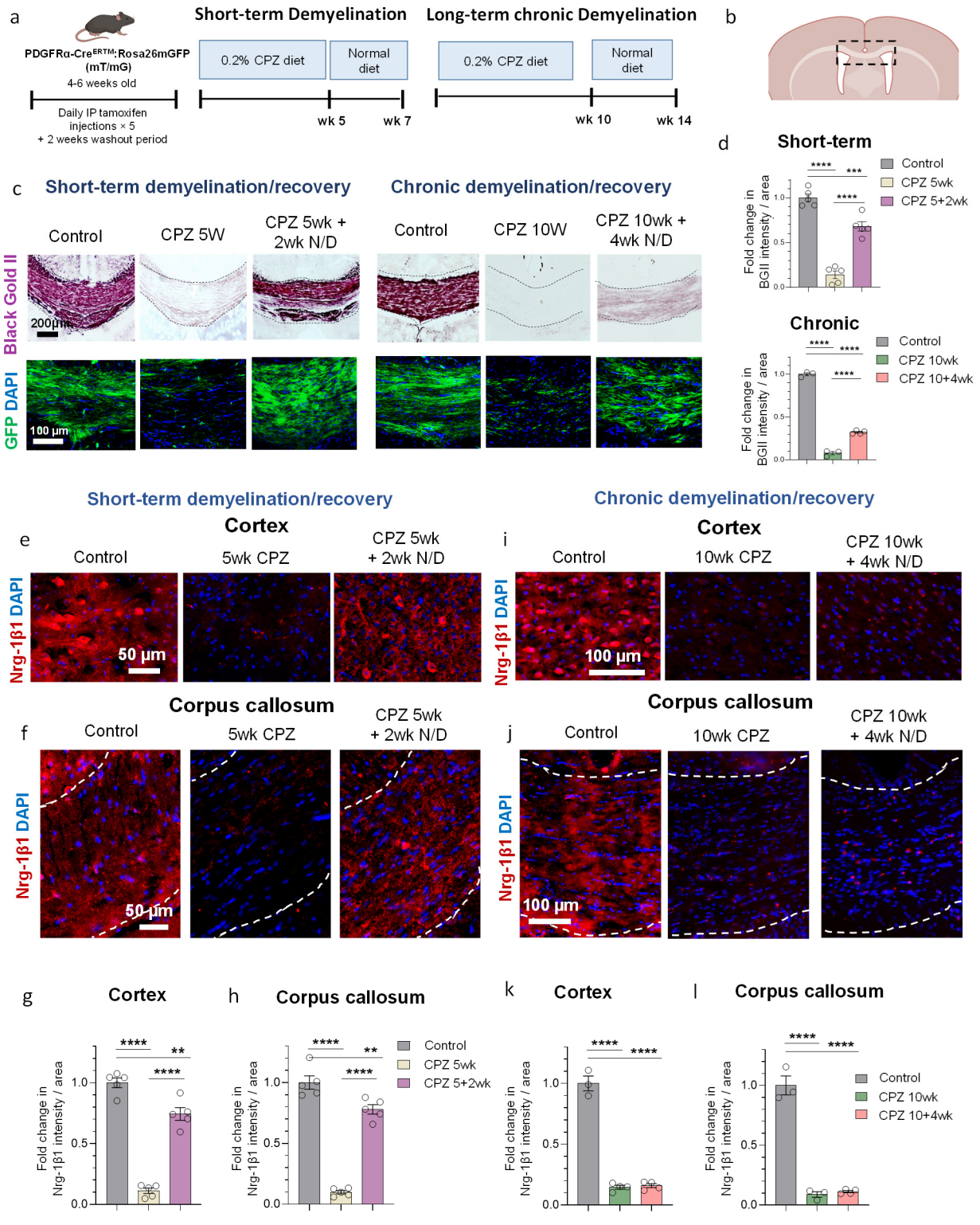


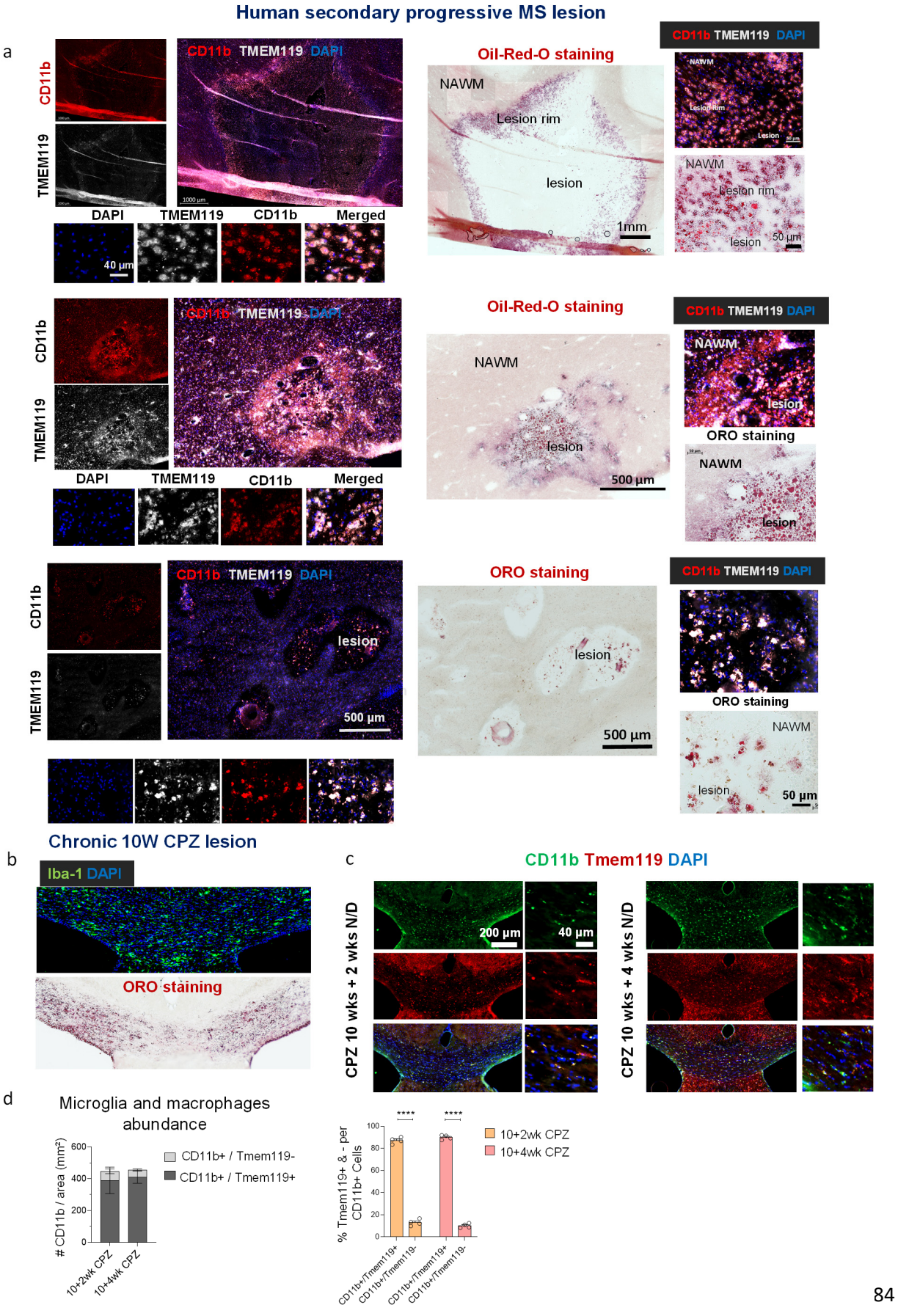
Figure 1: Impaired remyelination in chronic demyelinated lesions is associated with persistent dysregulation of Nrg-1. A) Schematic of experimental design for induction of short-

term and chronic long-term cuprizone (CPZ) demyelination in tamoxifen inducible PDGFR-Cre:Rosa mT/mG mice for tracking OPCs and oligodendrocytes by GFP expression. B) Corpus callosum (CC) was assessed for demyelination and remyelination. C-D) Black Gold II (BG II) staining of the CC shows short-term phase of CPZ-induced demyelination is followed with significant remyelination as early as 2 weeks after re-introduction to normal diet, while remyelination is impaired after prolonged demyelination, despite the presence of GFP+ OPC progeny. E-L) Immunohistochemical analysis of Nrg-1 expression in the cortex and CC revealed that Nrg-1 protein is downregulated significantly after short-term and chronic demyelination. While Nrg-1 expression was restored significantly during the recovery period after short-term demyelination, its severe downregulation persisted in chronic demyelinated lesions even up to 4 weeks after discontinuation of the CPZ diet. The data show the mean ± SEM. ** $p<0.01$, ***$p<0.001$, **** $p<0.0001$, One-way ANOVA; N=3-5 mice/group.

2.4.2 Abundance of Lipid laden microglia is a hallmark of chronic CPZ lesions and in human secondary progressive MS lesions

Microglia play critical roles in remyelination [156,157,213,302,318,319]. While microglia are abundant in chronic demyelinating lesions, their reparative capacity diminishes with MS progression as they acquire a disease-associated phenotype [95,108,175,189,190,195]. One hallmark of chronic demyelinated lesions is an accumulation of myelin-derived lipids within microglia [95,189,190,320]. Our assessments on post-mortem secondary progressive MS (SPMS) lesions also showed an abundant presence of lipid-laden foamy phagocytes within various lesion types, as indicated by Oil Red-O (ORO) staining that marks esterified lipid droplets [190,196] (Suppl. Fig. 1 A). We found that the majority of these lipid-laden phagocytes in SPMS lesions were CD11b+/TMEM119+ microglia. Likewise, in

CPZ-induced chronic lesions, our immunohistochemical staining further confirmed the abundance of ORO positive lipid-laden phagocytes marked by Iba-1 (Suppl. Fig. 1 B). Co-immunolabeling of CD11b with TMEM119 (a specific microglia marker) revealed a significant predominance of CD11b+/TMEM119+ microglia over CD11b+/TMEM119- macrophages within chronic demyelinated lesions after 2 weeks (87.1% ± 1.4 vs. 9.4% ± 1.4, respectively) and 4 weeks (90.0% ± 1.0 vs. 10.0% ± 1.0, respectively) recovery following CPZ withdrawal (Suppl. Fig. 1 C-D). These findings reveal that sufficient remyelination fails in chronic demyelinated lesions despite the abundance of microglia, suggesting that their reparative potential is suppressed within the dysregulated microenvironment of these lesions.


Human secondary progressive MS lesion
a
CD11b
TMEM119
CD11b TMEM119 DAPI
DAPI
TMEM119
CD11b
Merged
40 µm
Oil-Red-O staining
NAWM
Lesion rim
lesion
1mm
50 µm
500 µm
ORO staining
Chronic 10W CPZ lesion
b
Iba-1 DAPI
ORO staining
c
CD11b Tmem119 DAPI
CPZ 10 wks + 2 wks N/D
CPZ 10 wks + 4 wks N/D
200 µm
40 µm
d
Microglia and macrophages abundance
CD11b / area (mm²)
CD11b+ / Tmem119-
CD11b+ / Tmem119+
10+2wk CPZ
10+4wk CPZ
% Tmem119+ & - per CD11b+ Cells
****
10+2wk CPZ
10+4wk CPZ
CD11b+/Tmem119+
CD11b+/Tmem119-

Supplementary Figure 1: Lipid laden microglia are abundant in human progressive MS lesions and in chronic CPZ demyelinated lesions. A) Representative images from lesions at different stages from secondary progressive MS, immunostained for CD11b as a marker of microglia and macrophages and TMEM119 as a specific marker of microglia. We found abundance of CD11b+TMEM119+ microglia in these lesions. Qualitative ORO staining of the same tissue section showed intracellular accumulation of cholesterol in CD11b+TMEM119+ microglia at the rim and to some level, within the lesion, while cholesterol accumulations was not detected within the normal appearing white matter (NAWM). **B)** Iba-1 immunostaining and ORO staining of the chronically demyelinated CPZ lesions of corpus callosum suggest the abundance of phagocytes with myelin-derived lipid accumulation. **C-D)** Similarly, immunohistochemical analyses of CD11b+TMEM119+ cells showed that microglia are the predominant cells in the chronic demyelinated lesions of CPZ as compared to CD11b+TMEM119- macrophages. The data show the mean ± SEM. **** $p<0.0001$, *$p<0.05$, Two-way ANOVA; N=3-4 mice/group.

2.4.3 Nrg-1β1 availability promotes remyelination in chronic demyelinated lesions in a microglia dependent manner

To determine whether dysregulation of Nrg-1 in chronic demyelinated lesions underlies poor remyelination, we restored the diminished levels of Nrg-1β1 in PDGFRα-Cre mice after 10 weeks CPZ demyelination by daily subcutaneous injections of human recombinant Nrg-1β1 treatment (Fig. 2 A-B). CPZ mice received 600 ng of Nrg-1β1 per day (30µg/kg) as we previously employed in EAE mice [221]. Of note, Nrg-1β1 treatment was an 8 kDa peptide that is shown to pass the blood-brain-barrier [321]. Nrg-1β1 or vehicle (0.1% BSA in saline) treatment was administered during the recovery period post-CPZ withdrawal. Our quantitative analysis of myelin by BGII signal intensity

confirmed a robust and significant level of demyelination (~92% decrease) in corpus callosum of PDGFR-α Cre mice after 10 weeks of CPZ diet compared to age-matched control mice (Fig. 2 C-E). After 2 and 4 weeks of recovery period, the vehicle treated lesions showed low degree of spontaneous remyelination (25% and 32%, respectively) compared to the normal baseline of myelination. Intriguingly, Nrg-1β1-treated mice at both time-points showed a significantly higher level of remyelination compared to vehicle treated mice, with a more pronounced remyelination after 4 weeks of recovery that restored myelination to 85% of the baseline levels in age-matched healthy controls (Fig. 2 C-E).

We previously showed an immunomodulatory role for Nrg-1 in promoting a pro-regenerative and disease-modifying phenotype in microglia [221]. To investigate whether the beneficial effects of Nrg-1β1 treatment on chronic remyelination is through microglia regulation, we ablated microglia from the CNS by adding PLX5622, an inhibitor of CSF-1R (colony stimulating factor- 1 receptor) [311], to the diet of the mice starting from 1 week before CPZ-withdrawal and continued throughout the treatment and recovery period. We first confirmed the efficacy of PLX5622 in microglia ablation. Our immunohistochemical assessment of Iba-1 in the chronically demyelinated lesions of CPZ and PLX5622 fed mice showed remarkable depletion of microglia (89% decrease) in PLX5622 treated CPZ mice compared to non-PLX5622 treated counterparts (Fig. 2 B). Interestingly, these experiments showed that the beneficial effects of Nrg-1β1 treatment on remyelination was abolished in those chronic CPZ mice that received PLX5622 during the 2 and 4 weeks post-CPZ recovery to a level that was no longer statistically different than vehicle-treated CPZ mice (Fig. 2 C-E).

We further assessed remyelination and the ultrastructural properties of new myelin in the corpus callosum of chronic CPZ using transmission electron microscopy (TEM) (Fig. 2 F-K).

Electron-micrographs of the corpus callosum confirmed extensive demyelination after 10 weeks of CPZ diet (Fig. 2 G). Our assessment of myelin g-ratio showed significantly lower myelin thickness (indicated by higher g-ratio values) in vehicle treated group (0.806 ± 0.014) compared to normal age-matched control mice (0.685 ± 0.011) (Fig. 2 F, H), confirming our prior immunohistochemical findings of poor spontaneous remyelination in chronic demyelinated lesions. Importantly, Nrg-1β1 treatment significantly increased the overall thickness of myelin in chronic CPZ mice compared to vehicle-treated animals, evidenced by a reduced g-ratio (0.725 ± 0.008) that was no longer significantly different from the normal level observed in the age-matched control mice. In PLX5622 fed animals, Nrg-1β1 treatment did not show any beneficial effects on myelin g-ratio compared to vehicle (0.810 ± 0.006 in PLX+Nrg-1β1 vs. 0.811 ± 0.005 in PLX+Vehicle). The pooled g-ratio of all the axons quantified from every animal in each of the treatment groups also confirmed a significantly thinner myelin sheath in vehicle treated animals compared to control, which was restored by administration of Nrg-1β1; and this beneficial effect of Nrg-1β1 on myelin thickness could no longer be observed in PLX5622 microglia-depleted mice (Fig. 2 I). When we binned axons into different diameters, the same pattern in the g-ratio among different treatment groups could still be observed in all axon diameters (Fig.2 J). Notably, in the lower axon diameter bins, the difference between the treatment groups was more pronounced. By investigating the frequency distribution of myelin g-ratio, we found that the percentage of thinly myelinated axons with g-ratio of higher than 0.8 was lower in Nrg-1β1-treated mice in comparison to vehicle-treated mice (18.06% vs. 56.22%, respectively), while in PLX5622 fed groups, the percentage was comparable between Nrg-1β1 and vehicle-treated groups (56.50% vs. 61.93%, respectively) (Fig 2 K). Moreover, the percentage of heavily myelinated axons with g-ratio of lower than 0.6 was higher in Nrg-1β1 treated mice compared to vehicle (7.98% vs. 1.64%,

respectively) and closer to the normal control mice (18.80%). In the PLX5622 fed groups, the percentage was comparable between Nrg-1β1 and vehicle-treated mice (1.75% vs. 2.02%, respectively). Collectively, these findings provided compelling evidence that Nrg-1 promotes chronic remyelination through microglia-dependent mechanisms.

Lastly, we evaluated the effects of Nrg-1β1 treatment on remyelination in another chronic mouse model of MS, EAE, which represents an immunologically relevant disease model. In our previous work, we showed that Nrg-1β1 is depleted in EAE lesions and its therapeutic restoration at the peak of EAE positively modulates adaptive and innate immune response and attenuates disease severity by significant reduction in clinical scoring [221]. Here, we focused on the effects of Nrg-1β1 on remyelination of EAE lesions after for 4 weeks of treatment (600 ng/day or 30μg/kg, starting at the peak of the disease,14-16 days post EAE induction), as we previously described [221]. Assessment of myelin g-ratio on electron-micrograph images of the spinal cord demonstrate a significant improvement in remyelination of the EAE mice that received Nrg-1β1 treatment compared to vehicle-treated mice as indicated by a decrease in the overall myelin g-ratio (vehicle: 0.699 ± 0.006, Nrg-1β1 treated: 0.664 ± 0.007) (Fig. 2 L-O). Remarkably, Nrg-1β1 treatment restored myelination in EAE mice to the baseline of healthy non-EAE control mice (Fig. 2 L-M). Pooling of the calculated g-ratio from each animal within Nrg-1β1 vs. vehicle-treated groups further demonstrated a significant decrease in g-ratio across all axon diameters (Fig. 2 N). Additionally, evaluation of myelin g-ratio frequency distribution revealed significant changes in the distribution pattern of myelin thickness following Nrg-1β1 treatment (Fig. 2 O). Taken together, our results in two animal models of MS demonstrates the promise of Nrg-1β1 treatment a potent candidate for promoting remyelination.

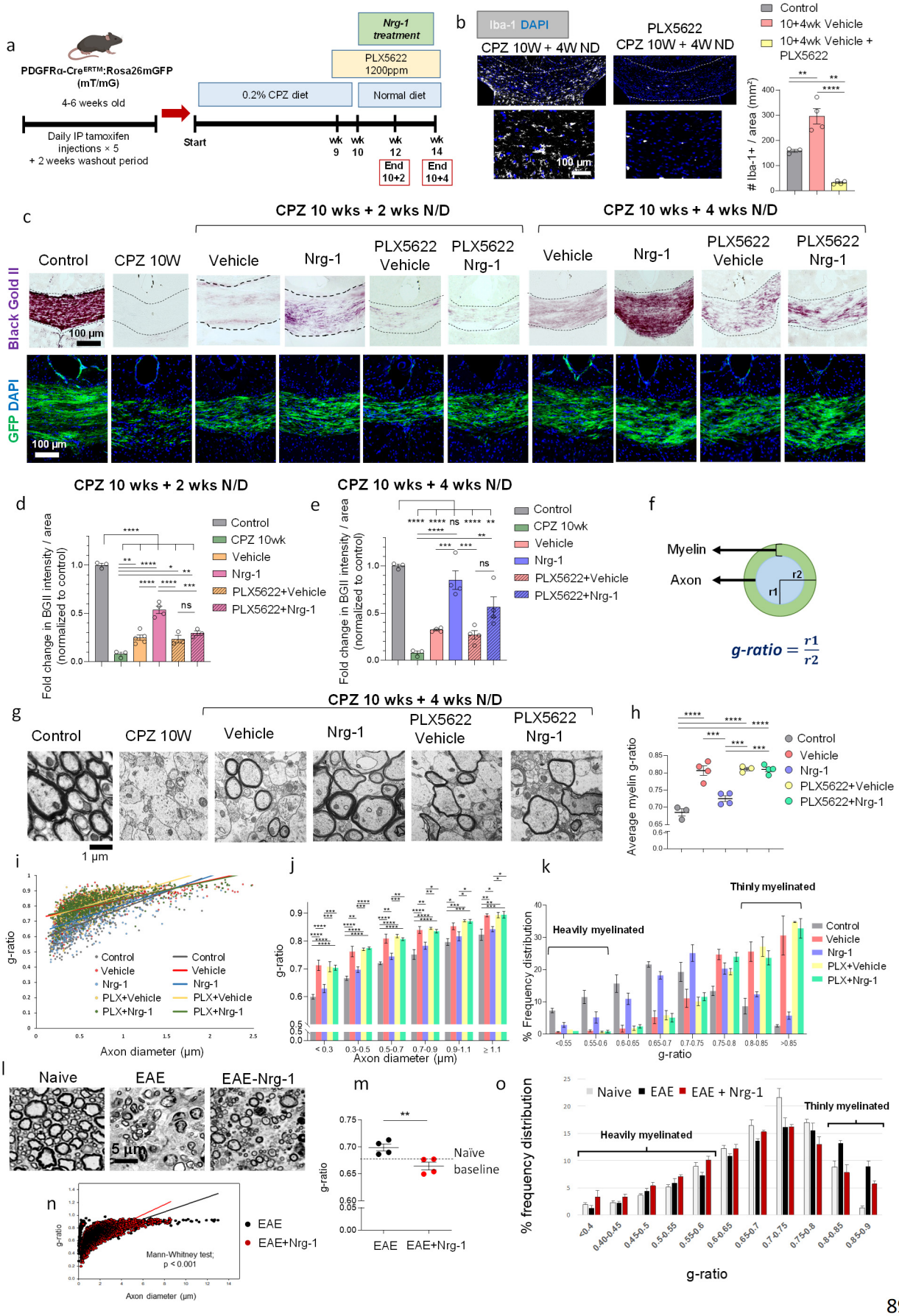

a
PDGFRα-CreERTM:Rosa26mGFP (mT/mG)
4-6 weeks old
Daily IP tamoxifen injections × 5 + 2 weeks washout period
Start
0.2% CPZ diet
Normal diet
Nrg-1 treatment
PLX5622 1200ppm
wk 9
wk 10
wk 12
wk 14
End 10+2
End 10+4
b
Iba-1 DAPI
CPZ 10W + 4W ND
PLX5622 CPZ 10W + 4W ND
100 μm
Control
10+4wk Vehicle
10+4wk Vehicle + PLX5622
Iba-1+ / area (mm²)
c
CPZ 10 wks + 2 wks N/D
CPZ 10 wks + 4 wks N/D
Control
CPZ 10W
Vehicle
Nrg-1
PLX5622 Vehicle
PLX5622 Nrg-1
Black Gold II
GFP DAPI
100 μm
d
CPZ 10 wks + 2 wks N/D
Fold change in BGII intensity / area (normalized to control)
Control
CPZ 10wk
Vehicle
Nrg-1
PLX5622+Vehicle
PLX5622+Nrg-1
e
CPZ 10 wks + 4 wks N/D
f
Myelin
Axon
r1
r2
g-ratio = r1/r2
g
CPZ 10 wks + 4 wks N/D
1 μm
h
Average myelin g-ratio
i
g-ratio
Axon diameter (μm)
j
k
Heavily myelinated
Thinly myelinated
% Frequency distribution
g-ratio
l
Naive
EAE
EAE-Nrg-1
5 μm
m
Naïve baseline
EAE
EAE+Nrg-1
n
Mann-Whitney test; p < 0.001
o
Naive
EAE
EAE + Nrg-1

Figure 2: Availability of Nrg-1 promotes remyelination in chronically demyelinated cuprizone lesions in a microglia dependent manner. A) Schematic of the design of the animal study. B) Representative images and quantification of Iba-1 immunostaining in the brain sections of PLX5622-treated mice confirms successful depletion of microglia from the CNS. C) Representative images of BGII myelin staining and GFP signal representing OPCs and their progeny within the CC of PDGFRα-Cre mice. D-E) Signal intensity assessment of BGII shows extensive demyelination of CC after 10 weeks of cuprizone diet, with poor spontaneous remyelination after 2 & 4 weeks of recovery, despite the presence of newly formed GFP+ OPC progeny. Nrg-1β1 treatment significantly increased remyelination in chronic demyelinated lesions that was more robust at the 4 week recovery time point compared to the 2 week period. Positive effects of Nrg-1β1 on remyelination was abolished in PLX5622 treated CPZ mice indicating that microglia mediate Nrg-1β1 effects. . F) Myelin g-ratio, the ratio of the inner diameter to the outer diameter of a myelinated axon, was calculated on electron-micrographs of CC to further characterize the ultrastructural properties of myelin. G-K) Extensive demyelination was confirmed in the CC after 10 weeks of cuprizone diet. Withdrawal of cuprizone led to low degree of remyelination with lower myelin thickness during spontaneous recovery in vehicle treated CPZ mice, indicating impaired remyelination. Nrg-1β1 treatment, however, increased overall myelin thickness, as shown by a reduced g-ratio. This effect was not observed in mice depleted of microglia. The g-ratio patterns were consistent across different axon diameters, with Nrg-1-treated mice having fewer thinly myelinated axons and more heavily myelinated axons compared to vehicle-treated mice. In microglia-depleted mice, the effect of Nrg-1β1 on myelin thickness was not significant. L-O) Nrg-1β1 treatment promotes remyelination in EAE lesions. L) Electron-micrograph of ventral bilateral spinal cord lesions of EAE mice compared to naïve mice. M)

Calculated overall myelin g-ratio shows a significant increase in the thickness of myelin in EAE lesions with Nrg-1β1 treatment reaching the baseline levels of naïve non-EAE mice. N) Pooling of the g-ratio values per axon diameters for each group shows the decrease in g-ratio with Nrg-1β1 treatment is present across all axon diameters. O) Frequency distribution of g-ratio values show an increase in heavily myelinated axons and a decrease in thinly myelinated fibers with Nrg-1β1 treatment. The data show the mean ± SEM. **** p<0.0001, ***p<0.001, ** p<0.01, *p<0.05, *(B, D, E, H, M)* One-way ANOVA; (*J, K, O*) Two-way ANOVA; (*N*) Mann-Whitney; N=3-5 mice/group.

