MiR-145-5p: its roles in oligodendrocyte differentiation and its contributions to the pathophysiology of demyelinating disease

Samantha F. Kornfeld

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Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

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Abstract

Multiple sclerosis (MS) is a debilitating disease in which demyelinated lesions form in the central nervous system (CNS). A specific microRNA, miR-145-5p, is dysregulated both in blood samples from RRMS patients and in chronic lesions from progressive MS patients. In the context of remyelination, miR-145-5p regulation may be important as it exhibits strong differential regulation in oligodendrocytes (OLs), the myelinating cells of the CNS, and is also expressed in other CNS glial cell types. Dysregulation of miR-145-5p may therefore play into pathologies observed in both relapsing-remitting (RRMS) and progressive MS. Using pre-clinical rodent models, we aimed to determine how altering normal expression of miR-145-5p specifically affects OL maturation, and how the dysregulation observed in MS may affect various aspects of disease.

First using a miR-145 knockdown model in primary rat OLs, we found in vitro that miR-145-5p plays a role both in maintaining oligodendrocyte progenitor cells (OPCs) in their proliferative state and preventing premature differentiation to OLs and that knockdown of miR-145 in OLs enhanced their differentiation. These effects were due at least in part to miR-145-5p regulation of a critical myelin gene transcription factor. The effects of miR-145-5p were further assessed in a miR-145 knockout mouse model in vivo. Contrary to in vitro assays, enhanced myelination was not detectable during development in these animals, nor when remyelination was assessed using the cuprizone toxic model of acute demyelination. However, chronic cuprizone exposure resulted in striking remyelination and functional recovery in miR-145 deficient animals. Sparse remyelination in wild-type animals with chronic cuprizone exposure was concomitant with upregulation of miR-145-5p, which was
not the case with acute exposure, identifying miR-145-5p dysregulation as a unique feature of chronic demyelination. Specific assessment of miR-145-5p overexpression in OLs *in vitro* resulted in severe differentiation deficits and eventual apoptosis, driven molecularly by altered expression of multiple pathways critical to successful OL differentiation and subsequent myelination.

Finally, we induced an inflammatory model of demyelination, experimental autoimmune encephalomyelitis (EAE), in our miR-145 knockout mouse to assess the role of miR-145-5p in autoimmune-mediated myelin damage. The clinical severity of EAE in miR-145 deficient animals was reduced, and this was accompanied by reduced loss of myelin and lessened immune cell infiltration in miR-145 knockout spinal cords. Alterations in both astrocytic and microglial activation were detected with loss of miR-145, suggesting that improved clinical outcomes in this model may be underpinned by changes in EAE-mediated neuroinflammation.

Collectively, these data suggest that miR-145-5p plays differing roles in both progressive and inflammatory MS, affecting multiple glial cell types in the CNS. Excitingly, loss of miR-145 expression in our mouse model of chronic demyelination allowed extensive remyelination and functional recovery following chronic demyelination, and in EAE improved clinical outcomes driven by underlying improvements in myelin retention and altered neuroinflammatory reactions. Thus, miR-145-5p merits further investigation as a potential therapeutic target to help overcome both remyelination failure in all forms of progressive MS and inflammation-driven demyelination in RRMS and early secondary progressive MS (SPMS).
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Abbreviations

Ach – acetylcholine
ASD – autism spectrum disorder
CNS – central nervous system
CNP – 2’3’-cyclic-nucleotide 3’-phosphodiesterase
CNTF – ciliary neurotrophic factor
CSPG – chondroitin sulphate proteoglycan
DMT – disease modifying therapy
EAE – experimental autoimmune encephalomyelitis
FAK – focal adhesion kinase
FB/MB – forebrain/midbrain
FGF2 – fibroblast growth factor 2
Cer/BS – cerebellum/brainstem
Hes5 – hair and enhancer of split 5
Id2 – inhibitor of DNA binding 2
Id4 – inhibitor of DNA binding 4
IS – ischemic stroke
MAG – myelin associated glycoprotein
Mash1 – mammalian achaete scute homolog 1
MBP – myelin basic protein
MS – multiple sclerosis
MYRF – myelin gene regulatory factor
MyT1 – myelin transcription factor 1
N-WASP – Wiscott-Aldrich syndrome protein
OPC – oligodendrocyte progenitor cell
OL – oligodendrocyte
PDGF-AA – platelet-derived growth factor AA
PDGFRα – platelet-derived growth factor receptor α
PLP – proteolipid protein
PPMS – primary progressive multiple sclerosis
RRMS – relapsing-remitting multiple sclerosis
SC – spinal cord
SCI – spinal cord injury
Sip1 – Smad-interacting protein 1
Sox2 – sex determining region Y box 2
Sox5 – sex determining region Y box 5
Sox6 – sex determining region Y box 6
Sox10 – sex determining region Y box 10
SPMS – secondary progressive multiple sclerosis
TBI – traumatic brain injury
T3 – triiodothyronine
Tcf7l2 – transcription factor 7 like 2
TPPP – tubulin polymerization promoting protein
WAVE1 – WASP family verprolin homologous
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Chapter 1