2.4.4 Nrg-1β1 treatment enhances OPC proliferation, oligodendrogenesis and oligodendrocyte maturation partially through microglia

It is postulated that remyelination failure in chronic MS lesions may occur due to a decline in the proliferation of OPCs or their ability to differentiate into mature myelinating oligodendrocytes [21]. To define the impact of Nrg-1β1 treatment on various steps of the remyelination process, we took advantage of PDGFRα-Cre mice to track OPCs progenies. Additionally, we tracked newly generated OPCs by daily injections of EdU during the early recovery period after CPZ withdrawal (Fig. 3 A). Our immunohistochemical assessment of the demyelinated lesions of corpus callosum for Olig2, a lineage marker of oligodendrocytes, confirmed a significant decrease in the number of Olig2+/GFP+ oligodendroglia after 10 weeks of CPZ diet (78% decrease) compared to age-matched healthy control, as expected (Fig. 3 B-C). After CPZ withdrawal the population of Olig2+/GFP+ cells within the corpus callosum increasingly recovered and reached the baseline control levels after 4 weeks recovery period, suggesting the spontaneous repopulation of the lesion site with OPCs. Nrg-1β1 treatment significantly accelerated repopulation of Olig2+/GFP+ cells

during the early phase of recovery at 2 weeks post-CPZ compared to vehicle treated counterparts (1.8 fold) that restored oligodendrogenesis to an even slightly higher than the baseline levels in healthy control mice. The same trend was observed at 4 weeks recovery period although the difference between Nrg-1β1 and vehicle did not reach statistical significance (Fig. 3 C). Interestingly, the effect of Nrg-1β1 persisted in mice that received PLX5622 suggesting that Nrg-1 effects on the overall number of oligodendrocyte population was not microglia dependent (Fig. 3 B-C). Furthermore, our assessment of Olig2+/GFP+/EdU+ cells during early phase of remyelination as a means to study OPC proliferation revealed 1.7-fold increase in proliferating OPCs with Nrg-1β1 treatment after 2 weeks of recovery period compared to vehicle treatment (Fig. 3 D). This trend persisted at the 4-week mark, where the population of Olig2+/GFP+/EdU+ cells increased by 1.8-fold in the vehicle-treated group compared to the 2-week recovery period. Additionally, Nrg-1β1 treatment further augmented the abundance of these newly generated cells (1.5-fold increase) at 4 weeks recovery timepoint compared to the vehicle group. This increase was also found to be independent of microglia, as PLX5622 did not alter the outcomes of Nrg-1β1 and vehicle treatment on the number of Olig2+/GFP+/EdU+ cells (Fig. 3 D). Interestingly, these results differ from Nrg-1-enhanced remyelination that is microglia-dependent as shown above, suggesting different roles of Nrg1 and microglia in OPC proliferation and oligodendrogenesis in chronic demyelinated lesion.

We next studied maturation of the newly generated oligodendroglia. Following a 10-week CPZ diet, there was a significant depletion of APC+/GFP+ mature oligodendrocytes within the corpus callosum consistent with demyelination. Two weeks after CPZ withdrawal, there was approximately 51% spontaneous restoration of APC+/GFP+ mature oligodendrocytes in the vehicle treated mice compared to the control group (Fig. 3 E-F). Of note, Nrg-1β1-treated mice

exhibited a significantly higher number of APC+/GFP+ oligodendrocytes compared to the vehicle-treated group, reaching 90% of the baseline in control conditions. Intriguingly, depletion of microglia using PLX5622 resulted in significant attenuation (50%) in the beneficial effects of Nrg-1β1 treatment on maturation of newly-formed oligodendrocytes. Moreover, we found similar results at the 4-week recovery time point. This data showed that Nrg-1β1 treatment enhanced the differentiation of OPCs to mature oligodendrocytes partially through microglia dependent mechanisms.

We further examined the extent of oligodendrocyte maturation among newly generated oligodendrocytes during early remyelination. Our analysis revealed a 1.7-fold increase in APC+/GFP+/EdU+ cells numbers with Nrg-1β1 treatment after 2 weeks of recovery compared to vehicle (Fig. 3 E, G). This trend persisted for the 4-week recovery time point, where Nrg-1β1 treatment enhanced oligodendrocyte maturation by a 1.55-fold increase. Importantly, this augmentation was found to be unaffected by microglial depletion in Nrg-1β1 treatment group at both 2 and 4 weeks recovery period. In summary, these findings demonstrate that Nrg-1β1 treatment fosters OPC proliferation, oligodendrogenesis and oligodendrocyte maturation, and that its effects on oligodendrocyte maturation and remyelination is microglia dependent. However, the earlier step of Nrg-1β1 on OPC proliferation is microglia-independent.

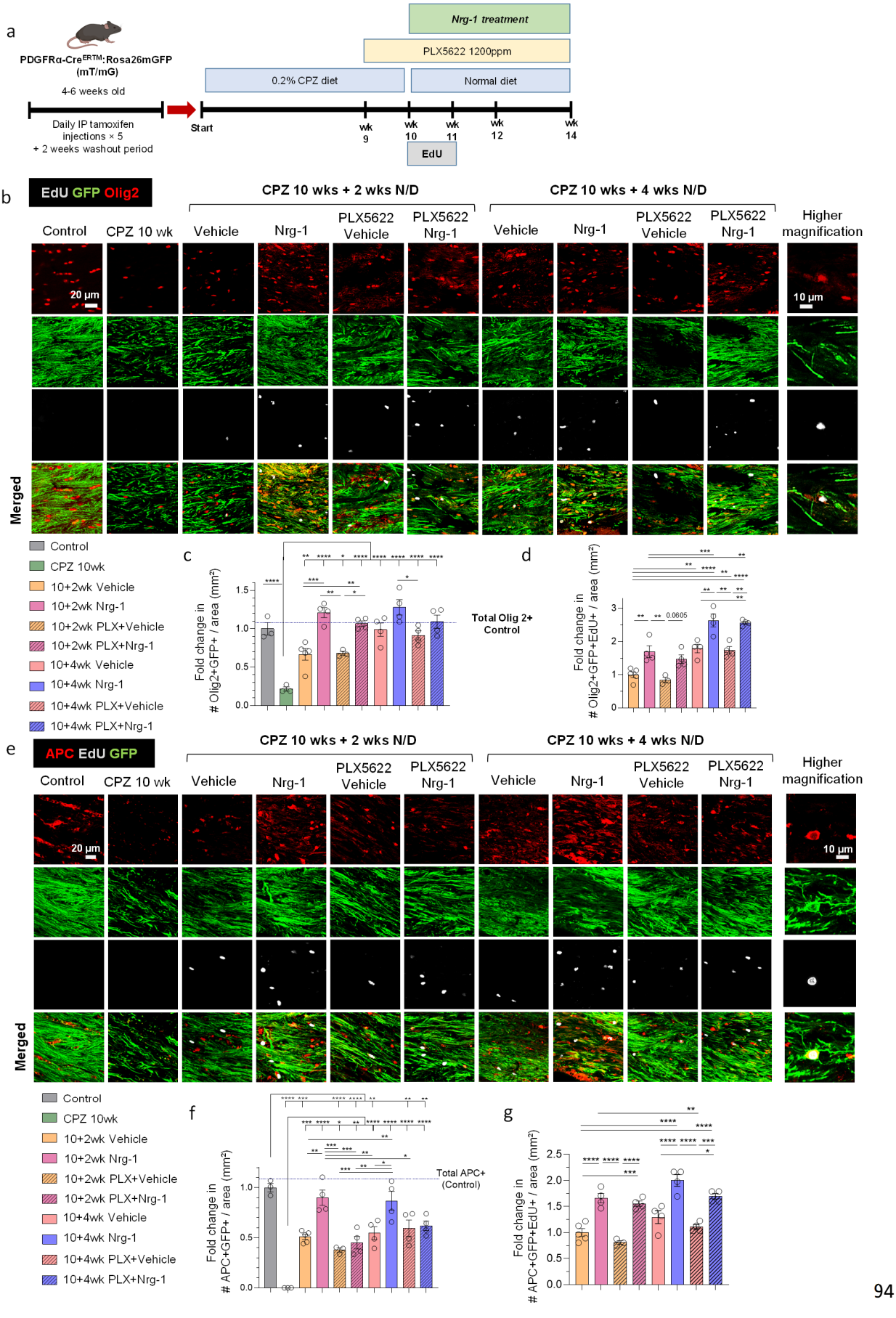

a
PDGFRα-CreERTM:Rosa26mGFP (mT/mG)
4-6 weeks old
Daily IP tamoxifen injections × 5 + 2 weeks washout period
Start
Nrg-1 treatment
PLX5622 1200ppm
0.2% CPZ diet
Normal diet
wk 9
wk 10
wk 11
wk 12
wk 14
EdU
b
EdU GFP Olig2
CPZ 10 wks + 2 wks N/D
CPZ 10 wks + 4 wks N/D
Control
CPZ 10 wk
Vehicle
Nrg-1
PLX5622 Vehicle
PLX5622 Nrg-1
Higher magnification
20 μm
10 μm
Merged
Control
CPZ 10wk
10+2wk Vehicle
10+2wk Nrg-1
10+2wk PLX+Vehicle
10+2wk PLX+Nrg-1
10+4wk Vehicle
10+4wk Nrg-1
10+4wk PLX+Vehicle
10+4wk PLX+Nrg-1
c
Fold change in # Olig2+GFP+ / area (mm²)
Total Olig 2+ Control
d
Fold change in # Olig2+GFP+EdU+ / area (mm²)
0.0605
e
APC EdU GFP
f
Fold change in # APC+GFP+ / area (mm²)
Total APC+ (Control)
g
Fold change in # APC+GFP+EdU+ / area (mm²)

Figure 3: **Nrg-1β1 treatment promotes oligodendrogenesis and oligodendrocyte maturation partially through microglia in chronically demyelinated CPZ lesions. A)** Schematic of the design of the animal study. **B)** representative images from the chronic CPZ lesions of CC at 12 week and 14 week time points from PDGFRα-Cre mice, showing newly generated GFP+ cells of oligodendrocyte lineage using Olig2 marker and EdU. **C)** Quantification of Olig2+/GFP+ cells shows a significant decrease in the number of oligodendrocytes after 10 weeks of CPZ diet compared to the age-matched control mice, which is consistent with demyelination. Of note, dotted line delineates the total count of Olig2-expressing cells in normal control mice, while the control column signifies the baseline of oligodendroglia renewal rate characterized by Olig2+/GFP+ cells. Nrg-1β1 treatment led to a significant elevation in Olig2+/GFP+ cells in the lesion at 2 weeks after withdrawal of CPZ compared to the vehicle counterpart group, which was irrespective of microglia depletion. The same pattern was observed at 4 weeks after CPZ withdrawal, but the effects of Nrg-1β1 treatment were not statistically significant. **D)** For assessment of OPC proliferation and new oligodendrogenesis at the early stage of remyelination, EdU was injected daily during the first week of recovery. Quantification of Olig2+GFP+EdU+ cells shows that Nrg-1β1 treatment promotes OPC proliferation and new oligodendrogenesis in a microglia independent manner. **E)** Representative images from the same lesions, showing newly formed mature oligodendrocytes marked by APC, EdU, and GFP. **F)** Quantification of APC+GFP+ cells also confirms loss of oligodendrocytes in demyelinated lesion after 10 weeks of CPZ diet. Treatment with Nrg-1β1 significantly enhanced repopulation of mature oligodendrocytes after 2 and 4 weeks of recovery in a microglia-dependent manner. **G)** Assessment of APC+GFP+EdU+ mature oligodendrocytes also shows that Nrg-1β1 treatment promoted the maturation of newly formed oligodendrocytes during both 2 and 4 weeks recovery

that was independent from the presence of microglia. The data show the mean ± SEM. **** p<0.0001, ***p<0.001, ** p<0.01, *p<0.05, One-way ANOVA; N=3-5 mice/group.

2.4.5 Ablation of endogenous Nrg-1 results in spontaneous demyelination and suppresses remyelination in chronic demyelinated lesions without altering the number of oligodendrocytes

Building on these findings, we sought to further elucidate the impact of endogenous Nrg-1 on remyelination of chronically demyelinated lesions using a loss of function approach. We ablated Nrg-1 in adult mice using a tamoxifen inducible Cre conditional Nrg-1 knockout (Nrg-1 cKO) model ($Nrg1^{fl/fl}$: UBC-Cre^{ERTM}). Upon Cre expression, exons 7-9 are excised, which encode EGF-like active domain that is crucial for Nrg-1 signaling [306]. We confirmed 79% and 80% reduction in the expression of Nrg-1 EGF-like domain in the cortex and corpus callosum of Nrg-1 cKO mice, respectively, compared to Cre negative wild type (WT) mice (Fig. 4 A-C). Intriguingly, our BGII myelin analysis showed that Nrg-1 ablation results in significant reduction in myelination of the brain spontaneously (Fig. 4 D-E). At 16 weeks post tamoxifen injection, we found 48% decrease in BGII myelin staining in the corpus callosum of Nrg-1 cKO mice compared to WT control. Under CPZ diet, Nrg-1 cKO mice also showed significantly less remyelination (60% decrease) during the 4 weeks recovery period post-CPZ withdrawal as compared to the vehicle treated WT CPZ mice. Notably, when treated with Nrg-1β1 peptide in a rescue strategy, remyelination was significantly enhanced (4.15 fold) in CPZ demyelinated lesions of Nrg-1 cKO mice, although it did not reach the levels observed in Nrg-1β1 treated CPZ WT mice.

Ultrastructural analyses of myelin g-ratio index also revealed that Nrg-1 ablation results in significant spontaneous thinning of the myelin sheath (Fig. 4 F-G). Under CPZ demyelination,

although there was an overall trend for higher g-ratio value in Nrg-1 cKO group, the difference was not statistically significant compared the WT CPZ mice. The pooled g-ratio of all quantified axons from each group revealed a pattern suggesting higher g-ratios across all the axons in Nrg-1 cKO mice compared to WT controls (Fig. 4 H). Interestingly, Nrg-1β1 treatment led to a significant increase in the overall myelin thickness in Nrg-1 cKO mice as indicated by a decrease in g-ratio (0.775 ± 0.011), demonstrating the efficacy of Nrg-1β1 treatment in promoting myelin thickness (Fig. 4 G-H). Binning the axons into different diameters revealed the same pattern of g-ratio changes across all axon diameters (Fig. 4 I), with more pronounced differences in the lower axon diameters. The frequency distribution of myelin g-ratio also showed an increased percentage of thinly myelinated and a decreased percentage of heavily myelinated axons in Nrg-1 cKO mice on a normal diet (43.85% ± 2.78 and 4.63% ± 1.03, respectively) compared to WT controls (17.62% ± 2.17 and 19.81% ± 2.70, respectively) (Fig. 4 J). Under CPZ demyelination and after 4 weeks of recovery period, the percentage of thinly myelinated axons was higher, in Nrg-1 cKO group compared to WT mice (68.56% ± 3.87 in WT vs. 83.85% ± 3.56 in Nrg-1 cKO). The percentage of heavily myelinated axons was negligible in these two groups (1.29% ± 0.57 in WT vs. 0.07% ± 0.07 in Nrg-1 cKO). However, with Nrg-1β1 treatment, Nrg-1 cKO mice in the rescue group showed a lower frequency of thinly myelinated and a higher percentage of heavily myelinated axons 4 weeks after CPZ withdrawal (42.07% ± 6.03 and 6.19% ± 2.48, respectively). These results confirms that endogenous Nrg-1 is important for proper brain myelination in adulthood and plays an important role in remyelination during chronic demyelinating episodes.

We next asked whether spontaneous disruption of myelin and reduced remyelination capacity in Nrg-1 cKO mice is a result of reduced oligodendrocyte numbers. Using immunohistochemical analysis for Olig2 and APC, we found a slight but not significant decrease

(21%) in the abundance of Olig2+ cells in Nrg-1 cKO mice on a normal diet compared to WT controls (Suppl. Fig. 2 A,C). Following a 10-week CPZ diet, the number of Olig2+ cells dropped significantly in both WT and Nrg-1 cKO mice, as expected. After a 4-week recovery period post-CPZ withdrawal, both WT and Nrg-1 cKO mice showed comparable replenishment in the number of Olig2+ cells, although the levels failed to return to baseline (47% and 44% of baseline, respectively). Treatment with Nrg-1β1 during the recovery period restored the number of Olig2+ cells to baseline levels in WT mice (92% of baseline). An increase was also observed in Nrg-1 cKO mice after Nrg-1β1 treatment (74% of baseline), although it was less pronounced than WT mice. Analysis of APC immunostaining also revealed comparable numbers of mature oligodendrocytes within the corpus callosum of Nrg-1 cKO mice on a normal diet compared to WT controls (Suppl. Fig. 2 B, D). After 10 weeks of CPZ diet, as expected, APC+ mature oligodendrocytes were scarcely present in WT and Nrg-1 cKO mice. Following 4 weeks of recovery, the number of APC+ cells in the vehicle treated WT mice and Nrg-1 cKO mice was not statistically different. Moreover, Nrg-1β1 treatment led to a comparable increase in the number of APC+ cells in both WT mice and Nrg-1 cKO mice reaching the levels close to the baseline healthy aged-matched control mice. Altogether, we show that while ablation of endogenous Nrg-1 interrupts myelination in the adult brain during homeostasis or in remyelination after a chronic demyelinating condition, it does not affect the number of oligodendrocytes within the 14-16 weeks period in this study.

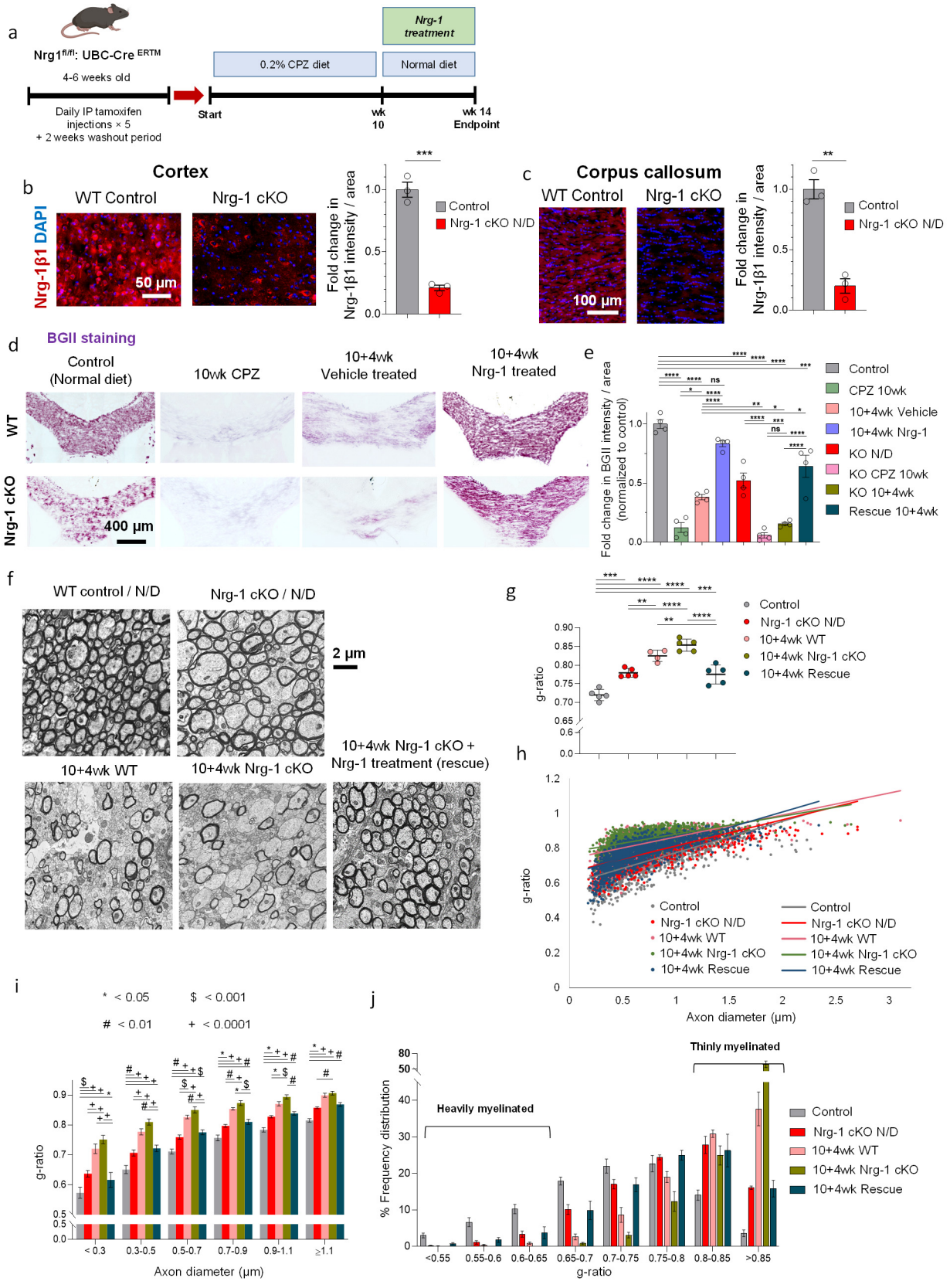
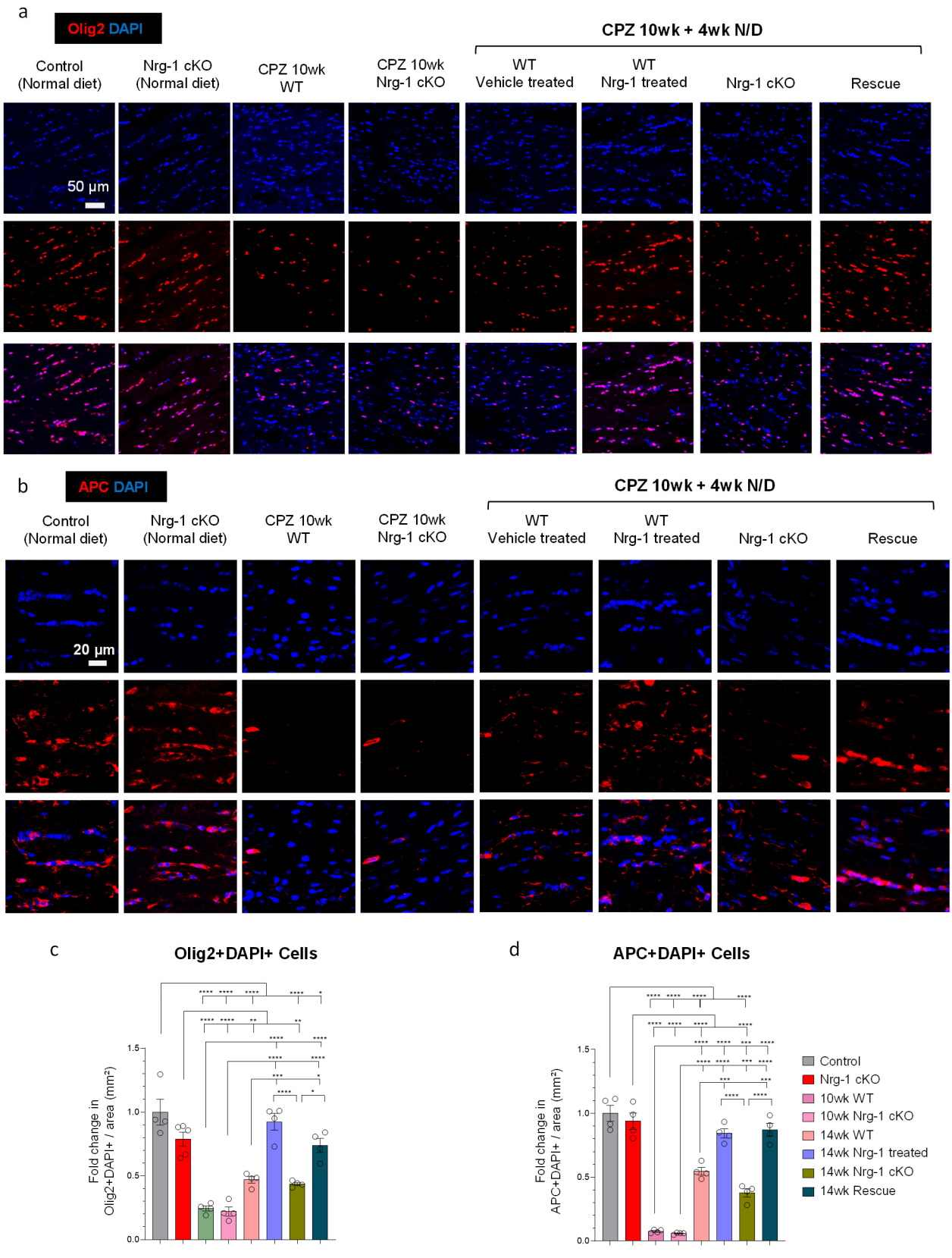

a
Nrg1fl/fl: UBC-Cre ERTM
4-6 weeks old
Daily IP tamoxifen injections × 5
+ 2 weeks washout period
Start
0.2% CPZ diet
Nrg-1 treatment
Normal diet
wk 10
wk 14 Endpoint
b
Cortex
WT Control
Nrg-1 cKO
Nrg-1β1 DAPI
50 μm
Fold change in Nrg-1β1 intensity / area
Control
Nrg-1 cKO N/D
c
Corpus callosum
WT Control
Nrg-1 cKO
100 μm
d
BGII staining
Control (Normal diet)
10wk CPZ
10+4wk Vehicle treated
10+4wk Nrg-1 treated
WT
Nrg-1 cKO
400 μm
e
Fold change in BGII intensity / area (normalized to control)
Control
CPZ 10wk
10+4wk Vehicle
10+4wk Nrg-1
KO N/D
KO CPZ 10wk
KO 10+4wk
Rescue 10+4wk
f
WT control / N/D
Nrg-1 cKO / N/D
2 μm
10+4wk WT
10+4wk Nrg-1 cKO
10+4wk Nrg-1 cKO + Nrg-1 treatment (rescue)
g
g-ratio
Control
Nrg-1 cKO N/D
10+4wk WT
10+4wk Nrg-1 cKO
10+4wk Rescue
h
g-ratio
Axon diameter (μm)
i
* < 0.05
$ < 0.001
# < 0.01
+ < 0.0001
g-ratio
Axon diameter (μm)
j
Heavily myelinated
Thinly myelinated
% Frequency distribution
g-ratio

Figure 4: Nrg-1 is important for remyelination and its genetic ablation results in spontaneous demyelination. A) Summary of the study design. Nrg-1 conditional knockout (cKO) mice were employed as well as Nrg-1 Cre negative mice as wild type (WT) controls. **B-C)** The knockout of Nrg-1 was confirmed in Nrg-1 cKO mice using immunohistochemical staining for EGF domain of Nrg-1β1. **D-E)** BG II staining in Nrg-1 cKO mice on a normal diet showed significant spontaneous demyelination in the corpus callosum at 16 weeks after tamoxifen injection as compared to age-matched control WT mice. After 10 weeks of CPZ diet, extensive demyelination was observed in both WT and Nrg-1 cKO mice. After 4 weeks of post-CPZ recovery, Nrg-1 cKO mice showed a higher degree of impaired remyelination as compared to WT counterparts. In a rescue experiment, Nrg-1β1 treatment in Nrg-1 cKO mice promoted remyelination. **F-J)** Electron-micrographs of the corpus callosum showed a significant decrease in overall myelin thickness (g-ratio) in Nrg-1 cKO mice on a normal diet at 16 weeks post-CPZ induction with tamoxifen, as compared to age-matched WT control mice. **G)** While myelin thickness was even lower in both WT and Nrg-1 cKO mice at 4 weeks after withdrawal of CPZ, Nrg-1β1 treatment in Nrg-1 cKO mice promoted remyelination and g-ratio as compared to vehicle treated knockout mice. **H, I)** The pattern of these changes was similar across axons of different diameters, while axons of lower diameters showed a more pronounced difference. Additionally, the frequency distribution of myelin g-ratio showed a lower percentage of thickly myelinated fibers in Nrg-1 cKO mice with normal diet as compared to WT controls. **J)** In CPZ mice and 4 weeks after withdrawal of CPZ, thinly myelinated fibers were more frequent in vehicle treated WT and Nrg-1 cKO mice, while thickly myelinated axons were more frequently present in Nrg-1β1 treated knockout mice. The data show the mean ± SEM. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, *$p<0.05$; (*C*) Unpaired t-test; (*E, G*) One-way ANOVA; (*I, J*) Two-way ANOVA; N=3-5 mice/group.


a
Olig2 DAPI
CPZ 10wk + 4wk N/D
Control (Normal diet)
Nrg-1 cKO (Normal diet)
CPZ 10wk WT
CPZ 10wk Nrg-1 cKO
WT Vehicle treated
WT Nrg-1 treated
Nrg-1 cKO
Rescue
50 µm
b
APC DAPI
CPZ 10wk + 4wk N/D
Control (Normal diet)
Nrg-1 cKO (Normal diet)
CPZ 10wk WT
CPZ 10wk Nrg-1 cKO
WT Vehicle treated
WT Nrg-1 treated
Nrg-1 cKO
Rescue
20 µm
c
Olig2+DAPI+ Cells
Fold change in Olig2+DAPI+ / area (mm²)
d
APC+DAPI+ Cells
Fold change in APC+DAPI+ / area (mm²)
Control
Nrg-1 cKO
10wk WT
10wk Nrg-1 cKO
14wk WT
14wk Nrg-1 treated
14wk Nrg-1 cKO
14wk Rescue

Supplementary Figure 2: Ablation of endogenous Nrg-1 does not affect the number of oligodendrocytes. A, C) Nrg-1 cKO mice show comparable number of Olig2+DAPI+ oligodendrocytes as observed in WT mice. Following 10 weeks of CPZ diet, both WT and Nrg-1 cKO mice show a significant reduction in oligodendrocytes. Nrg-1β1 treatment led to a significant increase in the number of oligodendrocytes after 4 weeks of recovery in both WT and Nrg-1 cKO mice compared to vehicle-treated mice; however, no significant differences were observed between WT and Nrg-1 cKO mice, whether treated with vehicle or Nrg-1β1. B, D) Mature APC+DAPI+ oligodendrocytes also showed comparable numbers in Nrg-1 cKO mice on a normal diet as the WT control mice. After 10 weeks of CPZ diet, both WT and Nrg-1 cKO mice show a significant depletion of oligodendrocytes, as expected. After 4 weeks of recovery, Nrg-1 cKO and WT mice showed similar repopulation of mature oligodendrocytes, and Nrg-1β1 treatment significantly promoted replacement of mature oligodendrocytes in both WT and knockout mice. The data show the mean ± SEM. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, *$p<0.05$, One-way ANOVA; N=4-5 mice/group.

2.4.6 Transcriptomic analysis reveals Nrg-1 treatment enhances microglial phagocytosis, cholesterol metabolism and cell survival in chronic cuprizone lesions

To elucidate how Nrg-1 treatment affects microglia population within chronic CPZ lesions, we conducted bulk RNA sequencing on MACS isolated CD11b+ microglia from corpus callosum of control and CPZ mice 2 weeks after withdrawal of CPZ. Of note, our immunohistochemical analysis of chronic CPZ lesions determined that ~90% of CD11b+ cells constitute microglia (marked by TMEM119) (Suppl. Fig. 1C-D), confirming that microglia constituted a large population of MACS isolated CD11b+ cells. Our RNAseq data identified 382 differentially

expressed genes in microglia from chronic CPZ lesions as compared to age-matched controls (Fig. 5, A). Among these transcripts, we detected an upregulation of genes related to phagocytosis and cell death and downregulation of genes involved in lipid metabolism and cell survival. When comparing the effects of Nrg-1 versus vehicle treatment on microglial transcripts in chronic CPZ lesions, 619 differentially expressed genes were detected (Fig. 5, B). Importantly, we observed a significant upregulation in multiple genes associated with phagocytosis (Fabp5, Sh3bp1, Pld2), cholesterol metabolism and biosynthesis (Srebf1, Lpl, Cyb5r1) and cholesterol transport and efflux (Abcg1, Arl4c, Abca9). Moreover, there was an upregulation in cell survival genes, while genes implicated in cell death (Dedd2, Rps6ka2, Lcn2) were downregulated in Nrg-1 treated mice compared to vehicle treated counterparts. These findings suggest that Nrg-1 treatment increases the capacity of microglia for phagocytosis and processing of myelin debris in chronic demyelinated lesions that promote their survival.

Figure 5

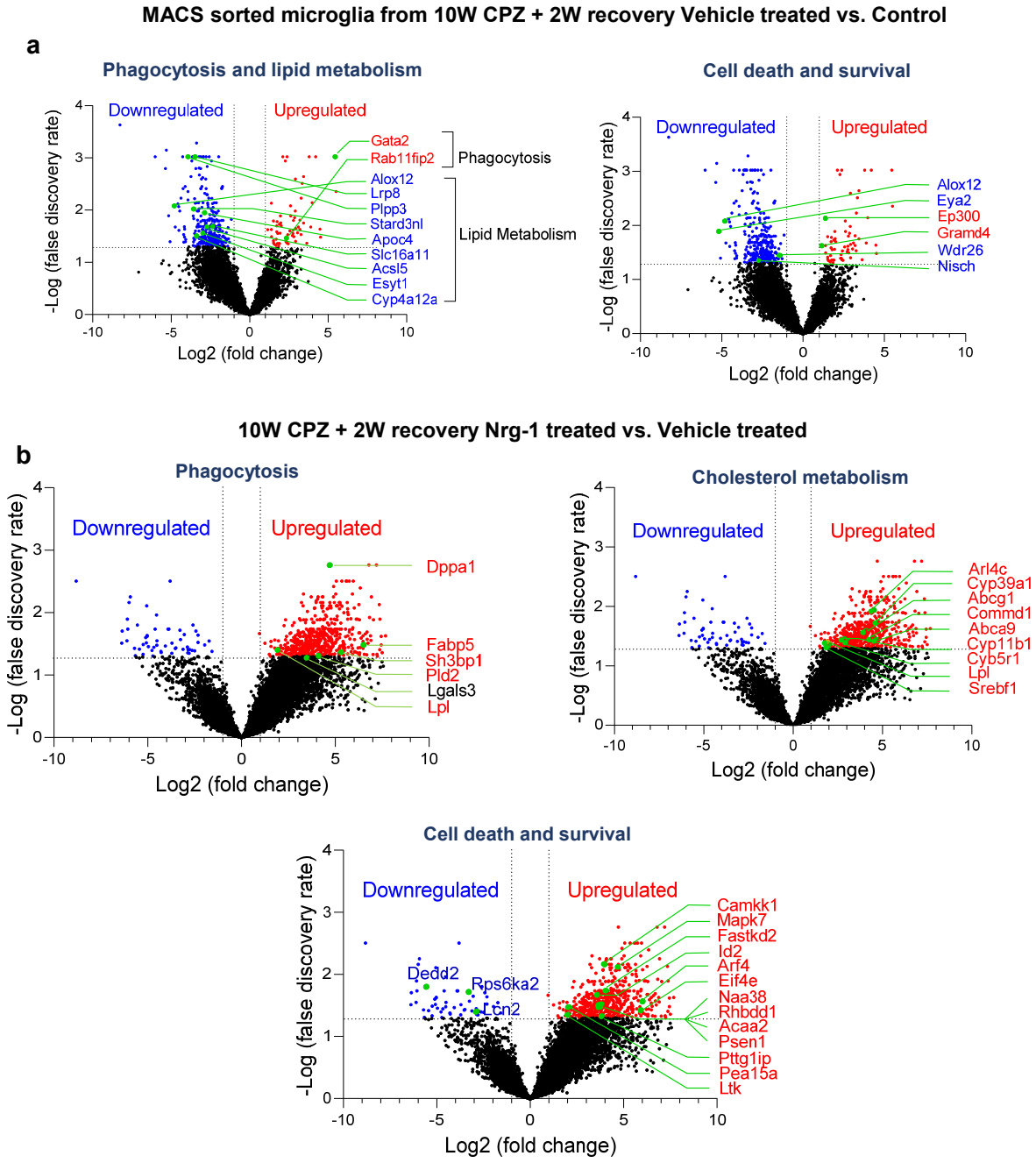


Figure 5: Transcriptomic analysis of microglia from chronic CPZ lesions reveals Nrg-1 treatment promotes microglia genes associated with phagocytosis, lipid and cholesterol metabolism, and cell survival. We isolated microglia by Magnetic Activated Cell Sorting

(MACS) from CPZ mice for RNAseq. A) Volcano plots demonstrate 382 differentially expressed genes (65 upregulated vs. 317 downregulated) in chronic CPZ mice 2 weeks after CPZ withdrawal in comparison to age-matched controls (FDR < 0.05, Benjamini–Hochberg; fold change > 2). These plots show significant differences in expression of genes involved in phagocytosis and cell survival and death. B) Volcano plots showing 619 differentially expressed genes (564 upregulated vs. 55 downregulated) in Nrg-1 treated vs. vehicle treated mice 2 weeks post-CPZ withdrawal (FDR < 0.05, Benjamini–Hochberg; fold change > 2). Nrg-1 treatment was shown to promote expression of phagocytosis and cholesterol metabolism related genes and alleviate expression of genes related to cell death while promoting cell survival related gene expression. N = 3-5 samples/group, each sample is the result of pooling of the brains of 2 mice.

2.4.7 Nrg-1β1 promotes the capacity of microglia for myelin clearance and cholesterol recycling and release through efflux transporters

Based on the findings in our transcriptomic experiments, we next aimed to identify the cellular and molecular mechanisms by which Nrg-1β1 treatment regulate microglia function during remyelination process using relevant *in vitro* platforms. In pure mouse primary cortical microglia cultures, we activated microglia, with lipopolysaccharide (LPS), a well-known pro-inflammatory stimulus, along with interferon-gamma (IFN-γ), an MS pathogenic cytokine. Nrg-1β1 treatment was added to the microglia in both resting and activated (LPS+IFN-γ) states. To assess the effects of Nrg-1β1 on the capacity of microglia for myelin debris phagocytosis, microglia were exposed to myelin particles extracted from YFP mice. Assessment of YFP signal intensity showed that LPS+IFN-γ activated microglia exhibited higher ability for myelin phagocytosis compared to resting microglia after 6 and 24 hours of myelin exposure (1.73 ± 0.15 fold at 6hr and 1.94 ± 0.24

fold at 24 hr) (Fig. 6 A-C, Suppl. Fig. 3 A). Nrg-1β1 treatment significantly promoted myelin phagocytosis of activated microglia (2.98 ± 0.21 fold at 6hr and 3.11 ± 0.13 fold at 24 hr), while it had no significant effect on resting microglia.

To determine whether the elevated level of myelin phagocytosis results in cholesterol accumulation within microglia, we stained microglia cultures under the same conditions with Filipin III (FIII) and ORO, which detect free cholesterol and esterified lipids, respectively (Fig. 6, C). We observed a significant increase in both FIII (4.6 fold) and ORO (2.1 fold) signal intensity in LPS+IFN-γ-activated microglia with Nrg-1β1 treatment after 24 hours of myelin exposure compared to non-treated activated microglia (Fig. 6, D-E). When myelin exposure was prolonged to 72 hours, FIII and ORO staining in activated microglia was higher compared to resting microglia (FIII: 1.74 ± 0.15 fold, ORO: 1.99 ± 0.26 fold) (Suppl. Fig. 3 B). However, in contrast to its promoting effects on cholesterol accumulation at 24 hours, at 72 hours Nrg-1β1-treated activated microglia had comparable FIII and ORO content similar to the levels detected in non-treated activated microglia (Suppl. Fig. 3 B). To examine whether the attenuation of the effects of Nrg-1β1 treatment on cholesterol accumulation over time is a result of enhanced cholesterol efflux, we assessed the expression levels of two key cholesterol efflux transporters, ABCA-1 and ABCG-1, in microglia as well as their cholesterol release to the media. Our quantitative qPCR on microglia for ABCA-1 and ABCG-1 revealed that exposure to myelin fragments triggered an upregulation in ABCA-1 and ABCG-1 expression in resting microglia (1.73 ± 0.14 fold in ABCA-1 and 8.25 ± 0.84 fold in ABCG-1) compared to resting microglia with no myelin exposure (Fig. 6 F). Interestingly, activation with LPS+IFN-γ resulted in a significant downregulation of these transporters in microglia with myelin exposure (36% decrease in ABCA-1 and 90% decrease in ABCG-1) compared to resting microglia exposed to myelin. Importantly, treatment with Nrg-1β1

was able to significantly restore the reduced expression levels of ABCA-1 (1.64 ± 0.18 fold) and ABCG-1 (4.48 ± 0.39 fold) in activated microglia (Fig. 6 F).

Next, we studied cholesterol release by microglia under various conditions. We found a significant elevation in the cholesterol concentration of microglia conditioned media (MCM) across all conditions after 72 hours following myelin exposure, indicating the ability of microglia in processing of cholesterol in myelin debris and its release extracellularly to their microenvironment (Fig. 6 G). Notably, upon activation with LPS+IFN-γ, myelin-exposed microglia exhibited a significant 20% decrease in their cholesterol release. Nrg-1β1 treatment resulted in a significant increase in cholesterol concentration (1.56 fold) in MCM from activated microglia, suggesting a positive role for Nrg-1β1 in enhancing cholesterol recycling and release by microglia during phagocytosis of myelin debris.

To further unravel the molecular mechanisms underlying the effects of Nrg-1β1 on cholesterol transport and release in microglia, we studied two key pathways involved in these processes, the Liver X Receptor (LXR) and the Peroxisome Proliferator-Activated Receptor Gamma (PPARγ). LXR is known to trigger the upregulation of cholesterol transporter genes such as ABCA-1 and ABCG-1, thereby facilitating the removal of intracellular sterols [322]. PPARγ is implicated in lipid metabolism in myeloid cells and can modulate the expression of LXRα, along with other genes involved in fatty acid and cholesterol metabolism [210,211,323]. To verify whether cholesterol excretion in LPS+IFN-γ activated microglia is indeed mediated through the LXR and PPARγ pathways, we functionally blocked these pathways in microglia by GSK2033 and GW9662, respectively. To determine the optimal concentration of these inhibitors, we first conducted a gradient cell toxicity assay (Suppl. Fig. 4 A-C). Our analyses identified 10 μM for GW9662 and 1 μM for GSK2033 as effective concentrations in our experiments. Next qPCR

analyses showed that blockade of LXR and PPARγ significantly suppresses ABCA-1 and ABCG-1 expression in activated microglia with or without Nrg-1β1 treatment, with the LXR antagonist GSK2033 exhibiting a more pronounced effect (Suppl. Fig. 4 D-E). Our functional assessment confirmed the involvement of LXR and PPARγ in Nrg-1 effects on cholesterol release by microglia because the beneficial effects of Nrg-1β1 were abrogated by blocking these pathways (Fig. 6 H). These findings identified that Nrg-1β1 promotes the capacity of microglia for cholesterol release through the LXR and PPAR-γ mediated cholesterol efflux machinery.