General Introduction
1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, autoimmune, inflammatory demyelinating disease of the central nervous system (CNS). The disease is characterized by recurrent focal inflammatory attack against myelin, leading to physical and cognitive disabilities. These areas of demyelination, known as lesions or plaques, are often remyelinated early in the disease via mechanisms of innate myelin regeneration; this in turn leads to functional recovery from acquired disabilities. However, remyelination diminishes with disease chronicity and in some cases of MS is negligible from disease onset.

Prevalence and presentation

Prevalence of MS in Canada ranks amongst the highest in the world, with ~100,000 Canadians affected (1). Globally, over two million people live with MS, with prevalence concentrated in Canada, northern United States, northern Europe, Israel, New Zealand and southeastern Australia – this highlights the latitude gradient associated with MS risk, with prevalence increasing with increasing distance from the equator (2). In addition to geographical disparities, sex asymmetry is also apparent with MS disproportionately affecting women over men at a ratio of ~3:1 (2-4).

Clinical signs of MS vary from patient to patient, and disease progression is highly heterogeneous. Demyelinated lesions most frequently occur in the periventricular regions, brain stem, optic nerve, and spinal cord, but may be found anywhere that myelin occurs in the CNS including grey matter, thus eliciting a gamut of symptoms dependent on the lesion location (5). The most commonly reported “first” symptoms include localized limb tingling,
numbness or weakness, and visual impairment; however, the true first symptoms are often insidious and are only recognized as MS-related in retrospect. MS is classified into various subtypes defined as relapsing remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS; Figure 1.1). RRMS, as the name suggests, is characterized by times of relapse – ie, onset and evolution of symptoms – followed by periods of remission – ie, recovery from symptoms. At diagnosis, about 85% of patients will present with RRMS (2). These periods of remission are a reflection of remyelination in lesioned areas. From a diagnostic perspective, SPMS and PPMS are differentiated from RRMS by the fact that periods of remission do not occur, and symptoms simply worsen over time without recovery. Historically, 50-60% of RRMS patients converted to SPMS at some point during their disease (2, 6). While this number is now diminishing with the advent of more successful immunomodulatory drugs to control MS inflammation, a large proportion of RRMS patients will still transition to SPMS. Patients with PPMS, which make up about 15% of all MS patients at diagnosis, experience continuous decline from onset without periods of remission (2).
Figure 1.1 Multiple sclerosis (MS) is classified into subtypes based on symptom progression. Relapsing-remitting MS (RRMS) is defined by periods of symptom presentation interspersed with periods of recovery. Progressive forms of MS are marked by accumulating disability without recovery; RRMS often transitions into secondary progressive MS (SPMS), whilst some patients present with progressive disease at onset, termed primary progress MS (PPMS).
Etiology and risk factors

The etiology of MS is extremely complex, and much remains poorly understood. However, many environmental and genetic risk factors have been identified. Viral infection, particularly Epstein-Barr virus, has long been implicated as a risk modifier for MS (7, 8). Smoking and obesity are also linked to increased MS risk (9, 10). As noted earlier, prevalence of MS is enriched in specific geographical areas, with greater risk positively correlated with residence in countries with more Northern or Southern latitudes (11, 12). This is thought to be linked to reduced UV exposure as a primary contributor, as well as the consequently reduced serum vitamin D levels (13). Similarly, childhood sunlight exposure and even exposure during pregnancy in like geographical areas appear to modify the risk of developing MS later in life (12, 14).

To date, more than two hundred genetic risk variants have also been discovered, including some involved in vitamin D metabolism (12, 15, 16). Most, however, are linked to genes involved in the regulation of both innate and acquired immune responses. Though having a first-degree relative with MS significantly increases the likelihood of acquiring the disease, monozygotic twin studies have shown only 17-18% coincidence in both siblings (17-19). Thus it is apparent that while genetics do play a role in MS risk, there is no specific genetic variant or even set of variants that will predict disease; instead, acquisition appears to depend on an accumulation of environmental factors in a genetically susceptible individual, in a manner as heterogeneous as disease progression.
Pathological hallmarks of MS