We next examined the effects of Nrg-1β1 treatment on microglia lipid load within chronically demyelinated lesions *in vivo.* We studied esterified lipids as an indicator of the accumulation of intracellular myelin-derived cholesterol, a characteristic phenotype of foamy microglia and macrophages [190,196]. Through ORO staining and intensity measurement, we observed a notable and significant increase in the overall levels of esterified lipids within the corpus callosum following 10 weeks of CPZ diet (5.66 ± 0.78 fold) compared to the age-matched healthy mice. During the recovery period, the levels of esterified lipids were decreased over time to a level closer to the control baseline (Fig. 6 I-J). To assess the amount of intracellular myelin-derived cholesterol accumulation per microglia, we normalized the ORO signal intensity to the number of Iba-1+ cells per area in each treatment group (Fig. 6 K). Our findings revealed a significant reduction (72%) in the esterified lipids in microglia following Nrg-1β1 treatment after a 2-week recovery period. This data show that Nrg-1β1 treatment attenuates the intracellular accumulation of lipids and foaminess in microglia within chronic demyelinated lesions.

We further investigated the potential impact of Nrg-1β1 treatment on the cholesterol efflux transporters in chronic demyelinated lesions. Immunohistochemical analysis of ABCA-1 expression levels in CD11b+ microglia revealed that Nrg-1β1 treatment results in a significant

increase (2.06 fold) in ABCA-1 levels per microglia at 2 weeks post-CPZ withdrawal compared to vehicle treated CPZ mice (Fig. 6 L-M). However, no significant differences were observed at the 4-week recovery endpoint. These findings indicate that Nrg-1β1 treatment plays a role in the clearance of myelin debris and modulation of the cholesterol efflux machinery by microglia in chronically demyelinated lesions.

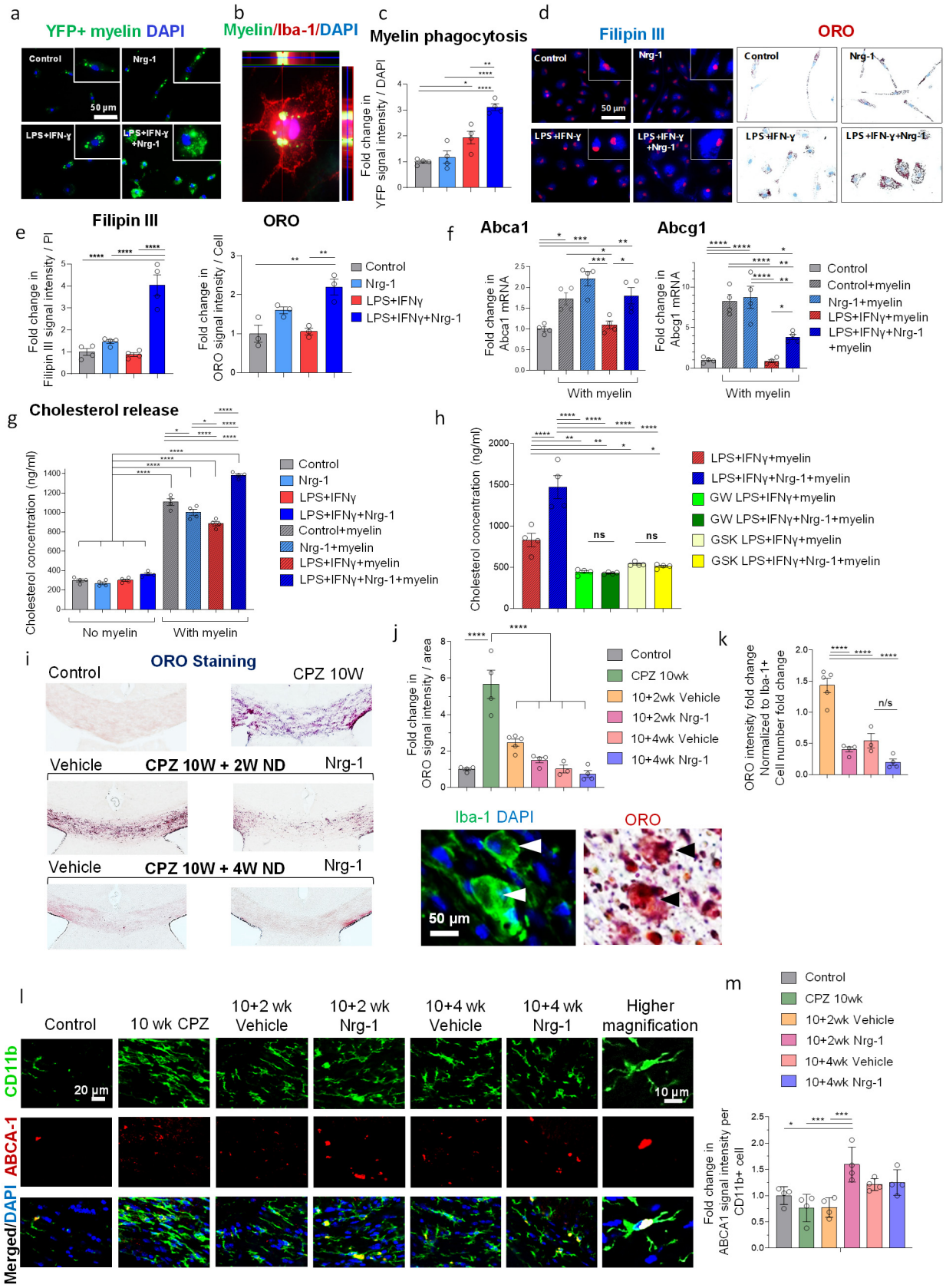
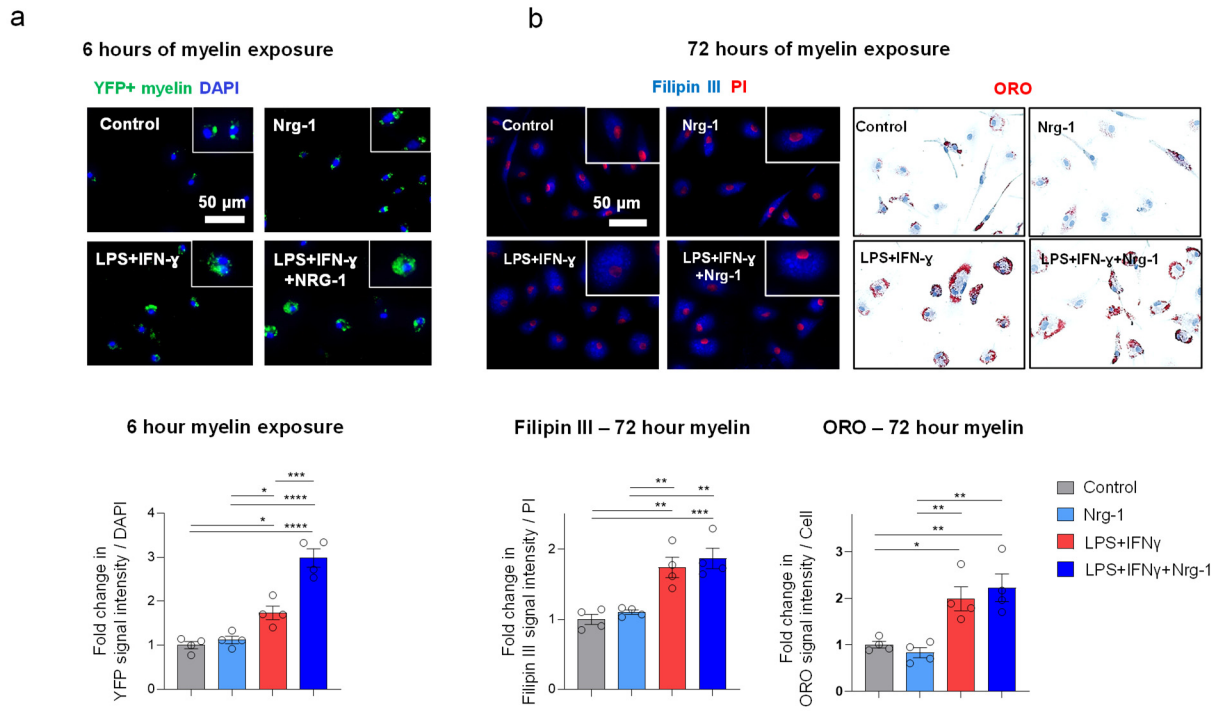

a
YFP+ myelin DAPI
Control
Nrg-1
LPS+IFN-γ
LPS+IFN-γ +Nrg-1
50 μm
b
Myelin/Iba-1/DAPI
c
Myelin phagocytosis
Fold change in YFP signal intensity / DAPI
d
Filipin III
ORO
e
Filipin III
Fold change in Filipin III signal intensity / PI
ORO
Fold change in ORO signal intensity / Cell
Control
Nrg-1
LPS+IFNγ
LPS+IFNγ+Nrg-1
f
Abca1
Fold change in Abca1 mRNA
Abcg1
Fold change in Abcg1 mRNA
With myelin
Control
Control+myelin
Nrg-1+myelin
LPS+IFNγ+myelin
LPS+IFNγ+Nrg-1 +myelin
g
Cholesterol release
Cholesterol concentration (ng/ml)
No myelin
With myelin
Control
Nrg-1
LPS+IFNγ
LPS+IFNγ+Nrg-1
Control+myelin
Nrg-1+myelin
LPS+IFNγ+myelin
LPS+IFNγ+Nrg-1+myelin
h
Cholesterol concentration (ng/ml)
ns
LPS+IFNγ+myelin
LPS+IFNγ+Nrg-1+myelin
GW LPS+IFNγ+myelin
GW LPS+IFNγ+Nrg-1+myelin
GSK LPS+IFNγ+myelin
GSK LPS+IFNγ+Nrg-1+myelin
i
ORO Staining
Control
CPZ 10W
Vehicle
CPZ 10W + 2W ND
Nrg-1
Vehicle
CPZ 10W + 4W ND
Nrg-1
j
Fold change in ORO signal intensity / area
Control
CPZ 10wk
10+2wk Vehicle
10+2wk Nrg-1
10+4wk Vehicle
10+4wk Nrg-1
k
ORO intensity fold change Normalized to Iba-1+ Cell number fold change
n/s
Iba-1 DAPI
ORO
50 μm
l
Control
10 wk CPZ
10+2 wk Vehicle
10+2 wk Nrg-1
10+4 wk Vehicle
10+4 wk Nrg-1
Higher magnification
CD11b
ABCA-1
Merged/DAPI
20 μm
10 μm
m
Control
CPZ 10wk
10+2wk Vehicle
10+2wk Nrg-1
10+4wk Vehicle
10+4wk Nrg-1
Fold change in ABCA1 signal intensity per CD11b+ cell

Figure 6: Nrg-1β1 treatment promotes microglial myelin phagocytosis and myelin-derived cholesterol recycling and release through cholesterol efflux transporters. **A)** Microglia are cultured in 4 different conditions: 1) resting (control), 2) resting treated with Nrg-1β1 (200 ng/ml), 3) LPS+IFN-ɣ treated (activated), 4) LPS+IFN-ɣ+Nrg-1β1 treated. The cells were exposed to YFP+ myelin added to their media. **B)** Z-stack imaging of stimulated microglia (YFP+ myelin: green, Iba-1+ microglia: red), confirming presence of myelin particles inside the phagocytic cell. **C)** After 24 hours, phagocytosis of myelin particle was measured by integrated density of YFP signal (myelin) in microglia. Nrg-1β1 treatment significantly enhanced myelin phagocytosis by activated microglia. **D)** The cells were stained with Filipin III and ORO to detect cellular accumulation of free cholesterol and esterified lipids, respectively. **E)** Nrg-1β1 treatment significantly enhanced accumulation of free cholesterols and esterified lipids in activated microglia that were exposed to myelin for 24 hours. **F)** qPCR for cholesterol efflux channels ABCA-1 and ABCG-1 revealed a reduction in their expression upon cytokine activation of myelin-exposed microglia, and Nrg-1β1 treatment significantly restored the expression of ABCA-1 and ABCG-1. **G)** Quantification of cholesterol levels in microglia conditioned media (MCM) across all treatment conditions revealed a significant rise in cholesterol concentration upon myelin exposure. Activation of myelin-exposed microglia led to a significant decrease in cholesterol release, while treatment with Nrg-1β1 effectively elevated the cholesterol concentration of activated MCM. **H)** Activated microglia were cultured in the presence of PPARγ antagonist GW9662 and LXR antagonist GSK2033 to functionally block upstream pathways to expression of cholesterol efflux channels. The capacity of Nrg-1β1 treatment in enhancing the cholesterol content of MCM was diminished by PPARγ and LXR blockade. **I-J)** ORO signal intensity shows a significant accumulation of esterified lipid within the demyelinated lesion of CC after 10 week of CPZ diet.

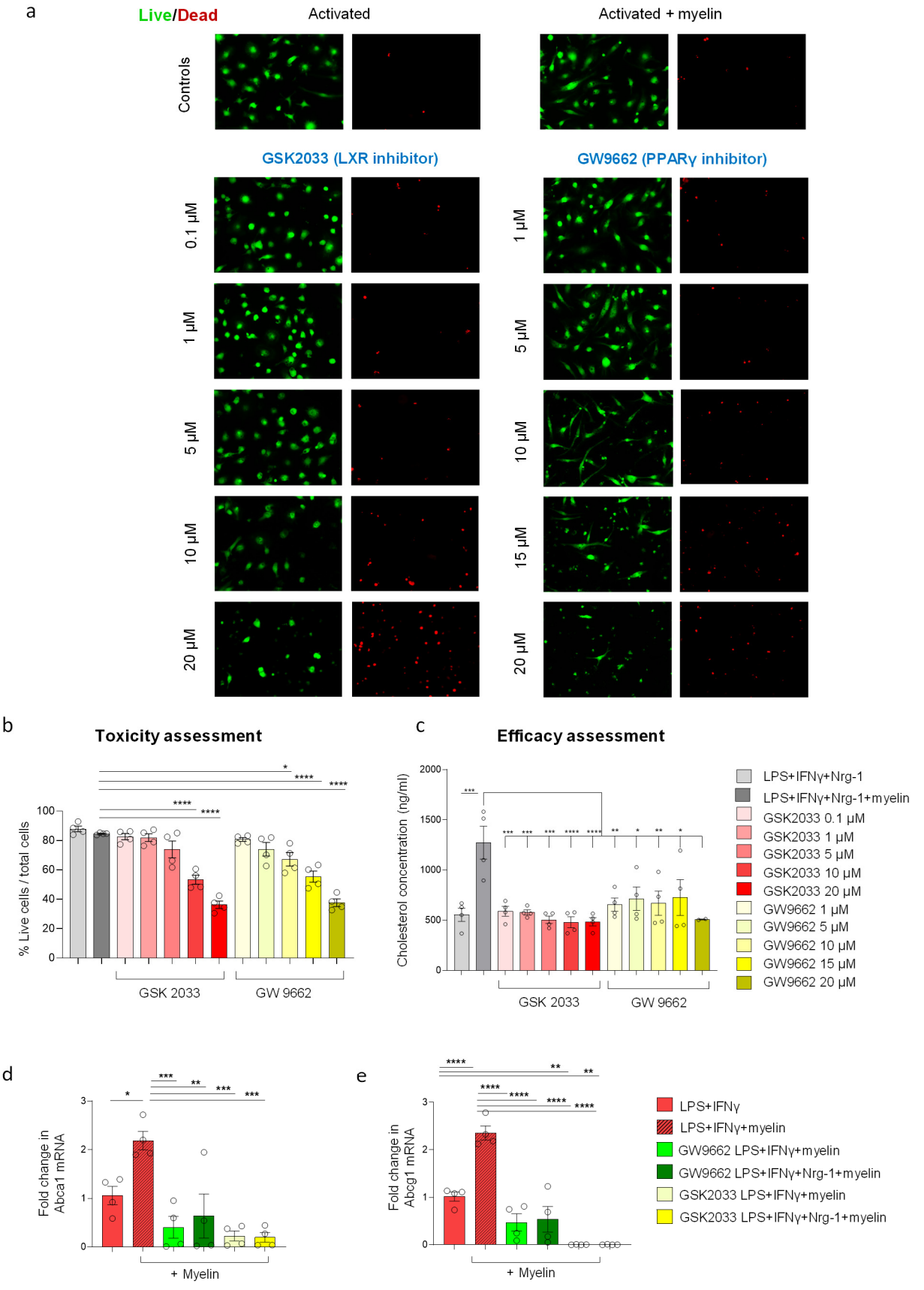
Following CPZ withdrawal, a gradual decrease in the accumulated lipids occurs in both vehicle and Nrg-1β1 treated CPZ mice. **K)** ORO signal intensity was normalized to the total number of Iba-1+ cells within the lesion at each time point to estimate the amount of esterified lipid accumulation per phagocyte. There was a high level of ORO in Iba-1+ cells at 12 weeks, while Nrg-1β1 treatment reduced the intracellular accumulation of esterified lipids. **L-M)** Immunohistochemical analysis of ABCA-1+/ CD11b+ cells showed a significant increase in the microglial levels of this cholesterol efflux transporter within chronic CPZ lesions with Nrg-1β1 treatment at 2 weeks post-CPZ withdrawal compared to vehicle treated mice. The data show the mean ± SEM. **** p<0.0001, ***p<0.001, ** p<0.01, *p<0.05, One-way ANOVA; (A-H) N=3-4 independent experiments/condition, (I-M) N=3-5 mice/group.



Supplementary Figure 3: Effects of Nrg-1β1 on myelin phagocytosis and lipid accumulation

A) Microglia were cultured under 4 specified conditions with 6 hours exposure to YFP+ myelin

particles. We found a significant enhancement of myelin phagocytosis in Nrg-1β1 treated LPS+IFN-γ stimulated microglia. B) Filipin III and ORO staining of activated microglia after 72 hours of myelin exposure showed a significant increase in signal intensity (i.e.: lipid accumulation within the cells). However, no significant increase in these intracellular lipid levels was observed with Nrg-1β1 treatment suggestive of enhance cholesterol release at this time point. The data show the mean ± SEM. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, *$p<0.05$, One-way ANOVA; N=3-4 independent experiments/condition.


a
Live/Dead
Activated
Activated + myelin
Controls
GSK2033 (LXR inhibitor)
GW9662 (PPARγ inhibitor)
0.1 µM
1 µM
5 µM
10 µM
20 µM
1 µM
5 µM
10 µM
15 µM
20 µM
b
Toxicity assessment
% Live cells / total cells
GSK 2033
GW 9662
c
Efficacy assessment
Cholesterol concentration (ng/ml)
GSK 2033
GW 9662
LPS+IFNγ+Nrg-1
LPS+IFNγ+Nrg-1+myelin
GSK2033 0.1 µM
GSK2033 1 µM
GSK2033 5 µM
GSK2033 10 µM
GSK2033 20 µM
GW9662 1 µM
GW9662 5 µM
GW9662 10 µM
GW9662 15 µM
GW9662 20 µM
d
Fold change in Abca1 mRNA
+ Myelin
e
Fold change in Abcg1 mRNA
+ Myelin
LPS+IFNγ
LPS+IFNγ+myelin
GW9662 LPS+IFNγ+myelin
GW9662 LPS+IFNγ+Nrg-1+myelin
GSK2033 LPS+IFNγ+myelin
GSK2033 LPS+IFNγ+Nrg-1+myelin

Supplementary Figure 4: Determining effective concentration of LXR and PPPAR-γ inhibitors in microglia culture. A-B) Functional blockers of LXR and PPAR-γ pathways, GSK2033 and GW9662 were used to verify the effects of Nrg-1β1 treatment on cholesterol efflux in microglia. To determine an effective concentration, a toxicity gradient assessment of these inhibitors using Live/Dead Assay was performed in activated Nrg-1β1 treated microglia after 72 hours of incubation. The results show that the survival rate of microglia was reduced significantly under concentrations above 5 μM for GSK2033 and 10 μM for GW9662. C) For evaluating the efficacy of these inhibitors, MCM from activated myelin-exposed microglia with Nrg-1β1 treatment was incubated with various concentrations of inhibitors for testing cholesterol content. The results showed that the level of cholesterol within the MCM was significantly lowered to baseline levels with all the concentrations used for both inhibitors. D-E) Using qPCR, we also confirmed significant downregulation of ABCA-1 and ABCG-1 genes in microglia after treatment with the selected concentration of GSK2033 and GW9662. The data show the mean ± SEM. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, *$p<0.05$, One-way ANOVA; N=3-4 independent experiments/condition.

2.4.8 Nrg-1β1 treatment promotes microglial integrity through attenuation of necroptosis

Excessive lipid accumulation in microglia by myelin phagocytosis and their impaired ability to process and release cholesterol induce cell death in foamy microglia [108,189,212]. Given availability of Nrg-1β1 treatment attenuated microglia foaminess, we next asked whether it promotes microglia integrity and survival in demyelinating lesions. We first conducted microglia count by co-immunostaining for CD11b and the microglia-specific marker P2y12 in chronic CPZ lesions across all experimental groups (Fig. 7 A-B). Our data confirmed a predominance of

CD11b+P2y12+ cells (indicative of microglia) over CD11b+P2y12- cells (indicative of macrophages) within these lesions at both 2 and 4 weeks post-CPZ withdrawal in vehicle and Nrg-1β1 treatments (Fig. 7 A-B). Interestingly, Nrg-1β1 treatment resulted in a significant increase in the abundance of CD11b+P2y12+ microglia at both 2 weeks (1.58 fold) and 4 weeks (1.44 fold) recovery time points compared to vehicle treatment (Fig. 7 B). The number of CD11b+P2y12- macrophages remained unaffected across all treatment groups and time points.

We next asked whether Nrg-1β1 treatment promoted microglial numbers within chronic demyelinated lesions by mitigating their cell death that is a characteristic of dysfunctional lipid droplet-rich foamy phagocytes [95,189]. Microglial cell death in remyelinating lesions is reported to be mediated through necroptosis [213]. Therefore, we assessed colocalization of CD11b+ microglia with the necroptosis marker RIP3. Our analyses revealed a significant increase (8.53 fold) in the percentage of necroptotic microglia in the corpus callosum of mice after 10 weeks of CPZ demyelination compared to age-matched control mice (Fig. 7 C-D). Importantly, we observed a significantly higher percentage of necroptotic microglia at 2 weeks (39.7% ± 2.4) and 4 weeks (36.3% ± 0.9) post-withdrawal of CPZ compared to 10 weeks of CPZ diet (28.8% ± 1.4). Interestingly, Nrg-1β1 treatment was able to significantly reduce the occurrence of RIP3+/CD11b+ cells at both the 2-week (16.5% ± 1.2) and 4-week (15.6% ± 1.6) recovery periods. These findings show that the presence of Nrg-1β1 attenuates microglial cell death and consequently results in an increase in the number of healthy microglial within chronic lesion, which is critical for the remyelination process.