The demyelinated lesions definitive of MS are initiated by focal inflammation. This inflammation is driven by aberrant entry of autoreactive lymphocytes into the brain and spinal cord parenchyma across the blood brain barrier (BBB). There are two primary sources of myelin damage. The first is B-cell mediated auto-antibody-driven targeting of myelin or of the myelin-producing cells, oligodendrocytes (OLs), culminating in complement aggregation and subsequent macrophage attack (20-22). The second is myelin/OL targeting by autoreactive T-cells, primarily CD4+ T helper 1 (Th1) cells and CD4+ T helper 17 (Th17) cells, causing OL cytotoxicity either through direct cell-cell interactions or secretion of myelin/OL damaging cytokines (20, 22). In addition to this primary loss of myelin, the arrival of non-CNS cells in this normally protected compartment prompts release of cytokines, chemokines, reactive oxygen species and reactive nitrogen species from CNS-resident macrophages and microglia that in turn produce secondary damage to myelin and OLs, furthering the cascade of demyelination (22-24).

While demyelination of course produces the obvious effect of reduced axonal impulse transmission and associated neurological defects in patients, myelin is not simply a passive insulator as was once thought. Via myelin, OLs are functionally connected to their associated axons, providing critical metabolic support without which axons suffer dysregulation of intracellular transport and unmet energy demands due to loss of OL-derived metabolites (25, 26). Additionally, without the physical protection of myelin ensheathment, immune cells active in the lesion site can damage and even transect axons (27, 28). While efficient remyelination, as is observed in earlier stages of RRMS, will protect
neurons from irreparable impairment, neurodegeneration subsequent to chronic loss of myelin underlies the permanent neurologic decline in progressive forms of MS.

**Therapeutic interventions**

With such an unclear and non-formulaic etiology, it is unsurprising that treating MS carries its own complexities. At this point in time, all treatments are immunomodulatory, aimed at reducing inflammation and consequently the frequency and severity of new lesion formation. This is approached from various avenues, including blocking leukocyte extravasation across the BBB, reducing T-cell activation, depleting B-cell populations, and total immune cell irradiation and autologous stem cell transplantation (29-31). A total of fifteen disease-modifying therapies (DMTs) are currently available in Canada, and stem cell treatments continue to advance towards Health Canada approval. While immunomodulatory therapies such as these continue to evolve and can be very effective in limiting relapse rate, they are only truly useful in RRMS where demyelination is driven by inflammation and remyelination capacity remains intact (32, 33). In cases where a DMT is approved for use in SPMS or PPMS, it is only in the earliest stages of progression where inflammation may still play a role in driving disease – none of the current DMTs prevent progression, and while they showed enough effect in clinical trial to garner bureaucratic approval their efficacy in patients is frustratingly modest. In short, similar to their actions in RRMS, these treatments may prevent accumulation of new damage but do not promote repair of existing damage. In progressive forms of the disease, neurodegeneration proceeds unchecked due to a lack of remyelination, which leaves neurons exposed to inflammatory
insults and without the necessary trophic support myelin provides. The development of successful treatments that promote remyelination remains a critically unmet need at the forefront of MS therapeutic research.

**Animal models for the study of MS**

Treatment development in MS, as in most therapeutic target research, is reliant on animal models. Due to the continued incomplete understanding of MS etiology and vast heterogeneity of disease, there is unfortunately no single gold-standard model, but instead a variety of models exist which partially recapitulate specific pathomechanisms of MS disease. The most well-known model is experimental autoimmune encephalomyelitis (EAE), which has been used to validate several DMTs now in use for RRMS (34, 35). This model mimics the autoimmune-based origins of MS and is a highly inflammatory model that most closely resembles RRMS. EAE can be actively induced by directly priming animals with crude CNS tissue or a specific myelin antigen, most commonly a fragment of myelin oligodendrocyte glycoprotein (MOG). This is introduced in conjunction with complete Freund’s adjuvant and often with pertussis toxin which acts both as an additional adjuvant and to weaken integrity of the BBB (36). Myelin antigen priming elicits effector T cell differentiation in lymphoid organs, primarily into Th1 and Th17 cells, followed by their entry into the CNS where they accumulate predominantly in the white matter of the spinal cord. Now sensitized to myelin, these cells are reactivated upon CNS entry by resident antigen presenting cells, leading to a widespread inflammatory cascade that allows recruitment of additional peripheral immune cells such as neutrophils and
monocytes/macrophages that further breakdown the BBB and participate in myelin attack, respectively (35, 37, 38). Passive EAE induction is also sometimes employed in which myelin antigen-specific T-cells from actively induced animals are transplanted into recipient animals, allowing more precise study of immune effector mechanisms since adjuvant is not required (35, 39). BBB integrity alterations and peripheral immune cell ingress into the CNS additionally results in activation of resident immune cells, namely microglia and astrocytes, which dichotomously and somewhat paradoxically contribute to both myelin damage and regeneration. Clinically, animals with EAE typically present with worsening ascending paralysis followed by partial recovery, coupled with significant weight loss. Functional motor impairments directly reflect the extent of immune cell entry and myelin damage.

The EAE model boasts many advantages; it is inducible in several species including rodents and non-human primates and can be induced under various conditions by altering the myelin antigen used, animal strain and age, and method of induction to allow study of specific aspects of disease. That said, it best recapitulates aspects of highly inflammatory active lesions, making it suitable for investigating pathologies associated with RRMS but not with progressive forms of MS in which remyelination failure occurs largely in the absence of peripheral inflammatory influence. Furthermore, demyelination and remyelination are often concurrent during the course of EAE, making it even less suitable for the directed study of remyelination or potential remyelinating therapies. Toxic models of demyelination are better suited for this purpose. Focal demyelination can be induced by directed injection of lysolecithin or ethidium bromide into a selected CNS region, resulting in specifically timed and predictable focal lesion formation and subsequent repair (40, 41). These models
are surgical in nature however, necessitating some degree of trauma which in itself stimulates an inflammatory response that may additionally complicate the study of remyelination in the context of chronic/progressive MS. For this reason, the most commonly used method of toxic demyelination for the study of remyelination is oral administration of the toxin cuprizone. This leads to regional death selectively of mature OLs, resulting in demyelination primarily in the corpus callosum, cortex and hippocampus. While cuprizone is best known as a copper ion chelator, its mode of action in inducing OL apoptosis is not completely understood as simultaneous copper supplementation does not negate demyelination (42). It does however vastly alter mitochondrial dynamics and disrupt energy metabolism in a manner independent of copper chelation and has more recently been shown to alter iron metabolism in OLs, a process requiring critical balance particularly in these cells, suggesting it may not selectively chelate only copper (43-45). Regardless of mechanism, it does reproducibly and reliably result in regional demyelination. Cuprizone administration can be performed over a shorter time course, typically 5-6 weeks, after which withdrawal from cuprizone results in spontaneous and efficient remyelination in affected areas. Conversely, prolonged exposure of 12 weeks leads to extremely limited remyelination even after cuprizone withdrawal (46-48). These differences in administration provide the opportunity not only to compare microenvironmental differences between tissues that are permissive versus reticent to remyelination, but also to investigate therapeutic targets specifically in a model in which remyelination does not readily proceed and is free of peripheral immune influence - an advantage in progressive MS research afforded by no other model to date.
1.2 Oligodendrocytes

The therapeutic promotion of remyelination must be underpinned by a thorough understanding of OL biology. These specialized glial cells are the sole producers of CNS myelin, and the differentiation program they undergo to reach maturity is at once complex, exquisite, and extremely susceptible to perturbation. The final product of OL differentiation is myelin, arranged in discontiguous internodes along axons. In addition to the trophic support and physical protection discussed earlier, the increased resistance and reduced capacitance conferred upon the underlying axon by the insulating properties of these internodes prevents depolarization of the axon membrane, and concentrates the factors required to generate action potentials into the unmyelinated spaces between internodes, the Nodes of Ranvier. This configuration of course leads to the saltatory nerve conduction required for efficient signal propagation along the axon. Together, these functions define the critical importance of myelin in the CNS.

Oligodendrocyte progenitor cells

The precursors to mature OLs are oligodendrocyte progenitor cells (OPCs). Developmentally, OPCs first originate from neuroepithelial stem cells in ventral ventricular zones of the neural tube, in close proximity to the floor plate (49-51). Later, secondary sources arise from dorsal telencephalic ventricular zones. Interestingly, specific regions of the CNS are preferentially myelinated by either dorsally- or ventrally-derived OPCs; however, ablation of one pool can be compensated for by the other, suggesting that despite their physical separation during development and discrete destinations as
myelinating cells, these two OPC populations are not biologically distinct from one another (52, 53). The adult CNS continues to generate OPCs, but these arise from neural stem cells of the subventricular zone (54). OPCs are found everywhere in the brain and spinal cord parenchyma throughout life, where they proliferate, migrate and differentiate based on environmental cues.