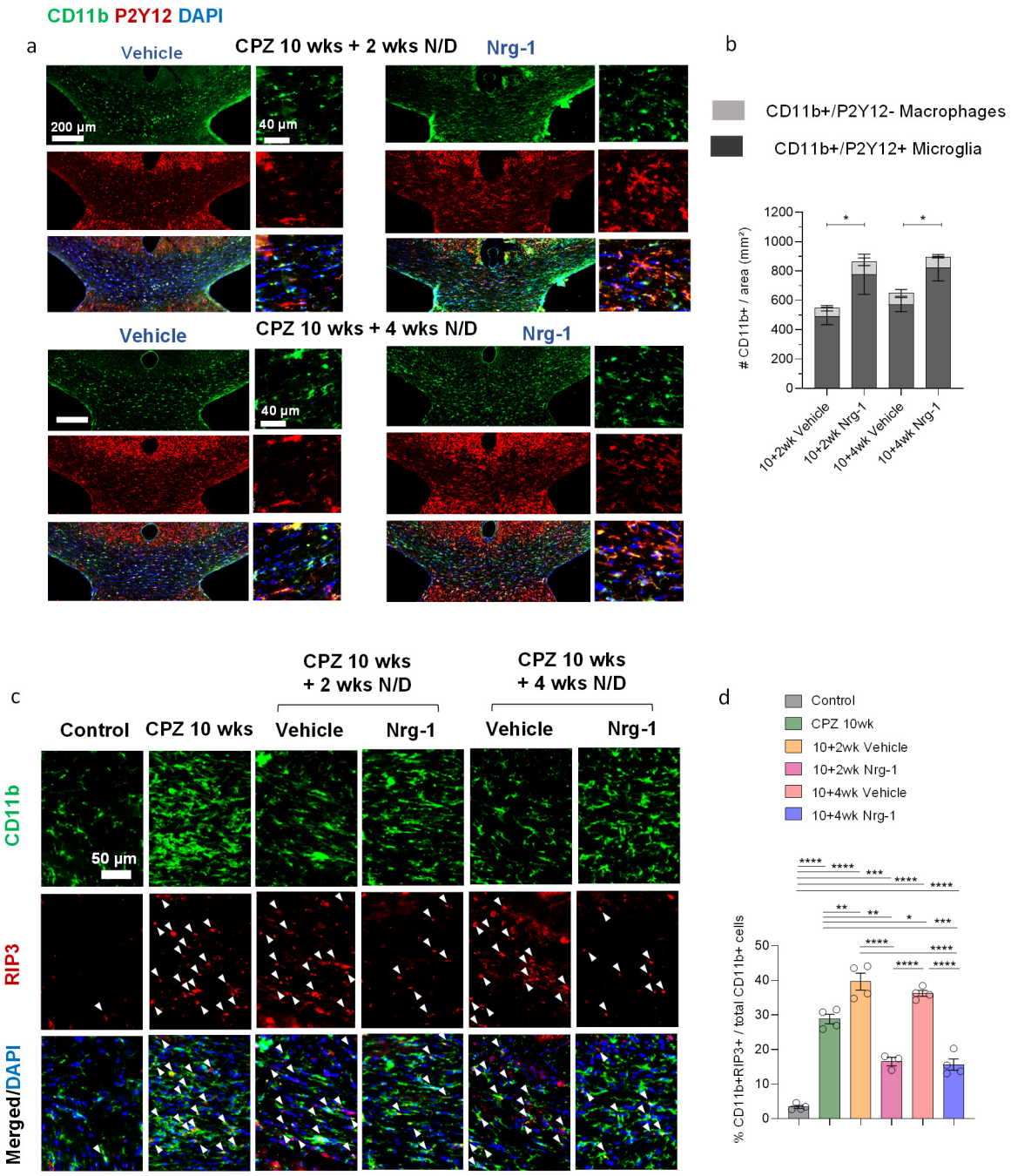


Figure 7: Nrg-1β1 treatment attenuates microglial cell death within chronically demyelinated lesions. A-B) Co-immunostaining of CD11b and P2y12 as a specific marker of microglia shows an increase in the number of microglia with Nrg-1β1 treatment at both 2 and 4 weeks recovery period following CPZ withdrawal. **C)** Assessment of microglia necroptosis within the demyelinated lesion using co-immunolabeling of RIP3 and CD11b reveals a significant

increase in necroptotic microglial cells at 12 and 14 week time points, which was attenuated by Nrg-1β1 treatment. The data show the mean ± SEM. **** p<0.0001, ***p<0.001, ** p<0.01, *p<0.05, (B) Two-way ANOVA, (D) One-way ANOVA; N=3-4 mice/group.

2.4.9 Nrg-1β1 treated microglia promote oligodendrocyte maturation and myelination via secreted factors

We further investigated whether the increased cholesterol efflux by microglia with Nrg-1β1 treatment promotes the capacity of OPCs for differentiation to mature myelinating oligodendrocytes. To this end, we designed a series of *in vitro* experiments in which we exposed murine primary cortical OPCs to MCM from different conditions as described above with or without myelin exposure. To rule out the potential direct effects of any remnant of Nrg-1β1 treatment in MCM on OPC maturation [222], we also included a control condition where OPCs were treated with Nrg-1β1. We first evaluated OPC transition into mature myelinating oligodendrocytes after 14 days in culture. Our analysis showed the transition of OPCs to oligodendrocytes *in vitro* as many cells expressed the oligodendrocyte marker O4 under various conditions (Fig. 8 A-B). Intensity measurement of O4 expression in these cells revealed a significant increase in the maturation level of oligodendrocytes (2.15 ± 0.09 fold) in cultures directly treated with Nrg-1β1 compared to the control cultures. We also found the direct effects of Nrg-1β1 on promoting the number (1.49 ± 0.07 fold) and expression level (3.00 ± 0.24 fold) of myelin basic protein (MBP) compared to the control cultures. These data confirmed the known positive effects of Nrg-1β1 on oligodendrocytes *in vitro* (Fig. 8 A-C). Importantly, our analysis of OPC cultures treated with MCM of LPS+IFN-γ+Nrg-1β1 treated activated microglia exposed to myelin exhibited significantly elevated levels of O4 expression (1.66 ± 0.05 fold) compared to the MCM of

LPS+IFN-γ+ activated microglia exposed to myelin and all other microglia conditions, albeit the number of O4+ cells was comparable (Fig. 8 B). Moreover, MCM of myelin+LPS+IFN-γ+Nrg-1β1 treated microglia significantly enhanced the expression levels of MBP in oligodendrocytes (1.68 fold) compared to the myelin+LPS+IFN-γ condition (Fig. 8 C).

Next, we sought to determine whether activated microglia treated with Nrg-1β1 can promote the myelination capacity of oligodendrocytes in a paracrine manner. To investigate this, we cultured OPCs with MCM from myelin exposed activated microglia with and without Nrg-1β1 treatment, on nanofibrous scaffolds designed to mimic axonal structures and provide a platform for oligodendrocytes to myelinate (Fig. 8 D-E). We maintained oligodendrocytes for 4 weeks to ensure adequate time for maturation and myelination. Our myelination assessment using MBP immunostaining revealed that MCM from Nrg-1β1 treated microglia significantly increased both the number (1.60 fold) and length (2.02 fold) of myelin sheaths per each wrapping oligodendrocyte. These results collectively suggest that the availability of Nrg-1β1 harnesses the potential of activated microglia to promote OPC maturation to myelinating oligodendrocytes through paracrine interactions.

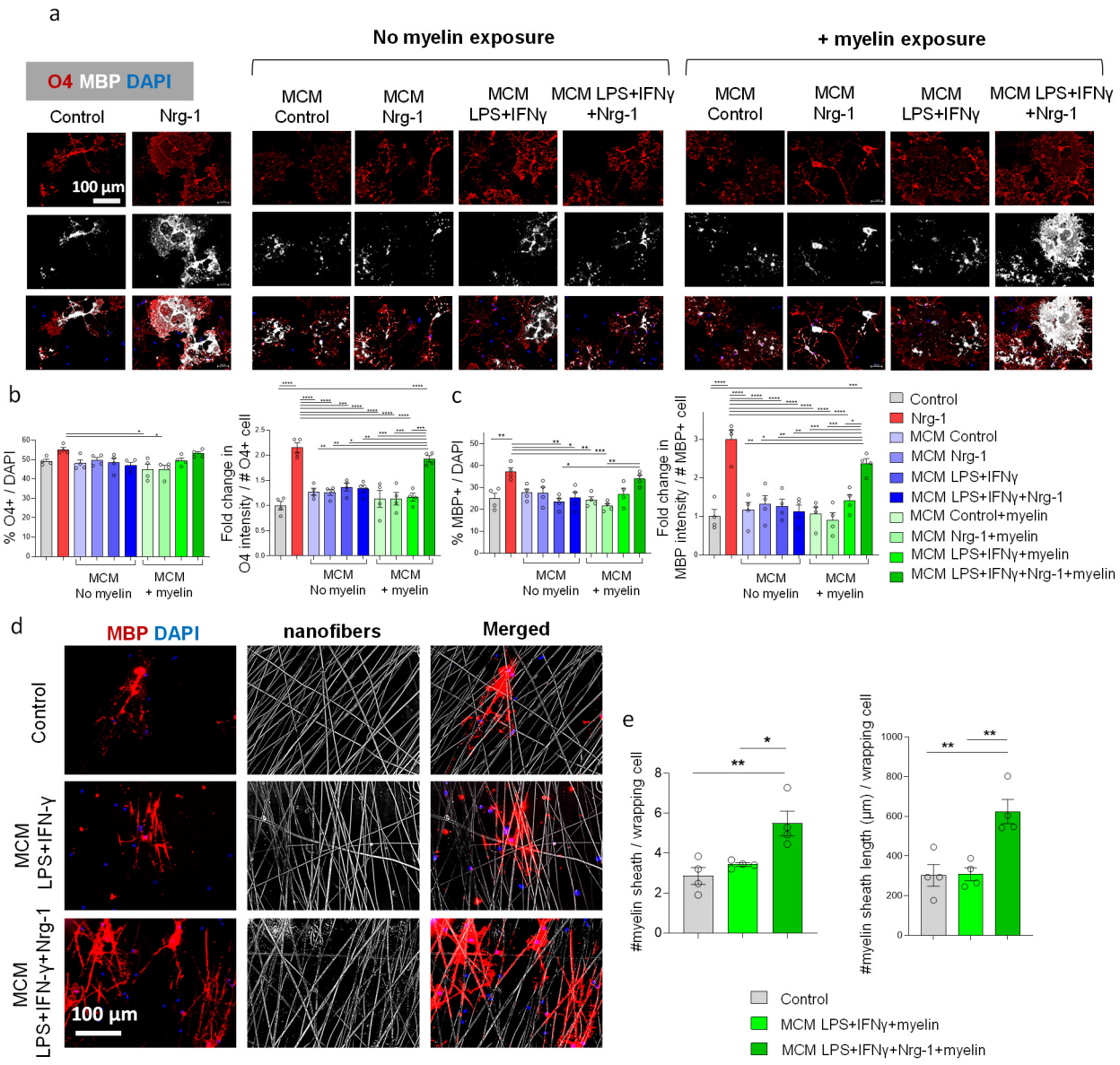


Figure 8: Nrg-1-treated microglia promote OPC maturation and myelination. **A)** OPCs were cultured in the presence of MCM for 14 days to assess their maturation and myelination phenotype using oligodendrocyte markers, O4 and MBP. Control conditions with only OPC media and direct Nrg-1β1 treatment (200ng/ml) were included. **B)** Direct addition of Nrg-1β1 to OPCs cultures resulted in a significant increase in O4 expression level in oligodendrocytes. Interestingly, OPCs cultured in the presence of MCM from activated microglia exposed to myelin and treated with Nrg-1β1 displayed markedly higher O4 expression in oligodendrocytes compared to control or

other MCM treated conditions, although the number of O4+ cells remained unchanged. **C)** Nrg-1β1 treated OPCs showed enhanced maturation, as evidenced by both an increase in the number of MBP+ cells and the expression of MBP. OPCs treated with MCM from activated and myelin-exposed Nrg-1β1-treated microglia showed a higher MBP signal intensity compared to other MCM conditions. **D-E)** OPCs were cultured on electro-spun nanofibrous scaffolds for 4 weeks to evaluate the ability of oligodendrocytes in myelinating the nanofibers. Myelination was assessed by MBP immunostaining. The quantity and length of myelin sheathes per each wrapping oligodendrocyte exhibited a significant increase in cultures treated with MCM from activated and myelin-exposed microglia treated with Nrg-1β1, as compared to MCM from non-Nrg-1 treated microglia. The data show the mean ± SEM. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, *$p<0.05$, One-way ANOVA; N=4 independent experiments/condition.

Chapter III: Discussion

3.1 General overview of findings and discussion

In this study, we demonstrate that spontaneous remyelination in the brain is impaired after a period of prolonged demyelination in mice. Remyelination failure is also a hallmark of chronic lesions in progressive MS, where it is primarily attributed to a maturation arrest of OPCs to myelinating oligodendrocytes [76,84–91]. Using a cuprizone (CPZ) model of chronic demyelination that mimics lesions of progressive MS, and transgenic PDGFRα-Cre mice, we show that remyelination fails despite repopulation of oligodendrocytes lineage cells in these lesions. We provide novel evidence that correlates impaired oligodendrocyte maturation and remyelination with persistent dysregulation of Nrg-1 in chronic demyelinating lesions, a pathology that we had previously reported in human MS lesions [221]. Interestingly, short-term CPZ demyelination also results in downregulation of Nrg-1 in the brain. However, following a recovery period, Nrg-1 expression is restored to its normal levels in acute demyelinated lesions that is associated with successful spontaneous remyelination. Collectively, these new findings identify Nrg-1 dysregulation as an endogenous mechanism that appears to underlie poor remyelination in chronic demyelinated lesions. Therapeutically, we show that restoration of Nrg-1 is sufficient to support remyelination of chronically demyelinated axons by facilitating the transition of newly generated oligodendrocytes into mature myelinating phenotype. Intriguingly, we reveal that Nrg-1β1 exerts its effects largely through modulation of microglia by shifting their phenotype from foamy dysfunctional to a reparative state. Interestingly, adult mice with conditional ablation of Nrg-1 also show spontaneous demyelination and poorer recovery following chronic CPZ-induced demyelination that further corroborates the impact of Nrg-1 dysregulation on remyelination failure in chronic MS lesions.

3.1.1 Nrg-1 treatment and remyelination

Our previous research provided evidence of Nrg-1 dysregulation within the lesions of human secondary progressive MS [221]. Here, we report a similar pathology within demyelinated lesions of chronic CPZ mice. Interestingly, both short-term and chronic CPZ demyelination induce downregulation of Nrg-1 within the affected areas of the brain. However, upon withdrawal of CPZ and following a recovery period, spontaneous remyelination in short-term lesions is correlated with restoration of Nrg-1 expression, while in chronic lesions, poor spontaneous remyelination is accompanied by persistent downregulation of Nrg-1. These findings suggest the importance of Nrg-1 for remyelination and its dysregulation in chronic lesions underlies remyelination failure. Given the similarities between chronic CPZ lesions and progressive MS lesions, this model allowed us to investigate the underlying mechanisms of remyelination failure and to explore potential therapeutic strategies aimed at promoting remyelination in progressive MS.

We report a significant enhancement of remyelination with Nrg-1β1 treatment in the chronic CPZ model of demyelination. We found that the effects of Nrg-1β1 treatment on remyelination is through promoting OPC proliferation and maturation, which is in agreement with our previous studies in which Nrg-1β1 enhanced OPC activities in LPC-induced short-term demyelinated lesions of spinal cord [222]. In this study, we have also confirmed the pro-remyelination effects of Nrg-1β1 treatment in the EAE mouse model that is a more immunologically relevant model of MS. A previous report in EAE also showed that treatment with an isoform of Nrg-1 (recombinant human glial growth factor 2 [rhGGF2]) promotes remyelination and clinical recovery of EAE mice [278]. We and others have also shown that Nrg-1 treatment significantly promotes oligodendrogenesis and Schwann cell mediated remyelination in spinal cord injury and focal demyelination in rodents [222,257,279].

3.1.2 Role of Nrg-1 in regulation of microglia function and survival

In the homeostatic adult brain, OPCs and oligodendrocytes maintain a low-rate level of cholesterol synthesis as they rely partially on other glia for the provision of cholesterol [99,100]. However, in chronic demyelinating lesions other sources such as astrocytes and myelin debris-clearing microglia become less efficient in supplying cholesterol to OPCs and oligodendrocytes contributing to sustained demyelination [95,108,189,190]. Microglia are highly plastic cells capable of adopting diverse phenotypes to perform various functions within the CNS [123,298,319,324]. In MS, microglia within active lesions are typically engaged in either promoting inflammation or resolution of neuroinflammation and facilitating the repair processes [73,147–151,175,320]. Microglia play critical roles in remyelination through clearance of myelin debris by phagocytosis [104,325] and intracellular recycling of myelin derived cholesterol [108,189,190], and supporting oligodendrocytes maturation and myelination [156,318,319] by paracrine secretion of cholesterol as well as growth factors and cytokines such as activing-A [157], IGF1 [326], IL1β [174], and TNFα [171]. Despite their roles as pro-inflammatory mediators, IL1β and TNFα knockout mice have shown an impaired capacity for remyelination, and this has been attributed to disruption in differentiation of OPCs to mature oligodendrocytes [171,174]. In lesions of progressive MS, microglia are abundantly present [195,196,303,304]. However, with aging and chronicity of MS, microglia lose their capacity to support remyelination [95,108,175,189,190]. This inability is attributed to accumulation of intracellular myelin-derived lipids and acquiring a foamy phenotype. The overloading of myelin debris and the burden of myelin processing eventually leads to microglial dysfunction, possibly due to senescence [95,108,189,190,194]. Recently, in a genetic model of remyelination failure, an upregulation in the lipid binding and lipid metabolism related genes, as well as an accumulation of neutral lipids were observed within microglia, further supporting the important role of microglial lipid metabolism in

successful remyelination [327]. Here, we show the abundance of lipid-laden dysfunctional foamy microglia within chronic lesions of human secondary progressive MS, as well as the brain of chronic CPZ mice. This is in agreement with previous studies that showed the existence of these cholesterol-laden foamy phagocytes within active MS lesions and in the rim of chronic mixed active/inactive lesions (chronic active lesions) of MS, both of which are present in the progressive phase of the disease [195,196,303,304].

We provide novel evidence that downregulation of Nrg-1 is an underlying mechanism that drive microglia dysfunction. Mechanistically, our transcriptomics, protein and functional studies collectively suggest that microglia within the corpus callosum of Nrg-1 treated CPZ mice demonstrate a pro-remyelination profile characterized by enhanced myelin phagocytosis, cholesterol metabolism and efflux that consequently protect microglia from necroptosis. This restoration of cholesterol recycling and excretion with Nrg-1 treatment likely underpins the reduction in foamy microglia in chronic CPZ lesions and enhanced remyelination that we have observed in this study. Recent evidence shows that an increase in the intracellular levels of cholesterol precursors and derivatives following myelin internalization activates LXR pathway, a key regulator of lipid homeostasis [95,108]. Activation of LXR as well as PPAR- γ pathways is shown to upregulate the expression of cholesterol efflux transporters such as ABCA-1 and ABCG-1 that can facilitate the removal of excess cholesterol and preventing the formation of toxic lipid droplets [108,210,211,328–330]. However, as MS lesions progress to a chronic state, microglia accumulate cholesterol and are laden with lipid droplets that form cholesterol crystals [108,187,189,190]. Formation of these foamy phagocytes is shown to be the result of incomplete recycling of lipids from the internalized myelin debris, partly due to insufficient LXR activation [95,108,189,190,331]. Our *in vitro*

findings also confirm an increase in phagocytosis of myelin debris along with a defective cholesterol efflux in pro-inflammatory primary microglia compared to homeostatic microglia.

We unravel that elevated cholesterol excretion under Nrg-1 treatment is achieved by restoration of microglial expression of cholesterol efflux transporters via modulation of LXR and PPAR-γ pathways. It has been recently shown that promoting the activity of ABCA-1 efflux transporters, or LXR pathway in general, can enhance remyelination of demyelinated lesions [215,332]. Previous studies in the cardiovascular system have also shown that Nrg-1 treatment upregulates ABCA-1 cholesterol transporter and cholesterol efflux in macrophages that attenuate formation of foamy macrophages and atherosclerosis [333]. The role of LXR and PPAR-γ in modulating microglial phenotype extends beyond cholesterol metabolism. These pathways also have anti-inflammatory roles through lowering the expression levels of pro-inflammatory cytokines via trans-repression of target genes of nuclear factor kappa B (NF-κB) [334–336]. Interestingly, we previously demonstrated that Nrg-1 treatment supports a phenotype shift in microglia and macrophages from pro-inflammatory to neuroprotective and pro-regenerative states in EAE, acute lysolecithin-induced demyelination, and spinal cord injury models [221–223]. Thereby, we propose that by activating the LXR and PPAR-γ pathways, Nrg-1 not only augments cholesterol efflux but also shifts microglia inflammatory profile towards a phenotype conducive to tissue repair and remyelination. This dual effect was indeed observed in our *in vitro* experiments, where the conditioned media collected from activated microglia treated with Nrg-1 promoted maturation of OPCs and supported myelination by the resulting oligodendrocytes. Altogether, presence of foamy microglia is not only a marker of chronic demyelination but also contributes to sustained neuroinflammation [95,108,189,190,331]. Overall, these findings underscore the therapeutic

potential of Nrg-1 for remyelination of chronically demyelinated white matter in MS by reversing microglia dysfunction.

Although our *in vivo* and *in vitro* findings established microglia-mediated mechanisms for the beneficial effects of Nrg-1 treatment on remyelination, we cannot rule out its potential effects on astrocytes and their role in promoting remyelination in chronic CPZ lesions. Recent research has demonstrated that astrocytic downregulation of cholesterol biosynthesis pathways can lead to remyelination failure [337]. Our previous studies also established that Nrg-1 treatment modulates astrocyte reactivity and attenuates astrogliosis including expression of chondroitin sulfate proteoglycans (CSPGs) [267] that are known to inhibit remyelination [284,285]. Nonetheless, while astrocyte-mediated effects of Nrg-1 on remyelination is plausible, our current data from chronic CPZ lesions suggest a prominent role for microglia as PLX5622 largely reversed Nrg-1β1 beneficial effects. Moreover, our lesion analysis showed the abundant presence of foamy microglia within the chronic demyelinating CPZ lesions that was significantly attenuated by Nrg-1 availability suggesting microglia appear to be a main target for Nrg-1 treatment.

Remarkably, Nrg-1 treatment reduces microglial necroptosis, leading to an increase in microglial numbers within remyelinating lesions. Recent evidence suggests that necroptosis is a main pathway causing inflammatory microglial cell death in demyelinated lesions [213]. Necroptosis is mediated by receptor-interacting protein kinase 1 and 3 (RIPK1, RIPK3) and mixed lineage kinase domain-like protein (MLKL), and is associated with inflammation and cell lysis [214]. Blocking necroptosis through inhibition of RIPK1 has been shown to modulate neuroinflammation and promote remyelination [338,339]. Interestingly, a recent study on skin flap transplantations has shown that Nrg-1 treatment successfully inhibited necroptosis and pyroptosis in ischemic flaps [340]. This effect was through inhibition of stimulator of interferon genes (STING) activity, a protein

known to be involved in secretion of several pro-inflammatory cytokines, by AKT phosphorylation, a well-known downstream pathway for Nrg-1 signaling [224,340,341]. Activation of Akt through Nrg-1 signaling is also shown to prevent neurotoxicity [342,343]. In the present study, reduction in necroptotic microglia with Nrg-1 treatment suggests the availability of Nrg-1 is important for preserving microglial health and function, and thereby provides a supportive environment for remyelination.