Morphologically, OPCs display a relatively simple form with only a few processes (Figure 1.2 A). Their membranes include some components largely unique to OPCs, such as the chondroitin sulphate proteoglycan (CSPG) NG2 and the ganglioside A2B5 (50, 55). They are additionally molecularly defined by the expression of genes that act to first specify their identity, and second to respond to particular growth factors (Figure 1.2 B). Multiple transcription factors are required to control regulation of OPC genes such as Olig1, Olig2, myelin transcription factor 1 (MyT1), mammalian achaete scute homolog 1 (Mash1), hair and enhancer of split 5 (Hes5), sex determining region Y box 5, 6 and 10 (Sox5, Sox6, Sox10), and inhibitor of DNA binding 2 and 4 (Id2 and Id4) (56-61). Of these, Olig2 uniquely specifies OPC identity, while the balance collectively promote proliferation/inhibit differentiation of these cells (56, 62).

The ability to respond to growth factors is the other defining characteristic of OPCs. Two growth factors especially critical in maintaining OPCs in their proliferative and migratory state are platelet-derived growth factor AA (PDGF-AA) and fibroblast growth factor 2 (FGF2 or bFGF) (57, 63-65). In addition to these, other FGFs such as FGF8, FGF17 and FGF18 also contribute to maintenance of the OPC identity, as well as acetylcholine (Ach (65, 66)). Control over sensitivity to these growth factors is dictated by the expression of
the cell-surface receptors that bind them. PDGF receptor α (PDGFRα), FGF receptor 1 (FGFR1), FGFR3 and muscarinic receptors M1, M2 and M3, binding PDGF-AA, FGF2, FGF8/17/18 and Ach respectively, are all highly expressed in OPCs and significantly downregulated in maturing OLs.

**OL differentiation and CNS myelination**

The transition from mitotic, migratory OPC to maturing OL requires a quick and complex reorganization of the cellular molecular landscape. Upon exposure to extrinsic factors such as triiodothyronine (T3), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF-1), the expression of almost all OPC-specific genes is overridden and the OL differentiation program is initiated (Figure 1.2 B). Upregulation of a host of new transcription factors occurs, including transcription factor 7 like 2 (Tcf7l2), myelin gene regulatory factor (MYRF), Smad-interacting protein 1 (Sip1), and homeobox protein Nkx2.2 (67-70). Most factors associated with OPC identity, including Hes5, Sox5, Sox6, Id2, Id4 and all growth factor receptors are urgently and strongly downregulated upon transition to maturing OL. Some however, such as Olig1, Olig2, Myt1, Mash1 and Sox10 are constitutively expressed, though they may serve different roles during differentiation than they did in OPCs.
Figure 1.2 Oligodendrocytes undergo complex differentiation both morphologically and molecularly. A. Morphological OL differentiation is characterized by a change from simple oligodendrocyte progenitor cells (OPCs) to maturing OLs that become increasingly ramified until they contact and wrap axons to form compact myelin internodes. B. Molecular OL differentiation is underpinned by a downregulation of OPC-specific factors (blue bars) including both proteins and miRNAs, followed by upregulation of OL-specific proteins and miRNAs (red bars) temporally regulated as OLs progress through the maturation program. A subset of transcription factors is constitutively expressed in both OPCs and OLs (purple bar).
This extensive reprogramming of the transcriptome with the switch from OPC to maturing OL drives equally extensive reorganization of the cell’s morphology (Figure 1.2 A). To go from a relatively simply morphological OPC to an OL that contacts, wraps and produces internode-forming myelin, the cells must extend increasingly complex processes mediated by both microfilament and microtubule reorganization (71, 72). Nascent membrane protrusions are driven by localized actin polymerization and extension of microfilaments to form filopodia. Additional microfilaments are constructed to extend filopodia into lamellipodia, at which point microtubules are extended into the process to continue its elongation. Primary branches, which extend directly from the cell body, become further ramified producing secondary, tertiary and quaternary branches until a complex network has been established to maximize the OL’s ability to locate and contact axons. The cytoskeletal drivers of this process include Src family kinase Fyn, Wiscott-Aldrich syndrome protein (N-WASP), WASP family verprolin homologous 1 (WAVE1) and focal adhesion kinase (FAK), which drive actin dynamics and microfilament assembly, as well as tubulin polymerization promoting protein (TPPP/p25) to regulate microtubule formation (73-77).

As OLs develop both morphologically and molecularly, they are of course most importantly characterized by their expression of myelin proteins. The developing OL membrane will eventually contact and wrap axons in concentric circles in an inside-outside manner, building layers of an internode with the continued growth of an inner tongue directly next to the axon that continuously pushes existing layers outwards, and building width of an internode by lateral membrane expansion as concentric layers are laid down.
Following exit from the cell cycle and commitment to terminal differentiation as an OL, one of the first myelin proteins expressed is 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP). CNP is involved in the earliest events of myelination by promoting microtubule-driven process extension, and later in balancing compaction in myelin to maintain integrity of cytosolic channels required both for intracellular transport and delivery of nutrients to an underlying axon (79, 80). Myelin associated glycoprotein (MAG) and myelin proteolipid protein (PLP) are next expressed, followed by myelin basic protein (MBP). MAG, though quantitatively minor in myelin, is critical to OL-axon interactions and in mature OLs is found embedded in the innermost membrane layer of internodes in direct contact with the axon (81). Both PLP and MBP constitute major components of myelin, and both serve to compact myelin by drawing adjacent layers of membrane towards each other on the extracellular and intracellular sides of the membrane lipid bilayer, respectively (72, 82). PLP is a transmembrane protein; when wrapping of the membrane around an axon occurs, the exoplasmic portions of PLP interact as wrapping membranes stack on top of each other, drawing the outer leaflets of apposed bilayers together. Within the cytoplasm, MBP functions by spanning the space between and adhering to both inner leaflets of the membrane bilayer, then drawing them together. This compaction leads to significant extrusion of cytoplasm from inside the myelin membrane as well as exclusion of extracellular fluid, further enhancing the lipid-rich character that confers the insulating properties of myelin (78). Finally, the ultimately expressed myelin protein is MOG; similar to MAG, it constitutes a minor proportion of the myelin proteins. It is a member of the IgG superfamily and is embedded only in the outer membrane of compacted myelin (83).
Interestingly, loss of MOG in animal models does not lead to any alterations in myelin ultrastructure or function (83). MOG function was previously unknown, but recently it was identified as a binder of nerve growth factor (NGF) and was shown to modulate axon outgrowth in localized areas of the spinal cord (84).

Historically, all OPCs and OLs were believed to be equal; that is, cells at similar stages of development were thought to be molecularly homogeneous by comparison. Advances in single cell RNA sequencing recently identified no fewer than twelve different subpopulations of OPCs, immature and mature OLs as characterized by their transcriptional signatures, suggesting there is a much greater degree of heterogeneity amongst these cells than was previously understood (85). While these data open up critical discussion, it is important to note that functional differences have yet to be identified between subgroups of OPCs or OLs at similar stages of differentiation. Interestingly, a greater argument can be made that the purported subpopulations are more reflective of plasticity than true heterogeneity, as mounting evidence shows that both OPC and OL phenotypes are highly influenced by environmental cues, which can differ for reasons as clear cut as the difference between white and grey matter, or as subtle as small changes in neuronal activity (86-90). Thus, the identified subpopulations may indicate transcriptional changes as a consequence of extrinsic influences, not intrinsically-driven heterogeneity, and that cells of supposedly different subpopulations may in fact become phenotypically homogeneous if assessed in the same environment. Whatever the conclusion of continued investigations in this area, it is clear that the course of OL differentiation is governed by an array of both
intrinsic and extrinsic influences that must act in highly regulated concert to achieve successful OL maturation and myelination.