3.1.3 Ablation of Nrg-1 signaling and its role in OPC maturation and remyelination

Our Nrg-1 knockout studies show that endogenous Nrg-1 plays a crucial role in myelination and maintaining myelin integrity. We found that conditional ablation of Nrg-1 in adult mice results in lower myelination in the brain and myelin thinning over time, and when these mice were challenged with chronic CPZ demyelination, they show an even lower degree of remyelination in the absence of Nrg-1 signaling, which could be rescued by exogenous Nrg-1 in chronic CPZ lesions of these mice. An early study also showed that a decrease in Nrg-1 type III expression in Nrg-1 $^{+/-}$ heterozygote mice leads to hypo-myelination in the peripheral nervous system (PNS) [250]. Another evidence from earlier studies also showed deficiency of Bace1, an enzyme essential for Nrg-1 processing and ligand release, leads to disruption of myelination in both PNS and CNS of Bace1 *null* mice [232,233]. Moreover, Nrg-1 signaling through interactions with its receptors ErbB2, 3, and 4 plays important roles in maturation of Schwann cells [224,344] and oligodendrocytes [222,257,262,345]. Although these developmental studies suggest the importance of endogenous Nrg-1 signaling in CNS myelination, the direct role of Nrg-1 in oligodendrocyte development and myelination is still controversial. While removal of oligodendrocytic Nrg-1 signaling through knockout of Nrg-1 or its receptors is shown to result in their maturation arrest

and hypomyelination of the CNS [253,345], studies by Brinkmann et al. revealed that knockout of Nrg-1/ErbB signaling from oligodendrocytes did not have any effects on CNS myelination, while overexpression of Nrg-1 resulted in hypermyelination of the CNS [263]. Of note, the majority of these studies are in developmental models. Here, in adult mice, we show that knockout of Nrg-1 does not affect the abundance of oligodendrocytes lineage within the brain white matter; however, it eventually leads to disruption in myelination. More specifically, our data suggest that the maturation of oligodendrocytes is negatively impacted by Nrg-1 ablation. Of note, in the adult CNS, Nrg-1 is primarily expressed by neurons and is axonally localized in white matter and to some extent is expressed by oligodendrocytes [224]. Overall, further studies using oligodendrocyte and neuron specific Nrg-1 knockout are required to deconstruct the ramifications of Nrg-1 ablation from each cell type. Nonetheless, our current study in chronic CPZ lesions and previous studies in EAE and MS white matter lesions[37], Nrg-1 is depleted from both axons and oligodendrocytes, which is recapitulated by the knockout model used in this study.

3.2. Study limitations

A limitation in this study, similar to other animal studies for MS, is the differences in pathophysiology of these models with that of MS [32]. MS pathogenesis involves an orchestrated auto-immune response to myelin proteins, specifically mediated by helper and cytotoxic T cells and involvement of B cells, and a simultaneous crucial contribution from innate immunity [19]. However, in CPZ animal model, the major cause of demyelination is the toxic effects of CPZ on oligodendrocytes, that is followed by a robust immune response from CNS innate immunity [41,42]. Despite this limitation, the chronic CPZ model mimics a number of important features of chronic lesions typical of progressive MS, which we focus on in this project, including a similar decline in

the capacity for spontaneous remyelination, presence of axonal damage and a neurodegenerative condition, dominance of innate immune response compared to adaptive immune response, abundance of lipid-laden dysfunctional foamy phagocytes, and as we found in this study, a persistent dysregulation in Nrg-1 expression within the lesion [21,46,47]. These characteristics deems the chronic CPZ model the most suitable choice for this study, as other widely used models have their own limitations.

Although our *in vivo* and *in vitro* assays established microglia-mediated mechanisms for the beneficial effects of Nrg-1 treatment on remyelination, we cannot rule out its potential effects on astrocytes and their role in promoting remyelination in chronic CPZ lesions. Recent research has demonstrated that astrocytic downregulation of cholesterol biosynthesis pathways can lead to remyelination failure [337]. Our previous studies have shown that Nrg-1 treatment modulates astrocyte reactivity and attenuates astrogliosis including expression of chondroitin sulfate proteoglycans (CSPGs) [267] that are known to inhibit remyelination . Nonetheless, while astrocytes mediated effects of Nrg-1 on remyelination is plausible, our data from 12 weeks CPZ study suggest a prominent role for microglia as PLX5622 largely reversed Nrg-1β1 beneficial effects. Moreover, our lesion analysis showed the abundant presence of foamy microglia within the chronic demyelinating CPZ lesions suggesting microglia appear to be a main target for Nrg-1.

Another limitation of this study is the potential off-target effects of PLX5622, which was used for microglial depletion [346]. Recent findings suggest that PLX5622 may have off-target effects on the cholesterol metabolism of endothelial cells within the CNS [347]. However, our study primarily focused on remyelination within white matter lesions of the corpus callosum, and endothelial cholesterol metabolism has not been reported to play a significant role in this process. While this

minimizes the potential impact of these off-target effects on our findings, it remains an important consideration when interpreting the results.

A technical limitation with this study is that our transcriptomic data does not distinguish different populations of microglia within the normal CNS and the chronic demyelinating lesions. Despite the specificity of the data for microglial population, this transcriptomic data is the result of pooling all the distinct microglia phenotypes [298,300]. As a result of this, it is not possible to precisely define which populations of microglia are influenced by Nrg-1 treatment.

A minor technical limitation in this study is the strategy to stimulate microglia *in vitro*, where we used a combination of LPS as an inflammatory stimulus and IFNγ as an inflammatory cytokine. While IFNγ is one of the most important and disease-relevant inflammatory cytokines implicated in MS pathogenesis, LPS is not directly involved in the disease process. However, due to the strong inflammatory response that LPC induces in microglia and macrophages, it is frequently used in the literature for *in vitro* activation of microglia, in combination with other pro-inflammatory cytokines, to recapitulate the inflammatory microenvironment of MS [108,190].

3.3. Conclusion:

In conclusion, our study unravels that dysregulation of Nrg-1 underpins remyelination failure in chronic demyelinated lesions. Restoration of Nrg-1 levels facilitates remyelination of chronically demyelinated white matter by modulating the dysfunctional myelin-derived lipid-laden foamy microglia and shifting their phenotype towards a reparative state by promoting lipid metabolism and cholesterol efflux. Given the abundance of microglia in chronic MS lesions, therapeutic restoration of their functions provides an exciting opportunity for MS treatment.

3.4 Clinical implications and translational impact of this study

From a clinical point of view, many people with MS eventually transition to a progressive form of the disease in which disabilities accumulate due to impaired remyelination. There are currently minimal treatment options available to successfully support remyelination in chronic lesions and alleviate neurodegeneration and the burden of progressive MS [295]. Our study reveals a potential link between Nrg-1 dysregulation and MS progression, which opens a new avenue for further investigations. Nrg-1 is also known as a susceptibility gene in schizophrenia and bipolar disorder, where a group of affected individuals show abnormalities within white matter [225–227,348–350]. Therapeutically, Nrg-1 peptide therapy is approved by the US Food and Drug Agency (NCT03388593) for Phase II/III trials in cardiac repair [351] indicating its safety for human use. Nrg-1β1 also freely passes the blood-CNS-barrier through receptor mediated processes [321] in which is a major consideration for pharmacological based therapies for the CNS. Hence, Nrg-1 shows high translational potential as a disease relevant therapeutic target for progressive MS.

References:

1. Jakimovski, D. *et al.* Multiple sclerosis. *The Lancet* vol. 403 183–202 (2024).

2. Wallin, M. T. & Culpepper, W. J. Global, regional, and national burden of multiple sclerosis 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016 GBD 2016 Multiple Sclerosis Collaborators*. *Artic. Lancet Neurol* **18**, 269–85 (2019).

3. Baranzini, S. E. & Oksenberg, J. R. The Genetics of Multiple Sclerosis: From 0 to 200 in 50 Years. *Trends in Genetics* vol. 33 960–970 (2017).

4. Patsopoulos, N. A. *et al.* Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science (80-.).* **365**, (2019).

5. Dunn, S. E., Lee, H., Pavri, F. R. & Zhang, M. A. Sex-based differences in multiple sclerosis (Part I): Biology of disease incidence. in *Current Topics in Behavioral Neurosciences* vol. 26 29–56 (Springer, Cham, 2015).

6. Tomassini, V. *et al.* Sex hormones modulate brain damage in multiple sclerosis: MRI evidence. *J. Neurol. Neurosurg. Psychiatry* **76**, 272–275 (2005).

7. Bansil, S., Lee, H. J., Jindal, S., Holtz, C. R. & Cook, S. D. Correlation between sex hormones and magnetic resonance imaging lesions in multiple sclerosis. *Acta Neurol. Scand.* **99**, 91–94 (1999).

8. Golden, L. C. *et al.* Parent-of-origin differences in DNA methylation of X chromosome genes in T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 26779–26787 (2019).

9. Jacobs, B. M., Noyce, A. J., Giovannoni, G. & Dobson, R. BMI and low vitamin D are causal factors for multiple sclerosis: A Mendelian Randomization study. *Neurol.*

Neuroimmunol. NeuroInflammation **7**, (2020).

10. Lanz, T. V. *et al.* Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. *Nat. 2022 6037900* **603**, 321–327 (2022).

11. Bjornevik, K. *et al.* Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. *Science (80-.).* **375**, 296–301 (2022).

12. Prosperini, L. *et al.* Shift of multiple sclerosis onset towards older age. *J. Neurol. Neurosurg. Psychiatry* **93**, 1137–1139 (2022).

13. Bebo, B. *et al.* The Economic Burden of Multiple Sclerosis in the United States: Estimate of Direct and Indirect Costs. *Neurology* **98**, E1810–E1817 (2022).

14. Lublin, F. D. *et al.* Defining the clinical course of multiple sclerosis: The 2013 revisions. *Neurology* **83**, 278–286 (2014).

15. Klineova, S. & Lublin, F. D. Clinical course of multiple sclerosis. *Cold Spring Harb. Perspect. Med.* **8**, (2018).

16. Makhani, N. & Tremlett, H. The multiple sclerosis prodrome. *Nature Reviews Neurology* vol. 17 515–521 (2021).

17. Okuda, D. T. *et al.* Incidental MRI anomalies suggestive of multiple sclerosis: The radiologically isolated syndrome. *Neurology* **72**, 800–805 (2009).

18. Miller, D., Barkhof, F., Montalban, X., Thompson, A. & Filippi, M. Clinically isolated syndromes suggestive of multiple sclerosis, part I: Natural history, pathogenesis, diagnosis, and prognosis. *Lancet Neurology* vol. 4 281–288 (2005).

19. Baecher-Allan, C., Kaskow, B. J. & Weiner, H. L. Multiple Sclerosis: Mechanisms and

Immunotherapy. *Neuron* vol. 97 742–768 (2018).

20. Griffiths, I. *et al.* Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science* **280**, 1610–1613 (1998).

21. Franklin, R. J. M. & Ffrench-Constant, C. Remyelination in the CNS: From biology to therapy. *Nature Reviews Neuroscience* vol. 9 839–855 (2008).

22. Nave, K. A. Myelination and support of axonal integrity by glia. *Nature* vol. 468 244–252 (2010).

23. Tillema, J. M. & Pirko, I. Neuroradiological evaluation of demyelinating disease. *Ther. Adv. Neurol. Disord.* **6**, 249 (2013).

24. Yong, H. Y. F. & Yong, V. W. Mechanism-based criteria to improve therapeutic outcomes in progressive multiple sclerosis. *Nature Reviews Neurology* vol. 18 40–55 (2022).

25. X, M. *et al.* Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *N. Engl. J. Med.* **376**, 30–31 (2017).

26. Sorensen, P. S. & Blinkenberg, M. The potential role for ocrelizumab in the treatment of multiple sclerosis: Current evidence and future prospects. *Therapeutic Advances in Neurological Disorders* vol. 9 44–52 (2016).

27. Margoni, M., Preziosa, P., Tortorella, P., Filippi, M. & Rocca, M. A. Does Ocrelizumab Limit Multiple Sclerosis Progression? Current Evidence from Clinical, MRI, and Fluid Biomarkers. *Neurotherapeutics* **19**, 1216 (2022).

28. Kappos, L. *et al.* Siponimod versus placebo in secondary progressive multiple sclerosis

(EXPAND): a double-blind, randomised, phase 3 study. *Lancet (London, England)* **391**, 1263–1273 (2018).

29. Cree, B. A. C. *et al.* Siponimod: Disentangling disability and relapses in secondary progressive multiple sclerosis. *Mult. Scler.* **27**, 1564–1576 (2021).

30. Lassmann, H. & van Horssen, J. Oxidative stress and its impact on neurons and glia in multiple sclerosis lesions. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1862**, 506–510 (2016).

31. Faissner, S. *et al.* Systematic screening of generic drugs for progressive multiple sclerosis identifies clomipramine as a promising therapeutic. *Nat. Commun.* **8**, (2017).

32. Ransohoff, R. M. Animal models of multiple sclerosis: The good, the bad and the bottom line. *Nat. Neurosci.* **15**, 1074–1077 (2012).

33. 'T Hart, B. A. *et al.* Histopathological Characterization of Magnetic Resonance Imaging-Detectable Brain White Matter Lesions in a Primate Model of Multiple Sclerosis: A Correlative Study in the Experimental Autoimmune Encephalomyelitis Model in Common Marmosets (Callithrix jacchus). *Am. J. Pathol.* **153**, 649–663 (1998).

34. Dal Canto, M. C., Melvold, R. W., Kim, B. S. & Miller, S. D. Two models of multiple sclerosis: Experimental allergic encephalomyelitis (EAE) and theiler's murine encephalomyelitis virus (TMEV) infection. A pathological and immunological comparison. *Microsc. Res. Tech.* **32**, 215–229 (1995).

35. Mokhtarian, F., McFarlin, D. E. & Raine, C. S. Adoptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. *Nature* **309**,

356–358 (1984).

36. Zamvil, S. *et al.* T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. *Nature* **317**, 355–358 (1985).

37. Jarjour, A. A., Zhang, H., Bauer, N., Ffrench-Constant, C. & Williams, A. In vitro modeling of central nervous system myelination and remyelination. *GLIA* vol. 60 1–12 (2012).

38. Hall, S. M. The effect of injections of lysophosphatidyl choline into white matter of the adult mouse spinal cord. *J. Cell Sci.* **10**, 535–546 (1972).

39. Woodruff, R. H. & Franklin, R. J. M. Demyelination and remyelination of the caudal cerebellar peduncle of adult rats following stereotaxic injections of lysolecithin, ethidium bromide, and complement/anti-galactocerebroside: A comparative study. *Glia* **25**, 216–228 (1999).

40. Plemel, J. R. *et al.* Mechanisms of lysophosphatidylcholine-induced demyelination: A primary lipid disrupting myelinopathy. *Glia* **66**, 327–347 (2018).

41. Torkildsen, Brunborg, L. A., Myhr, K. M. & Bø, L. The cuprizone model for demyelination. *Acta Neurologica Scandinavica* vol. 117 72–76 (2008).

42. Bénardais, K. *et al.* Cuprizone [bis(cyclohexylidenehydrazide)] is selectively toxic for mature oligodendrocytes. *Neurotox. Res.* **24**, 244–250 (2013).

43. McTigue, D. M. & Tripathi, R. B. The life, death, and replacement of oligodendrocytes in the adult CNS. *J. Neurochem.* **107**, 1–19 (2008).

44. Thorburne, S. K. & Juurlink, B. H. J. Low glutathione and high iron govern the

susceptibility of oligodendroglial precursors to oxidative stress. *J. Neurochem.* **67**, 1014–1022 (1996).

45. Zirngibl, M., Assinck, P., Sizov, A., Caprariello, A. V. & Plemel, J. R. Oligodendrocyte death and myelin loss in the cuprizone model: an updated overview of the intrinsic and extrinsic causes of cuprizone demyelination. *Mol. Neurodegener. 2022 171* **17**, 1–28 (2022).

46. Hibbits, N., Pannu, R., Wu, T. J. & Armstrong, R. C. Cuprizone demyelination of the corpus callosum in mice correlates with altered social interaction and impaired bilateral sensorimotor coordination. *ASN Neuro* **1**, 153–164 (2009).

47. Praet, J., Guglielmetti, C., Berneman, Z., Van der Linden, A. & Ponsaerts, P. *Cellular and molecular neuropathology of the cuprizone mouse model: Clinical relevance for multiple sclerosis*. vol. 47 485–505 (Pergamon, 2014).

48. Dal Canto, M. C., Kim, B. S., Miller, S. D. & Melvold, R. W. Theiler's murine encephalomyelitis virus (TMEV)-induced demyelination: A model for human multiple sclerosis. *Methods A Companion to Methods Enzymol.* **10**, 453–461 (1996).

49. Oleszak, E. L., Chang, J. R., Friedman, H., Katsetos, C. D. & Platsoucas, C. D. Theiler's Virus Infection: a Model for Multiple Sclerosis. *Clin. Microbiol. Rev.* **17**, 174 (2004).

50. Readhead, C. & Hood, L. The dysmyelinating mouse mutations shiverer (shi) and myelin deficient (shimld). *Behav. Genet.* **20**, 213–234 (1990).

51. Dedoni, S. *et al.* An overall view of the most common experimental models for multiple sclerosis. *Neurobiology of Disease* vol. 184 106230 (2023).

52. Monje, M. Myelin Plasticity and Nervous System Function. *Annu. Rev. Neurosci.* **41**, 61–76 (2018).

53. Yalçın, B. & Monje, M. Microenvironmental interactions of oligodendroglial cells. *Dev. Cell* **56**, 1821–1832 (2021).

54. Jahn, O., Tenzer, S. & Werner, H. B. Myelin proteomics: Molecular anatomy of an insulating sheath. *Molecular Neurobiology* vol. 40 55–72 (2009).

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

56. Saab, A. S. *et al.* Oligodendroglial NMDA Receptors Regulate Glucose Import and Axonal Energy Metabolism. *Neuron* **91**, 119–132 (2016).

57. Bercury, K. K. & Macklin, W. B. Dynamics and mechanisms of CNS myelination. *Dev. Cell* **32**, 447–458 (2015).

58. Vincze, A., Mázló, M., Seress, L., Komoly, S. & Ábrahám, H. A correlative light and electron microscopic study of postnatal myelination in the murine corpus callosum. *Int. J. Dev. Neurosci.* **26**, 575–584 (2008).

59. Liu, J. *et al.* Impaired adult myelination in the prefrontal cortex of socially isolated mice. *Nat. Neurosci.* **15**, 1621–1623 (2012).

60. Xiao, Y. & Czopka, T. Myelination-independent functions of oligodendrocyte precursor cells in health and disease. *Nat. Neurosci.* **26**, 1663–1669 (2023).

61. Huang, J. K. *et al.* Myelin Regeneration in Multiple Sclerosis: Targeting Endogenous Stem Cells. *Neurotherapeutics* vol. 8 650–658 (2011).

62. Xing, Y. L. *et al.* Adult neural precursor cells from the subventricular zone contribute significantly to oligodendrocyte regeneration and remyelination. *J. Neurosci.* **34**, 14128–14146 (2014).

63. Lubetzki, C. & Stankoff, B. Demyelination in multiple sclerosis. in *Handbook of Clinical Neurology* vol. 122 89–99 (Elsevier, 2014).

64. Neumann, H., Medana, I. M., Bauer, J. & Lassmann, H. Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends in Neurosciences* vol. 25 313–319 (2002).

65. Sun, D. *et al.* Myelin Antigen-Specific CD8+ T Cells Are Encephalitogenic and Produce Severe Disease in C57BL/6 Mice. *J. Immunol.* **166**, 7579–7587 (2001).

66. Stromnes, I. M. & Goverman, J. M. Passive induction of experimental allergic encephalomyelitis. *Nat. Protoc.* **1**, 1952–1960 (2006).

67. Hauser, S. L. *et al.* B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N. Engl. J. Med.* **358**, 676–688 (2008).

68. Kappos, L. *et al.* Ocrelizumab in relapsing-remitting multiple sclerosis: A phase 2, randomised, placebo-controlled, multicentre trial. *Lancet* **378**, 1779–1787 (2011).

69. Disanto, G., Morahan, J. M., Barnett, M. H., Giovannoni, G. & Ramagopalan, S. V. The evidence for a role of B cells in multiple sclerosis. *Neurology* vol. 78 823–832 (2012).

70. Distéfano-Gagné, F., Bitarafan, S., Lacroix, S. & Gosselin, D. Roles and regulation of microglia activity in multiple sclerosis: insights from animal models. *Nature Reviews Neuroscience* vol. 24 397–415 (2023).

71. Absinta, M. *et al.* A lymphocyte–microglia–astrocyte axis in chronic active multiple sclerosis. *Nature* **597**, 709–714 (2021).

72. Szalai, A. J., Hu, X., Adams, J. E. & Barnum, S. R. Complement in experimental autoimmune encephalomyelitis revisited: C3 is required for development of maximal disease. *Mol. Immunol.* **44**, 3132–3136 (2007).

73. Haider, L. *et al.* Oxidative damage in multiple sclerosis lesions. *Brain* **134**, 1914–1924 (2011).

74. Kotter, M. R., Li, W. W., Zhao, C. & Franklin, R. J. M. Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. **26**, 328–332 (2006).

75. Setzu, A. *et al.* Inflammation stimulates myelination by transplanted oligodendrocyte precursor cells. *Glia* **54**, 297–303 (2006).

76. Goldschmidt, T., Antel, J., König, F. B., Brück, W. & Kuhlmann, T. Remyelination capacity of the MS brain decreases with disease chronicity. *Neurology* **72**, 1914–1921 (2009).

77. Neumann, B., Segel, M., Chalut, K. J. & Franklin, R. J. M. Remyelination and ageing: Reversing the ravages of time. *Multiple Sclerosis Journal* vol. 25 1835–1841 (2019).

78. Patrikios, P. *et al.* Remyelination is extensive in a subset of multiple sclerosis patients. *Brain* **129**, 3165–3172 (2006).

79. Patani, R., Balaratnam, M., Vora, A. & Reynolds, R. Remyelination can be extensive in multiple sclerosis despite a long disease course. *Neuropathol. Appl. Neurobiol.* **33**, 277–

287 (2007).

80. Mason, J. L. *et al.* Oligodendrocytes and progenitors become progressively depleted within chronically demyelinated lesions. *Am. J. Pathol.* **164**, 1673–1682 (2004).

81. Niehaus, A. *et al.* Patients with Active Relapsing-Remitting Multiple Sclerosis Synthesize Antibodies Recognizing Oligodendrocyte Progenitor Cell Surface Protein: Implications for Remyelination. *Ann Neurol* **48**, 362–371 (2000).

82. Williams, A. *et al.* Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis? *Brain* **130**, 2554–2565 (2007).

83. Franklin, R. J. M. & Ffrench-Constant, C. Regenerating CNS myelin - From mechanisms to experimental medicines. *Nature Reviews Neuroscience* vol. 18 753–769 (2017).

84. Wolswijk, G. Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J. Neurosci.* **18**, 601–609 (1998).

85. Chang, A., Nishiyama, A., Peterson, J., Prineas, J. & Trapp, B. D. NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J. Neurosci.* **20**, 6404–6412 (2000).

86. Chang, A., Tourtellotte, W. W., Rudick, R. & Trapp, B. D. Premyelinating Oligodendrocytes in Chronic Lesions of Multiple Sclerosis. *N. Engl. J. Med.* **346**, 165–173 (2002).

87. Kuhlmann, T. *et al.* Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain* **131**, 1749–1758 (2008).

88. Reynolds, R. *et al.* The response of NG2-expressing oligodendrocyte progenitors to

demyelination in MOG-EAE and MS. *Journal of Neurocytology* vol. 31 523–536 (2002).

89. Wang, S. *et al.* Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **21**, 63–75 (1998).

90. Jurynczyk, M., Jurewicz, A., Bielecki, B., Raine, C. S. & Selmaj, K. Inhibition of Notch signaling enhances tissue repair in an animal model of multiple sclerosis. *J. Neuroimmunol.* **170**, 3–10 (2005).

91. Back, S. A. *et al.* Hyaluronan accumulates in demyelinated lesions and inhibits oligodendrocyte progenitor maturation. *Nat. Med.* **11**, 966–972 (2005).

92. Sim, F. J., Zhao, C., Penderis, J. & Franklin, R. J. M. The Age-Related Decrease in CNS Remyelination Efficiency Is Attributable to an Impairment of Both Oligodendrocyte Progenitor Recruitment and Differentiation. *J. Neurosci.* **22**, 2451–2459 (2002).