**Remyelination and OLs in the MS lesion microenvironment**

In healthy white matter, myelination continues to take place throughout adulthood to replace myelin lost in the course of normal oligodendrocyte death (91). Mature oligodendrocytes are not commonly involved in this process. Instead, a demyelinating event is followed by the recruitment of OPCs distributed throughout the CNS to the demyelinated area; these cells then differentiate and remyelinate denuded axons (Figure 1.3 A) (92, 93). While newly established internodes tend to be shorter, thinner and more numerous within a given axon length in comparison to myelin sheaths created during development, they appear to be equally efficient in allowing conduction of saltatory nerve impulses (91, 94). Importantly, in addition to restoring saltatory conduction, remyelination is critical to protecting axons from degeneration (95, 96). Remyelination also occurs in RRMS, but at some point, this capacity disappears and there is complete remyelination failure – i.e., the transition to SPMS. Periods of recovery for RRMS patients coincide with remyelination within lesions, which occurs with greatest efficiency early in the disease (97). As remyelination declines and eventually ceases to occur, fewer and less complete remission phases arise, and patients typically transition to an SPMS presentation. Further, patients who present with PPMS exhibit little to no remyelination from disease onset. This paucity of remyelination in progressive MS leads to neurodegeneration and permanent disability (95).
Figure 1.3 RRMS and progressive MS exhibit disparate OPC behaviours at the site of myelin damage. A. In healthy white matter and in RRMS lesions, OPCs recruited to the site of myelin damage undergo successful differentiation and remyelinate denuded axons. B. In progressive MS, OPCs are recruited to the site of myelin damage, but fail to differentiate and thus fail to remyelinate denuded axons. Axons left unmyelinated degenerate, resulting in irreversible neurological damage.
Tackling the therapeutic promotion of remyelination must first beg the question of why, in progressive forms of MS, does remyelination fail? Progression, particularly in SPMS, does coincide with chronicity of disease and therefore an aging patient; because of this, many believed that age itself was the culprit as remyelination does become less efficient with time, suggesting that older oligodendrocyte progenitor cells (OPCs) are less capable of remyelination (98, 99). However, that explanation does not satisfy why some RRMS patients convert to SPMS in their 50s while others last into their 70s; nor does it explain the inability of PPMS patients to remyelinate at any point when diagnosis typically occurs in their 40s or 50s (100). In fact, the notion that aged OPCs cannot remyelinate has been strongly challenged with evidence to the contrary (94, 101). Reasons for this remyelination failure may be multi-faceted. Recruitment of OPCs to the area may be impaired due to an overexpression of chemorepellants such as Semaphorin A (102); however, many lesions in chronic and progressive disease are replete with OPCs and early OLs that simply never differentiate, indicating that a lack of OPCs is not the cause for loss of remyelination (103-106). Instead, what appears to affect the capacity for remyelination is the environment in which OPCs are challenged to remyelinate; factors such as chondroitin sulphate proteoglycans (CSPGs), polysialylated neural adhesion molecule (PSA-NCAM), leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 1 (LINGO-1), Galectin-4, Jagged, and inflammatory cytokines along with myelin debris itself are present in lesion tissue, and all negatively regulate OL differentiation (33, 94, 107). Additional inhibitory factors continue to be uncovered. Various small molecules have been discovered which promote the differentiation of OPCs and initially generated excitement as
potential therapeutic targets to promote remyelination (108, 109). Unfortunately, none of these small molecules are able to promote remyelination in the face of challenges similar to those present in the progressive lesion environment (110). As such, remyelination therapies still signify an important opportunity for MS treatment. Such therapies could benefit not only patients with SPMS and PPMS for whom there are currently no effective strategies to slow disease progression, but also RRMS patients through combination of therapeutics to both relieve immune-mediated attack on myelin and enhance remyelination.

1.3 MicroRNAs

MicroRNAs (miRNAs) are short, non-coding RNAs that post-transcriptionally regulate gene expression. Typically 18-25 nucleotides long, they bind most often to the 3’ untranslated region (UTR) of mRNA, leading to degradation or inhibition of translation of the target (111, 112). Any given miRNA can have multiple targets, and any given mRNA may be targeted by multiple miRNAs. It is estimated that miRNAs are involved in regulating up to 60% of mammalian mRNAs, and are critical in development, apoptosis, cell-cycle control, stress responses and cellular differentiation (113-117). Their roles in gene expression are critical to the point that dysregulation of microRNA expression is linked to disease states including cancer, neurodevelopmental disease, cardiovascular disease and autoimmune disease, amongst others (111, 118).
Biogenesis and function

There are both canonical and non-canonical miRNA biogenesis pathways. Typical biogenesis begins with transcription of a primary miRNA (pri-miRNA) by RNA polymerase. Pri-miRNAs contain hundreds of nucleotides that fold upon themselves to adopt a hairpin secondary structure. They are then further processed in the nucleus by the microprocessor complex made up of RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and ribonuclease III enzyme Drosha which collectively cleave the pri-miRNA hairpin (111, 119, 120). The approximately 60 nucleotide product, pre-miRNA, is then exported to the cytoplasm by the Exportin 5 (XPO5)/RanGTP complex embedded in the nuclear membrane (111, 121). Pre-miRNAs retain a terminal loop, which is then cleaved in the cytoplasm by RNase III endonuclease Dicer to produce a short double-stranded RNA fragment (111). Only one strand is then loaded into the miRNA-induced silencing complex, or miRISC. This protein complex effects miRNA:mRNA binding and the subsequent translational inhibition or degradation of mRNA. The core member of the miRISC is a member of the Argonaut family (Ago), which binds a single mature miRNA strand while the other is discarded and then degraded. Non-canonical processing occurs of pre-miRNAs that arise from introns after mRNA splicing in the nucleus; these species, called mirtrons, are directly shuttled out of the nucleus by Exportin-5 without passing through DGCR8/Drosha, and are further processed in the cytoplasm as detailed above beginning with Dicer-mediated conversion to a double-stranded RNA fragment. Additionally, non-canonical m$^7$G-capped pre-miRNAs are transcribed and then exported from the nucleus by Exportin-1, where they then also undergo the balance of the canonical pathway beginning with Dicer processing. Mature
miRNAs from both canonical and non-canonical sources are indistinguishable from each other and function in the same manner.