93. Zhao, C., Li, W. W. & Franklin, R. J. M. Differences in the early inflammatory responses to toxin-induced demyelination are associated with the age-related decline in CNS remyelination. *Neurobiol. Aging* **27**, 1298–1307 (2006).

94. Shields, S. A., Gilson, J. M., Blakemore, W. F. & Franklin, R. J. M. Remyelination occurs as extensively but more slowly in old rats compared to young rats following gliotoxin-induced CNS demyelination. *Glia* **28**, 77–83 (1999).

95. Berghoff, S. A., Spieth, L. & Saher, G. Local cholesterol metabolism orchestrates remyelination. *Trends in Neurosciences* vol. 45 272–283 (2022).

96. Saher, G. *et al.* High cholesterol level is essential for myelin membrane growth. *Nat. Neurosci.* **8**, 468–475 (2005).

97. Dietschy, J. M. Central nervous system: Cholesterol turnover, brain development and neurodegeneration. *Biol. Chem.* **390**, 287–293 (2009).

98. Björkhem, I., Leoni, V. & Meaney, S. Genetic connections between neurological disorders and cholesterol metabolism. *Journal of Lipid Research* vol. 51 2489–2503 (2010).

99. Camargo, N. *et al.* Oligodendroglial myelination requires astrocyte-derived lipids. **15**, e1002605 (2017).

100. Wang, H. & Eckel, R. H. What are lipoproteins doing in the brainα. *Trends in Endocrinology and Metabolism* vol. 25 8–14 (2014).

101. de la Fuente, A. G. *et al.* Changes in the oligodendrocyte progenitor cell proteome with ageing. *Mol. Cell. Proteomics* **19**, 1281–1302 (2020).

102. Boisvert, M. M., Erikson, G. A., Shokhirev, M. N. & Allen, N. J. The Aging Astrocyte Transcriptome from Multiple Regions of the Mouse Brain. *Cell Rep.* **22**, 269–285 (2018).

103. Franceschi, C., Garagnani, P., Parini, P., Giuliani, C. & Santoro, A. Inflammaging: a new immune–metabolic viewpoint for age-related diseases. *Nature Reviews Endocrinology* vol. 14 576–590 (2018).

104. Lampron, A. *et al.* Inefficient clearance of myelin debris by microglia impairs remyelinating processes. *J. Exp. Med.* **212**, 481–495 (2015).

105. Rawji, K. S., Gonzalez Martinez, G. A., Sharma, A. & Franklin, R. J. M. The Role of Astrocytes in Remyelination. *Trends in Neurosciences* vol. 43 596–607 (2020).

106. Prineas, J. W. & Parratt, J. D. E. Multiple Sclerosis: Microglia, Monocytes, and Macrophage-Mediated Demyelination. *J. Neuropathol. Exp. Neurol.* **80**, 975–996 (2021).

107. Spann, N. J. *et al.* Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell* **151**, 138–152 (2012).

108. Berghoff, S. A. *et al.* Microglia facilitate repair of demyelinated lesions via post-squalene sterol synthesis. *Nat. Neurosci.* **24**, 47–60 (2021).

109. Voskuhl, R. R. *et al.* Gene expression in oligodendrocytes during remyelination reveals cholesterol homeostasis as a therapeutic target in multiple sclerosis. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 10130–10139 (2019).

110. Tay, T. L., Savage, J. C., Hui, C. W., Bisht, K. & Tremblay, M. È. Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. *J. Physiol.* **595**, 1929–1945 (2017).

111. Frost, J. L. & Schafer, D. P. Microglia: Architects of the Developing Nervous System. *Trends Cell Biol.* **26**, 587–597 (2016).

112. Ginhoux, F. *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**, 841–845 (2010).

113. Olson, M. C. *et al.* PU. 1 is not essential for early myeloid gene expression but is required for terminal myeloid differentiation. *Immunity* **3**, 703–714 (1995).

114. Zusso, M. *et al.* Regulation of Postnatal Forebrain Amoeboid Microglial Cell Proliferation and Development by the Transcription Factor Runx1. *J. Neurosci.* **32**, 11285–11298 (2012).

115. Butovsky, O. *et al.* Identification of a unique TGF-β–dependent molecular and functional signature in microglia. *Nat. Neurosci. 2013 171* **17**, 131–143 (2013).

116. Wang, Y. *et al.* IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nat. Immunol. 2012 138* **13**, 753–760 (2012).

117. Greter, M. *et al.* Stroma-derived interleukin-34 controls the development and maintenance of langerhans cells and the maintenance of microglia. *Immunity* **37**, 1050–1060 (2012).

118. Réu, P. *et al.* The Lifespan and Turnover of Microglia in the Human Brain. *Cell Rep.* **20**, 779–784 (2017).

119. Askew, K. *et al.* Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain. *Cell Rep.* **18**, 391–405 (2017).

120. Tay, T. L. *et al.* A new fate mapping system reveals context-dependent random or clonal expansion of microglia. *Nat. Neurosci. 2017 206* **20**, 793–803 (2017).

121. Ajami, B., Bennett, J. L., Krieger, C., Tetzlaff, W. & Rossi, F. M. V. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat. Neurosci. 2007 1012* **10**, 1538–1543 (2007).

122. Hickman, S. E. *et al.* The microglial sensome revealed by direct RNA sequencing. *Nat. Neurosci.* **16**, 1896–1905 (2013).

123. Prinz, M., Jung, S. & Priller, J. Microglia Biology: One Century of Evolving Concepts. *Cell* **179**, 292–311 (2019).

124. Marín-Teva, J. L. *et al.* Microglia Promote the Death of Developing Purkinje Cells. *Neuron* **41**, 535–547 (2004).

125. Swinnen, N. *et al.* Complex invasion pattern of the cerebral cortex bymicroglial cells during development of the mouse embryo. *Glia* **61**, 150–163 (2013).

126. Cunningham, C. L., Martínez-Cerdeño, V. & Noctor, S. C. Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *J. Neurosci.* **33**, 4216–4233 (2013).

127. Walton, N. M. *et al.* Microglia instruct subventricular zone neurogenesis. *Glia* **54**, 815–825 (2006).

128. Ueno, M. *et al.* Layer V cortical neurons require microglial support for survival during postnatal development. *Nat. Neurosci.* **16**, 543–551 (2013).

129. Shigemoto-Mogami, Y., Hoshikawa, K., Goldman, J. E., Sekino, Y. & Sato, K. Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone. *J. Neurosci.* **34**, 2231–2243 (2014).

130. Arnò, B. *et al.* Neural progenitor cells orchestrate microglia migration and positioning into the developing cortex. *Nat. Commun.* **5**, (2014).

131. Monje, M. L., Toda, H. & Palmer, T. D. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* **302**, 1760–1765 (2003).

132. Goshen, I. *et al.* Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. *Mol. Psychiatry* **13**, 717–728 (2008).

133. Bernardino, L. *et al.* Tumor necrosis factor-alpha modulates survival, proliferation, and neuronal differentiation in neonatal subventricular zone cell cultures. *Stem Cells* **26**, 2361–2371 (2008).

134. Das, S. & Basu, A. Inflammation: a new candidate in modulating adult neurogenesis. *J.*

Neurosci. Res. **86**, 1199–1208 (2008).

135. Russo, I., Barlati, S. & Bosetti, F. Effects of neuroinflammation on the regenerative capacity of brain stem cells. *J. Neurochem.* **116**, 947–956 (2011).

136. Watts, R. J., Schuldiner, O., Perrino, J., Larsen, C. & Luo, L. Glia engulf degenerating axons during developmental axon pruning. *Curr. Biol.* **14**, 678–684 (2004).

137. Paolicelli, R. C. *et al.* Synaptic pruning by microglia is necessary for normal brain development. *Science* **333**, 1456–1458 (2011).

138. Schafer, D. P. *et al.* Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* **74**, 691–705 (2012).

139. Tremblay, M. Ě., Lowery, R. L. & Majewska, A. K. Microglial interactions with synapses are modulated by visual experience. *PLoS Biol.* **8**, (2010).

140. Ji, K., Miyauchi, J. & Tsirka, S. E. Microglia: an active player in the regulation of synaptic activity. *Neural Plast.* **2013**, (2013).

141. Béchade, C., Cantaut-Belarif, Y. & Bessis, A. Microglial control of neuronal activity. *Front. Cell. Neurosci.* **7**, (2013).

142. Nemes-Baran, A. D., White, D. R. & DeSilva, T. M. Fractalkine-Dependent Microglial Pruning of Viable Oligodendrocyte Progenitor Cells Regulates Myelination. *Cell Rep.* **32**, (2020).

143. Hughes, A. N. & Appel, B. Microglia phagocytose myelin sheaths to modify developmental myelination. *Nat. Neurosci.* **23**, 1055–1066 (2020).

144. Djannatian, M. *et al.* Myelination generates aberrant ultrastructure that is resolved by

microglia. *J. Cell Biol.* **222**, (2023).

145. Borst, K., Dumas, A. A. & Prinz, M. Microglia: Immune and non-immune functions. *Immunity* **54**, 2194–2208 (2021).

146. Butovsky, O. & Weiner, H. L. Microglial signatures and their role in health and disease. *Nat. Rev. Neurosci. 2018 1910* **19**, 622–635 (2018).

147. Lassmann, H., Raine, C. S., Antel, J. & Prineas, J. W. Immunopathology of multiple sclerosis: Report on an international meeting held at the Institute of Neurology of the University of Vienna. *J. Neuroimmunol.* **86**, 213–217 (1998).

148. Henderson, A. P. D., Barnett, M. H., Parratt, J. D. E. & Prineas, J. W. Multiple sclerosis: Distribution of inflammatory cells in newly forming lesions. *Ann. Neurol.* **66**, 739–753 (2009).

149. Trapp, B. D. *et al.* Axonal Transection in the Lesions of Multiple Sclerosis. *N. Engl. J. Med.* **338**, 278–285 (1998).

150. Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T. & Brück, W. Acute axonal injury in multiple sclerosis: Correlation with demyelination and inflammation. *Brain* **123**, 1174–1183 (2000).

151. Fischer, M. T. *et al.* NADPH oxidase expression in active multiple sclerosis lesions in relation to oxidative tissue damage and mitochondrial injury. *Brain* **135**, 886–899 (2012).

152. Heppner, F. L. *et al.* Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nat. Med. 2005 112* **11**, 146–152 (2005).

153. Goldmann, T. *et al.* A new type of microglia gene targeting shows TAK1 to be pivotal in

CNS autoimmune inflammation. *Nat. Neurosci. 2013 1611* **16**, 1618–1626 (2013).

154. Nikić, I. *et al.* A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. *Nat. Med. 2011 174* **17**, 495–499 (2011).

155. Sherafat, A., Pfeiffer, F., Reiss, A. M., Wood, W. M. & Nishiyama, A. Microglial neuropilin-1 promotes oligodendrocyte expansion during development and remyelination by trans-activating platelet-derived growth factor receptor. *Nat. Commun.* **12**, (2021).

156. Baaklini, C. S. *et al.* Microglia promote remyelination independent of their role in clearing myelin debris. *Cell Rep.* **42**, (2023).

157. Miron, V. E. *et al.* M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat. Neurosci.* **16**, 1211–1218 (2013).

158. Dong, Y. *et al.* Oxidized phosphatidylcholines found in multiple sclerosis lesions mediate neurodegeneration and are neutralized by microglia. *Nat. Neurosci.* **24**, 489–503 (2021).

159. Rawji, K. S. *et al.* Niacin-mediated rejuvenation of macrophage/microglia enhances remyelination of the aging central nervous system. *Acta Neuropathol.* **139**, 893–909 (2020).

160. Neumann, H., Kotter, M. R. & Franklin, R. J. M. Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain* **132**, 288–295 (2009).

161. Plemel, J. R., Manesh, S. B., Sparling, J. S. & Tetzlaff, W. Myelin inhibits oligodendroglial maturation and regulates oligodendrocytic transcription factor expression. *Glia* **61**, 1471–1487 (2013).

162. Syed, Y. A. *et al.* Antibody-mediated neutralization of myelin-associated EphrinB3

accelerates CNS remyelination. *Acta Neuropathol.* **131**, 281–298 (2016).

163. Healy, L. M. *et al.* MerTK Is a Functional Regulator of Myelin Phagocytosis by Human Myeloid Cells. *J. Immunol.* **196**, 3375–3384 (2016).

164. Cantoni, C. *et al.* TREM2 regulates microglial cell activation in response to demyelination in vivo. *Acta Neuropathol.* **129**, 429–447 (2015).

165. Poliani, P. L. *et al.* TREM2 sustains microglial expansion during aging and response to demyelination. *J. Clin. Invest.* **125**, 2161–2170 (2015).

166. Grajchen, E. *et al.* CD36-mediated uptake of myelin debris by macrophages and microglia reduces neuroinflammation. *J. Neuroinflammation* **17**, 224 (2020).

167. Cabral Da Costa, C., Van Der Laan, L. J. W., Dijkstra, C. D. & Brück, W. The Role of the Mouse Macrophage Scavenger Receptor in Myelin Phagocytosis. *Eur. J. Neurosci.* **9**, 2650–2657 (1997).

168. Reichert, F. & Rotshenker, S. Complement-receptor-3 and scavenger-receptor-AI/II mediated myelin phagocytosis in microglia and macrophages. *Neurobiol. Dis.* **12**, 65–72 (2003).

169. Rotshenker, S. Microglia and macrophage activation and the regulation of complement-receptor-3 (CR3/MAC-1)-mediated myelin phagocytosis in injury and disease. *J. Mol. Neurosci.* **21**, 65–72 (2003).

170. Crowley, M. T. *et al.* A Critical Role for Syk in Signal Transduction and Phagocytosis Mediated by Fcγ Receptors on Macrophages. *J. Exp. Med.* **186**, 1027–1039 (1997).

171. Arnett, H. A. *et al.* TNFα promotes proliferation of oligodendrocyte progenitors and

remyelination. *Nat. Neurosci.* **4**, 1116–1122 (2001).

172. Giera, S. *et al.* Microglial transglutaminase-2 drives myelination and myelin repair via GPR56/ADGRG1 in oligodendrocyte precursor cells. *Elife* **7**, (2018).

173. Suh, H. S., Zhao, M. L., Derico, L., Choi, N. & Lee, S. C. Insulin-like growth factor 1 and 2 (IGF1, IGF2) expression in human microglia: differential regulation by inflammatory mediators. *J. Neuroinflammation* **10**, (2013).

174. Mason, J. L., Suzuki, K., Chaplin, D. D. & Matsushima, G. K. Interleukin-1β promotes repair of the CNS. *J. Neurosci.* **21**, 7046–7052 (2001).

175. Kent, S. A. & Miron, V. E. *Microglia regulation of central nervous system myelin health and regeneration*. *Nature Reviews Immunology* vol. 24 49–63 (Nature Publishing Group, 2024).

176. Streit, W. J., Graeber, M. B. & Kreutzberg, G. W. Functional plasticity of microglia: A review. *Glia* vol. 1 301–307 (1988).

177. Michelucci, A., Heurtaux, T., Grandbarbe, L., Morga, E. & Heuschling, P. Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyloid-beta. *J. Neuroimmunol.* **210**, 3–12 (2009).

178. Paolicelli, R. C. *et al.* Microglia states and nomenclature: A field at its crossroads. *Neuron* **110**, 3458–3483 (2022).

179. Hammond, T. R. *et al.* Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes. *Immunity* **50**,

253-271.e6 (2019).

180. Li, Q. *et al.* Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing. *Neuron* **101**, 207-223.e10 (2019).

181. Kracht, L. *et al.* Human fetal microglia acquire homeostatic immune-sensing properties early in development. *Science* **369**, 530–537 (2020).

182. Keren-Shaul, H. *et al.* A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell* **169**, 1276-1290.e17 (2017).

183. Hou, J. *et al.* Transcriptomic atlas and interaction networks of brain cells in mouse CNS demyelination and remyelination. *Cell Rep.* **42**, (2023).

184. Streit, W. J., Xue, Q. S., Tischer, J. & Bechmann, I. Microglial pathology. *Acta Neuropathol. Commun.* **2**, 1–17 (2014).

185. Mosher, K. I. & Wyss-Coray, T. Microglial dysfunction in brain aging and Alzheimer's disease. *Biochem. Pharmacol.* **88**, 594–604 (2014).

186. Yousef, H. *et al.* Aged blood impairs hippocampal neural precursor activity and activates microglia via brain endothelial cell VCAM1. *Nat. Med.* **25**, 988–1000 (2019).

187. Marschallinger, J. *et al.* Lipid-droplet-accumulating microglia represent a dysfunctional and proinflammatory state in the aging brain. *Nat. Neurosci.* **23**, 194–208 (2020).

188. Childs, B. G. *et al.* Senescent intimal foam cells are deleterious at all stages of atherosclerosis. *Science* **354**, 472–477 (2016).

189. Cantuti-Castelvetri, L. *et al.* Defective cholesterol clearance limits remyelination in the aged central nervous system. *Science (80-.).* **359**, 684–688 (2018).

190. Bogie, J. F. J. *et al.* Stearoyl-CoA desaturase-1 impairs the reparative properties of macrophages and microglia in the brain. *J. Exp. Med.* **217**, (2020).

191. Boven, L. A. *et al.* Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. *Brain* **129**, 517–526 (2006).

192. Bogie, J. F. J. *et al.* Myelin alters the inflammatory phenotype of macrophages by activating PPARs. *Acta Neuropathol. Commun.* **1**, (2013).

193. Bogie, J. F. J. *et al.* Myelin-derived lipids modulate macrophage activity by liver X receptor activation. *PLoS One* **7**, (2012).

194. Safaiyan, S. *et al.* Age-related myelin degradation burdens the clearance function of microglia during aging. *Nat. Neurosci.* **19**, 995–998 (2016).

195. Kuhlmann, T. *et al.* An updated histological classification system for multiple sclerosis lesions. *Acta Neuropathol.* **133**, 13–24 (2017).

196. Grajchen, E., Hendriks, J. J. A. & Bogie, J. F. J. *The physiology of foamy phagocytes in multiple sclerosis*. vol. 6 124 (2018).

197. Luo, J., Yang, H. & Song, B. L. Mechanisms and regulation of cholesterol homeostasis. *Nat. Rev. Mol. Cell Biol. 2019 214* **21**, 225–245 (2019).

198. Viaud, M. *et al.* Lysosomal cholesterol hydrolysis couples efferocytosis to anti-inflammatory oxysterol production. *Circ. Res.* **122**, 1369–1384 (2018).

199. Dang, E. V., McDonald, J. G., Russell, D. W. & Cyster, J. G. Oxysterol Restraint of Cholesterol Synthesis Prevents AIM2 Inflammasome Activation. *Cell* **171**, 1057-1071.e11 (2017).

200. Thomas, D. G. *et al.* LXR Suppresses Inflammatory Gene Expression and Neutrophil Migration through cis-Repression and Cholesterol Efflux. *Cell Rep.* **25**, 3774-3785.e4 (2018).

201. Itoh, N. *et al.* Cell-specific and region-specific transcriptomics in the multiple sclerosis model: Focus on astrocytes. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E302–E309 (2017).

202. Berghoff, S. A. *et al.* Neuronal cholesterol synthesis is essential for repair of chronically demyelinated lesions in mice. *Cell Rep.* **37**, 109889 (2021).

203. Hong, C. & Tontonoz, P. Liver X receptors in lipid metabolism: opportunities for drug discovery. *Nat. Rev. Drug Discov. 2014 136* **13**, 433–444 (2014).

204. Wang, Z. *et al.* Nuclear Receptor NR1H3 in Familial Multiple Sclerosis. *Neuron* **90**, 948–954 (2016).

205. Miyazaki, M., Kim, Y. C., Gray-Keller, M. P., Attie, A. D. & Ntambi, J. M. The biosynthesis of hepatic cholesterol esters and triglycerides is impaired in mice with a disruption of the gene for stearoyl-CoA desaturase 1. *J. Biol. Chem.* **275**, 30132–30138 (2000).

206. Sun, Y. *et al.* Stearoyl-CoA desaturase inhibits ATP-binding cassette transporter A1-mediated cholesterol efflux and modulates membrane domain structure. *J. Biol. Chem.* **278**, 5813–5820 (2003).

207. Wang, Y. & Oram, J. F. Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a protein kinase C delta pathway. *J. Lipid Res.* **48**, 1062–1068 (2007).

208. Wang, Y. & Oram, J. F. Unsaturated fatty acids inhibit cholesterol efflux from

macrophages by increasing degradation of ATP-binding cassette transporter A1. *J. Biol. Chem.* **277**, 5692–5697 (2002).

209. Yang, Y., Jiang, Y., Wang, Y. & An, W. Suppression of ABCA1 by unsaturated fatty acids leads to lipid accumulation in HepG2 cells. *Biochimie* **92**, 958–963 (2010).

210. Chinetti, G. *et al.* PPAR-α and PPAR-γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat. Med. 2001 71* **7**, 53–58 (2001).

211. Wouters, E. *et al.* Altered PPARγ Expression Promotes Myelin-Induced Foam Cell Formation in Macrophages in Multiple Sclerosis. *Int. J. Mol. Sci.* **21**, 1–13 (2020).

212. Westerterp, M. *et al.* Cholesterol Efflux Pathways Suppress Inflammasome Activation, NETosis, and Atherogenesis. *Circulation* **138**, 898–912 (2018).

213. Lloyd, A. F. *et al.* Central nervous system regeneration is driven by microglia necroptosis and repopulation. *Nat. Neurosci.* **22**, 1046–1052 (2019).

214. Ye, K., Chen, Z. & Xu, Y. The double-edged functions of necroptosis. *Cell Death and Disease* vol. 14 1–12 (2023).

215. Meffre, D. *et al.* Liver X receptors alpha and beta promote myelination and remyelination in the cerebellum. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 7587–7592 (2015).

216. Muse, E. D. *et al.* Cell-specific discrimination of desmosterol and desmosterol mimetics confers selective regulation of LXR and SREBP in macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E4680–E4689 (2018).

217. Gu, Z. *et al.* Apolipoprotein E Mimetic Promotes Functional and Histological Recovery in

Lysolecithin-Induced Spinal Cord Demyelination in Mice. *J. Neurol. Neurophysiol.* **2014**, 10 (2014).

218. Miron, V. E., Schubart, A. & Antel, J. P. Central nervous system-directed effects of FTY720 (fingolimod). *J. Neurol. Sci.* **274**, 13–17 (2008).

219. Newton, J. *et al.* FTY720/fingolimod increases NPC1 and NPC2 expression and reduces cholesterol and sphingolipid accumulation in Niemann-Pick type C mutant fibroblasts. *FASEB J.* **31**, 1719 (2017).

220. Blumenfeld Kan, S., Staun-Ram, E., Golan, D. & Miller, A. HDL-cholesterol elevation associated with fingolimod and dimethyl fumarate therapies in multiple sclerosis. *Mult. Scler. J. - Exp. Transl. Clin.* **5**, (2019).

221. Kataria, H. *et al.* Neuregulin-1 beta 1 is implicated in pathogenesis of multiple sclerosis. *Brain* **144**, 162–185 (2021).

222. Kataria, H. *et al.* Neuregulin-1 promotes remyelination and fosters a pro-regenerative inflammatory response in focal demyelinating lesions of the spinal cord. *Glia* **66**, 538–561 (2018).

223. Alizadeh, A., Santhosh, K. T., Kataria, H., Gounni, A. S. & Karimi-Abdolrezaee, S. Neuregulin-1 elicits a regulatory immune response following traumatic spinal cord injury. *J. Neuroinflammation* **15**, 1–21 (2018).

224. Kataria, H., Alizadeh, A. & Karimi-Abdolrezaee, S. Neuregulin-1/ErbB network: An emerging modulator of nervous system injury and repair. *Progress in Neurobiology* vol. 180 (2019).

225. Thomson, P. A. *et al.* Association of Neuregulin 1 with schizophrenia and bipolar disorder in a second cohort from the Scottish population. *Mol. Psychiatry 2007 121* **12**, 94–104 (2006).

226. Georgieva, L. *et al.* Support for Neuregulin 1 as a Susceptibility Gene for Bipolar Disorder and Schizophrenia. *Biol. Psychiatry* **64**, 419–427 (2008).

227. Prata, D. P. *et al.* An association study of the neuregulin 1 gene, bipolar affective disorder and psychosis. *Psychiatr. Genet.* **19**, 113–116 (2009).

228. Falls, D. L. Neuregulins: Functions, forms, and signaling strategies. *EGF Recept. Fam. Biol. Mech. Role Cancer* 15–31 (2003) doi:10.1016/B978-012160281-9/50003-7.

229. Meyer, D. & Birchmeier, C. Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. *Proc. Natl. Acad. Sci.* **91**, 1064–1068 (1994).

230. Kramer, R. *et al.* Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc. Natl. Acad. Sci.* **93**, 4833–4838 (1996).

231. Meyer, D. & Birchmeier, C. Multiple essential functions of neuregulin in development. *Nature* **378**, 386–390 (1995).

232. Hu, X. *et al.* Bace1 modulates myelination in the central and peripheral nervous system. *Nat. Neurosci.* **9**, 1520–1525 (2006).

233. Willem, M. *et al.* Control of peripheral nerve myelination by the β-secretase BACE1. *Science (80-.).* **314**, 664–666 (2006).

234. Bao, J., Wolpowitz, D., Role, L. W. & Talmage, D. A. Back signaling by the Nrg-1

intracellular domain. *J. Cell Biol.* **161**, 1133–1141 (2003).

235. Olayioye, M. A., Neve, R. M., Lane, H. A. & Hynes, N. E. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* **19**, 3159–3167 (2000).

236. Olayioye, M. A. *et al.* ErbB-1 and ErbB-2 Acquire Distinct Signaling Properties Dependent upon Their Dimerization Partner. *Mol. Cell. Biol.* **18**, 5042–5051 (1998).

237. Kim, J. H., Saito, K. & Yokoyama, S. Chimeric receptor analyses of the interactions of the ectodomains of ErbB-1 with epidermal growth factor and of those of ErbB-4 with neuregulin. *Eur. J. Biochem.* **269**, 2323–2329 (2002).

238. Garrett, T. P. J. *et al.* The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol. Cell* **11**, 495–505 (2003).

239. Cho, H. S. *et al.* Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nat. 2003 4216924* **421**, 756–760 (2003).

240. Yarden, Y. & Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol. 2001 22* **2**, 127–137 (2001).

241. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A. & Carraway, K. L. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc. Natl. Acad. Sci.* **91**, 8132–8136 (1994).

242. Sierke, S. L., Cheng, K., Kim, H. H. & Koland, J. G. Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein. *Biochem. J.* **322**, 757–763 (1997).

243. Iwakura, Y. & Nawa, H. ErbB1-4-dependent EGF/neuregulin signals and their cross talk in the central nervous system: Pathological implications in schizophrenia and Parkinson's disease. *Front. Cell. Neurosci.* **7**, 1–35 (2013).

244. Li, K. X. *et al.* Neuregulin 1 regulates excitability of fast-spiking neurons through Kv1.1 and acts in epilepsy. *Nat. Neurosci. 2011 152* **15**, 267–273 (2011).

245. Mei, L. & Xiong, W. C. Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. *Nat. Rev. Neurosci. 2008 96* **9**, 437–452 (2008).

246. Si, J., Wang, Q. & Mei, L. Essential Roles of c-JUN and c-JUN N-Terminal Kinase (JNK) in Neuregulin-Increased Expression of the Acetylcholine Receptor ε-Subunit. *J. Neurosci.* **19**, 8498–8508 (1999).

247. Ni, C. Y., Murphy, M. P., Golde, T. E. & Carpenter, G. γ-secretase cleavage and nuclear locatization of ErbB-4 receptor tyrosine kinase. *Science (80-.).* **294**, 2179–2181 (2001).

248. Sardi, S. P., Murtie, J., Koirala, S., Patten, B. A. & Corfas, G. Presenilin-Dependent ErbB4 Nuclear Signaling Regulates the Timing of Astrogenesis in the Developing Brain. *Cell* **127**, 185–197 (2006).

249. Dong, Z. *et al.* Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat schwann cell precursors. *Neuron* **15**, 585–596 (1995).

250. Michailov, G. V. *et al.* Axonal Neuregulin-1 Regulates Myelin Sheath Thickness. *Science (80-.).* **304**, 700–703 (2004).

251. Vartanian, T., Goodearl, A., Viehöver, A. & Fischbach, G. Axonal Neuregulin Signals Cells of the Oligodendrocyte Lineage through Activation of HER4 and Schwann Cells

through HER2 and HER3. *J. Cell Biol.* **137**, 211–220 (1997).

252. Canoll, P. D. *et al.* GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. *Neuron* **17**, 229–243 (1996).

253. Vartanian, T., Fischbach, G. & Miller, R. Failure of spinal cord oligodendrocyte development in mice lacking neuregulin. **96**, 731–735 (1999).

254. Kim, J. Y., Sun, Q., Oglesbee, M. & Yoon, S. O. The Role of ErbB2 Signaling in the Onset of Terminal Differentiation of Oligodendrocytes In Vivo. *J. Neurosci.* **23**, 5561–5571 (2003).

255. Makinodan, M., Rosen, K. M., Ito, S. & Corfas, G. A critical period for social experience-dependent oligodendrocyte maturation and myelination. *Science (80-.).* **337**, 1357–1360 (2012).

256. Schmucker, J. *et al.* erbB3 is dispensable for oligodendrocyte development in vitro and in vivo. *Glia* **44**, 67–75 (2003).

257. Gauthier, M. K., Kosciuczyk, K., Tapley, L. & Karimi-Abdolrezaee, S. Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury. *Eur. J. Neurosci.* **38**, 2693–2715 (2013).

258. Rupert, C. E. & Coulombe, K. L. K. The roles of neuregulin-1 in cardiac development, homeostasis, and disease. *Biomark. Insights* **2015**, 1–9 (2015).

259. Erickson, S. L. *et al.* ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2- and heregulin-deficient mice. *Development* **124**, 4999–5011

(1997).

260. Gassmann, M. *et al.* Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nat. 1995 3786555* **378**, 390–394 (1995).

261. Lee, K. F. *et al.* Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nat. 1995 3786555* **378**, 394–398 (1995).

262. Taveggia, C. *et al.* Type III neuregulin-1 promotes oligodendrocyte myelination. *Glia* **56**, 284–293 (2008).

263. Brinkmann, B. G. *et al.* Neuregulin-1/ErbB Signaling Serves Distinct Functions in Myelination of the Peripheral and Central Nervous System. *Neuron* **59**, 581–595 (2008).

264. Carroll, S. L., Miller, M. L., Frohnert, P. W., Kim, S. S. & Corbett, J. A. Expression of Neuregulins and their Putative Receptors, ErbB2 and ErbB3, Is Induced during Wallerian Degeneration. *J. Neurosci.* **17**, 1642–1659 (1997).

265. Bermingham-Mcdonogh, O., Xu, Y. T., Marchionni, M. A. & Scherer, S. S. Neuregulin Expression in PNS Neurons: Isoforms and Regulation by Target Interactions. *Mol. Cell. Neurosci.* **10**, 184–195 (1997).

266. Stassart, R. M. *et al.* A role for Schwann cell–derived neuregulin-1 in remyelination. *Nat. Neurosci. 2012 161* **16**, 48–54 (2012).

267. Alizadeh, A. *et al.* Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury. *Glia* **65**, 1152–1175 (2017).

268. Xu, Z., Jiang, J., Ford, G. & Ford, B. D. Neuregulin-1 is neuroprotective and attenuates inflammatory responses induced by ischemic stroke. *Biochem. Biophys. Res. Commun.*

322, 440–446 (2004).

269. Shyu, W. C. *et al.* Neuregulin-1 reduces ischemia-induced brain damage in rats. *Neurobiol. Aging* **25**, 935–944 (2004).

270. Guo, W. P., Wang, J., Li, R. X. & Peng, Y. W. Neuroprotective effects of neuregulin-1 in rat models of focal cerebral ischemia. *Brain Res.* **1087**, 180–185 (2006).

271. Shahsavani, N., Alizadeh, A., Kataria, H. & Karimi-Abdolrezaee, S. Availability of neuregulin-1beta1 protects neurons in spinal cord injury and against glutamate toxicity through caspase dependent and independent mechanisms. *Exp. Neurol.* **345**, (2021).

272. Lasiene, J. *et al.* Neuregulin 1 confers neuroprotection in SOD1-linked amyotrophic lateral sclerosis mice via restoration of C-boutons of spinal motor neurons. *Acta Neuropathol. Commun.* **4**, 15 (2016).

273. Hama, Y. *et al.* Level of plasma neuregulin-1 SMDF is reduced in patients with idiopathic Parkinson's disease. *Neurosci. Lett.* **587**, 17–21 (2015).

274. Carlsson, T. *et al.* Systemic administration of neuregulin-1β1 protects dopaminergic neurons in a mouse model of Parkinson's disease. *J. Neurochem.* **117**, 1066–1074 (2011).

275. Depboylu, C., Rösler, T. W., De Andrade, A., Oertel, W. H. & Höglinger, G. U. Systemically administered neuregulin-1β1 rescues nigral dopaminergic neurons via the ErbB4 receptor tyrosine kinase in MPTP mouse models of Parkinson's disease. *J. Neurochem.* **133**, 590–597 (2015).

276. Ryu, J. *et al.* Neuregulin-1 attenuates cognitive function impairments in a transgenic mouse model of Alzheimer's disease. *Cell Death Dis. 2016 72* **7**, e2117–e2117 (2016).

277. Woo, R. S. *et al.* Neuregulin-1 protects against neurotoxicities induced by Swedish amyloid precursor protein via the ErbB4 receptor. *Neuroscience* **202**, 413–423 (2012).

278. Cannella, B. *et al.* The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model for multiple sclerosis. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 10100–10105 (1998).

279. Bartus, K. *et al.* Neuregulin-1 controls an endogenous repair mechanism after spinal cord injury. *Brain* **139**, 1394–1416 (2016).

280. Assinck, P. *et al.* Myelinogenic Plasticity of Oligodendrocyte Precursor Cells following Spinal Cord Contusion Injury. *J. Neurosci.* **37**, 8635–8654 (2017).

281. Bartus, K. *et al.* ErbB receptor signaling directly controls oligodendrocyte progenitor cell transformation and spontaneous remyelination after spinal cord injury. *Glia* **67**, 1036–1046 (2019).

282. Ziaee, S. M. & Karimi-Abdolrezaee, S. Emerging role of neuregulin-1beta1 in pathogenesis and progression of multiple sclerosis. *Neural Regen. Res.* **18**, 133–134 (2023).

283. Alizadeh, A., Dyck, S. M. & Karimi-Abdolrezaee, S. Traumatic spinal cord injury: An overview of pathophysiology, models and acute injury mechanisms. *Front. Neurol.* **10**, 441408 (2019).

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

285. Keough, M. B. *et al.* An inhibitor of chondroitin sulfate proteoglycan synthesis promotes

central nervous system remyelination. *Nat. Commun. 2016 71* **7**, 1–12 (2016).

286. Gensel, J. C. *et al.* Macrophages Promote Axon Regeneration with Concurrent Neurotoxicity. *J. Neurosci.* **29**, 3956–3968 (2009).

287. Jones, T. B. Lymphocytes and autoimmunity after spinal cord injury. *Exp. Neurol.* **258**, 78–90 (2014).

288. Wang, J. J. J. *et al.* Targeting microglia and macrophages: A potential treatment strategy for multiple sclerosis. *Front. Pharmacol.* **10**, 440854 (2019).

289. Dendrou, C. A., Fugger, L. & Friese, M. A. Immunopathology of multiple sclerosis. *Nature Reviews Immunology* vol. 15 545–558 (2015).

290. Lubetzki, C., Zalc, B., Williams, A., Stadelmann, C. & Stankoff, B. Remyelination in multiple sclerosis: from basic science to clinical translation. *The Lancet Neurology* vol. 19 678–688 (2020).

291. Shields, S. A., Gilson, J. M., Blakemore, W. & Franklin, R. J. Erratum: Remyelination occurs as extensively but more slowly in old rats compared to young rats following fliotoxin-induced CNS demyelination (Glia (1999) 28 (77-83)). *GLIA* vol. 29 102 (2000).

292. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* vol. 153 1194 (2013).

293. Woodruff, R. H., Fruttiger, M., Richardson, W. D. & Franklin, R. J. M. Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination. *Mol. Cell. Neurosci.* **25**, 252–262 (2004).

294. Nave, K. A. *Myelination and the trophic support of long axons*. vol. 11 275–283 (Nat Rev

Neurosci, 2010).

295. Filippi, M. *et al.* Multiple sclerosis. *Nat. Rev. Dis. Prim.* **4**, 1–27 (2018).

296. Lassmann, H., Van Horssen, J. & Mahad, D. Progressive multiple sclerosis: Pathology and pathogenesis. *Nature Reviews Neurology* vol. 8 647–656 (2012).

297. Trapp, B. D. & Nave, K. A. Multiple sclerosis: An immune or neurodegenerative disorder? *Annual Review of Neuroscience* vol. 31 247–269 (2008).

298. Masuda, T. *et al.* Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. *Nat. 2019 5667744* **566**, 388–392 (2019).

299. Prinz, M., Masuda, T., Wheeler, M. A. & Quintana, F. J. Microglia and Central Nervous System-Associated Macrophages-From Origin to Disease Modulation. *Annu. Rev. Immunol.* **39**, 251–277 (2021).

300. Zia, S. *et al.* Single-cell microglial transcriptomics during demyelination defines a microglial state required for lytic carcass clearance. *Mol. Neurodegener.* **17**, (2022).

301. Colonna, M. & Butovsky, O. Microglia function in the central nervous system during health and neurodegeneration. *Annual Review of Immunology* vol. 35 441–468 (2017).

302. McNamara, N. B. *et al.* Microglia regulate central nervous system myelin growth and integrity. *Nature* **613**, 120–129 (2023).

303. Frischer, J. M. *et al.* The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* **132**, 1175–1189 (2009).

304. Faissner, S., Plemel, J. R., Gold, R. & Yong, V. W. Progressive multiple sclerosis: from pathophysiology to therapeutic strategies. *Nature Reviews Drug Discovery* vol. 18 905–

922 (2019).

305. Kang, S. H., Fukaya, M., Yang, J. K., Rothstein, J. D. & Bergles, D. E. NG2+ CNS glial progenitors remain committed to the oligodendrocyte lineage in postnatal life and following neurodegeneration. *Neuron* **68**, 668–681 (2010).

306. Li, L. *et al.* The breast proto-oncogene, HRGα regulates epithelial proliferation and lobuloalveolar development in the mouse mammary gland. *Oncogene* **21**, 4900–4907 (2002).

307. Fricker, F. R. *et al.* Axonal neuregulin 1 is a rate limiting but not essential factor for nerve remyelination. *Brain* **136**, 2279–2297 (2013).

308. Hart, C. G. *et al.* Acute upregulation of bone morphogenetic protein-4 regulates endogenous cell response and promotes cell death in spinal cord injury. *Exp. Neurol.* **325**, (2020).

309. Buck, S. B. *et al.* Detection of S-phase cell cycle progression using 5-ethynyl-2′-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2′-deoxyuridine antibodies. *Biotechniques* **44**, 927–929 (2008).

310. Spangenberg, E. E. *et al.* Eliminating microglia in Alzheimer's mice prevents neuronal loss without modulating amyloid-β pathology. *Brain* **139**, 1265–1281 (2016).

311. Spangenberg, E. *et al.* Sustained microglial depletion with CSF1R inhibitor impairs parenchymal plaque development in an Alzheimer's disease model. *Nat. Commun.* **10**, 1–21 (2019).

312. Fan, J. *et al.* Galaxy integrated omics: Web-based standards-compliant workflows for

proteomics informed by transcriptomics. *Mol. Cell. Proteomics* **14**, 3087–3093 (2015).

313. Grüning, B. A. *et al.* The RNA workbench: Best practices for RNA and high-throughput sequencing bioinformatics in Galaxy. *Nucleic Acids Res.* **45**, W560–W566 (2017).

314. Blankenberg, D. & Hillman-Jackson, J. Analysis of next-generation sequencing data using galaxy. *Methods Mol. Biol.* **1150**, 21–43 (2014).

315. Ge, S. X., Son, E. W. & Yao, R. iDEP: An integrated web application for differential expression and pathway analysis of RNA-Seq data. *BMC Bioinformatics* **19**, (2018).

316. Lee, S. *et al.* A culture system to study oligodendrocyte myelination processes using engineered nanofibers. *Nat. Methods* **9**, 917–922 (2012).

317. Schmued, L. *et al.* Introducing Black-Gold II, a highly soluble gold phosphate complex with several unique advantages for the histochemical localization of myelin. *Brain Res.* **1229**, 210–217 (2008).

318. Sariol, A. *et al.* Microglia depletion exacerbates demyelination and impairs remyelination in a neurotropic coronavirus infection. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 24464–24474 (2020).

319. Plemel, J. R. *et al.* Microglia response following acute demyelination is heterogeneous and limits infiltrating macrophage dispersion. *Sci. Adv.* **6**, (2020).

320. Haase, S. & Linker, R. A. Inflammation in multiple sclerosis. *Therapeutic Advances in Neurological Disorders* vol. 14 (2021).

321. Kastin, A. J., Akerstrom, V. & Pan, W. Neuregulin-1-β1 enters brain and spinal cord by receptor-mediated transport. *J. Neurochem.* **88**, 965–970 (2004).

322. Kidani, Y. & Bensinger, S. J. Liver X receptor and peroxisome proliferator-activated receptor as integrators of lipid homeostasis and immunity. *Immunol. Rev.* **249**, 72–83 (2012).

323. Castrillo, A. & Tontonoz, P. Nuclear receptors in macrophage biology: At the crossroads of lipid metabolism and inflammation. *Annu. Rev. Cell Dev. Biol.* **20**, 455–480 (2004).

324. Stratoulias, V., Venero, J. L., Tremblay, M. & Joseph, B. Microglial subtypes: diversity within the microglial community. *EMBO J.* **38**, (2019).

325. Natrajan, M. S. *et al.* Retinoid X receptor activation reverses age-related deficiencies in myelin debris phagocytosis and remyelination. *Brain* **138**, 3581–3597 (2015).

326. Wlodarczyk, A. *et al.* A novel microglial subset plays a key role in myelinogenesis in developing brain. *EMBO J.* **36**, 3292–3308 (2017).

327. Duncan, G. J. *et al.* Remyelination protects neurons from DLK-mediated neurodegeneration. *Nat. Commun. 2024 151* **15**, 1–19 (2024).

328. Repa, J. J. *et al.* Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* vol. 289 1524–1529 (2000).

329. Repa, J. J. *et al.* Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors α and β. *J. Biol. Chem.* **277**, 18793–18800 (2002).

330. Schulman, I. G. Liver X receptors link lipid metabolism and inflammation. *FEBS Letters* vol. 591 2978–2991 (2017).

331. Huang, J. K. *et al.* Retinoid X receptor gamma signaling accelerates CNS remyelination. *Nat. Neurosci.* **14**, 45–55 (2011).

332. Vanherle, S. *et al.* The ApoA-I mimetic peptide 5A enhances remyelination by promoting clearance and degradation of myelin debris. *Cell Rep.* **41**, (2022).

333. Xu, G. *et al.* Preventive effects of heregulin-β1 on macrophage foam cell formation and atherosclerosis. *Circ. Res.* **105**, 500–510 (2009).

334. Joseph, S. B., Castrillo, A., Laffitte, B. A., Mangelsdorf, D. J. & Tontonoz, P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat. Med.* **9**, 213–219 (2003).

335. Ghisletti, S. *et al.* Parallel SUMOylation-Dependent Pathways Mediate Gene- and Signal-Specific Transrepression by LXRs and PPARγ. *Mol. Cell* **25**, 57–70 (2007).

336. Ito, A. *et al.* LXRs link metabolism to inflammation through Abca1-dependent regulation of membrane composition and TLR signaling. *Elife* **4**, (2015).

337. Molina-Gonzalez, I. *et al.* Astrocyte-oligodendrocyte interaction regulates central nervous system regeneration. *Nat. Commun.* **14**, 1–18 (2023).

338. Mifflin, L. *et al.* A RIPK1-regulated inflammatory microglial state in amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. U. S. A.* **118**, (2021).

339. Ma, X. R. *et al.* Inhibition of RIPK1 by ZJU-37 promotes oligodendrocyte progenitor proliferation and remyelination via NF-κB pathway. *Cell death Discov.* **8**, (2022).

340. Zhu, X. *et al.* Neuregulin-1, a member of the epidermal growth factor family, mitigates STING-mediated pyroptosis and necroptosis in ischaemic flaps. *Burn. Trauma* **12**, (2024).

341. Decout, A., Katz, J. D., Venkatraman, S. & Ablasser, A. The cGAS–STING pathway as a therapeutic target in inflammatory diseases. *Nature Reviews Immunology* vol. 21 548–569

(2021).

342. Mòdol-Caballero, G., Santos, D., Navarro, X. & Herrando-Grabulosa, M. Neuregulin 1 reduces motoneuron cell death and promotes neurite growth in an in vitro model of motoneuron degeneration. *Front. Cell. Neurosci.* **11**, (2018).

343. Yoo, J. Y., Kim, H. B., Baik, T. K., Lee, J. H. & Woo, R. S. Neuregulin 1/ErbB4/Akt signaling attenuates cytotoxicity mediated by the APP-CT31 fragment of amyloid precursor protein. *Exp. Mol. Pathol.* **120**, (2021).

344. Newbern, J. & Birchmeier, C. Nrg1/ErbB signaling networks in Schwann cell development and myelination. *Seminars in Cell and Developmental Biology* vol. 21 922–928 (2010).

345. Park, S. K., Miller, R., Krane, I. & Vartanian, T. The ErbB2 gene is required for the development of terminally differentiated spinal cord oligodendrocytes. *J. Cell Biol.* **154**, 1245–1258 (2001).

346. Mangale, V. *et al.* Microglia influence host defense, disease, and repair following murine coronavirus infection of the central nervous system. *Glia* **68**, 2345–2360 (2020).

347. Profaci, C. P. *et al.* Microglia are not necessary for maintenance of blood-brain barrier properties in health, but PLX5622 alters brain endothelial cholesterol metabolism. *Neuron* **0**, 1–12 (2024).

348. Stefansson, H. *et al.* Neuregulin 1 and Susceptibility to Schizophrenia. *Am. J. Hum. Genet.* **71**, 877–892 (2002).

349. Law, A. J. *et al.* Neuregulin 1 transcripts are differentially expressed in schizophrenia and

regulated by 5′ SNPs associated with the disease. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 6747–6752 (2006).

350. Corfas, G., Roy, K. & Buxbaum, J. D. Neuregulin 1-erbB signaling and the molecular/cellular basis of schizophrenia. *Nature Neuroscience* vol. 7 575–580 (2004).

351. Gao, R. et al. Survival Study of the Recombinant Human Neuregulin-1β in Subjects With Chronic Heart Failure. *Clinical trials.gov* https://clinicaltrials.gov/study/NCT03388593 (2018).