In animals, the degree of miRNA:mRNA binding complementarity is conferred not by the miRNA sequence in its entirety but by a short sequence at the 5’ end of miRNA known as the seed region. It comprises the first 6-8 nucleotides of the miRNA, and several known patterns of nucleotide pairings between the seed and 3’ UTR of the target can elicit regulatory control over that mRNA’s fate. Though binding sites in regions other than the 3’ UTR do exist, generally miRNA-binding outside the path of the ribosome allows the most efficient regulation by miRNAs (113). Interestingly, the extent that the seed region pairs with the target site influences the type of regulation prompted by miRNA binding (122, 123). Perfect complementarity incites direct endonucleolytic activity of miRISC-associated proteins such as Ago2 (123, 124). More commonly, partial complementarity leads to degradation of the target mRNA via deadenylation which in turn leads to mRNA uncapping (123-125); this leaves the mRNA vulnerable to cytoplasmic exonucleases with the general job of degrading any susceptible single-stranded RNA fragments (125). Inhibition of translation without mRNA degradation is also possible, resulting in reduced target protein but not target mRNA by mechanisms which remain under investigation (122).

The influences of miRNAs are much more complex and nuanced than was originally understood in the early years after their discovery. Control over the timing and location of miRNA-mediated gene regulation extends beyond transcription and biogenesis; sub-cellular localization as well as cellular export via small vesicles such as exosomes respectively act to sequester miRNAs to specific regions or organelles inside the cell or export them into the
extracellular space from whence they can be taken up and become active in other cells or even other tissues (126). Not only has this expanded comprehension of how miRNA control over gene expression is itself regulated, but has also highlighted their potential as biomarkers in various diseases including MS, amongst many others, and as therapeutically-deliverable molecules due to their ease of packaging and cellular uptake in exosomes and similar biomimetic vehicles (127-129).

**miRs are required for OL differentiation**

During the course of oligodendrocyte differentiation, miRNAs add an additional layer of regulation to an already complex molecular landscape. Conditional knockout of *Dicer1* in neuronal precursors of late embryonic mice, leading to a complete lack of mature miRNA biogenesis, results in insufficient numbers of OPCs and mature OLs generated from the ventral spinal cord pool (130). When knocked out specifically from OPCs, *Dicer1* deletion results in severe OL differentiation and myelination defects during CNS development (131, 132). Lack of *Dicer1* in mature OLs in adult mice reduces OL numbers and drives demyelination, leading to neurodegeneration in already-established major white matter tracts (133).

Some specific miRNA species have been identified that exhibit strong differential regulation across the OL differentiation program (Figure 1.2 B). In human OPCs, miR-9, miR-214, miR-199a-5p and miR-145-5p are highly expressed relative to maturing and mature OLs (134). Collectively, these target or are predicted to target myelin-specific factors whose expression would trigger differentiation or membrane compaction – functions not desirable
in OPCs until appropriate conditions arise (134, 135). Conversely, miRs -219, -338, -138 and -297c are differentially upregulated as OPCs transition to OLs (131, 132, 134, 136). These miRNAs jointly serve two functions; the first, to repress promoters of proliferation such as growth factor receptors PDGFRα and FGFR1, and transcription factors Hes5 and Sox6. Secondly, they target factors that actively block differentiation and myelin formation, such as phosphatase and tensin homolog (PTEN) and lamin B1 (LMNB1). Importantly, exogenous individual application of each of these pro-differentiation miRNAs is sufficient to promote differentiation of OPCs to OLs both in vitro and in vivo (131, 132, 136).

**miRs are dysregulated in MS**

In contrast with the carefully timed and executed expression of miRNAs over the course of OPC proliferation and OL differentiation, miRNAs exhibit widespread dysregulation in MS. This dysregulation can be found in lesions, cerebrospinal fluid (CSF), serum, B cells, multiple types of T cells, and most recently even in the gut microbiome (137-142). A host of specific miRNAs have been specifically investigated in this context, while the roles of even more have yet to be determined. Studies in this area show not only compartment-specific changes in miRNA expression profiles, but that many of these changes are specific to RRMS versus SP or PPMS, allowing differentiation of MS subtypes from each other as well as from healthy control (143, 144).

Interestingly, retrospective investigation has revealed that many already-established DMTs for RRMS have some important effects on miRNA expression. Fingolimod, natalizumab and interferon-β all appear to have both direct and indirect effects on the
miRNA expression profiles of CD4+ T cells and, in some cases, serum (145-150). Each affects the expression of several different miRNAs involved in suppressing the differentiation of Th17 cells, which are heavily involved in the autoreactive response against CNS myelin in RRMS. Further, after time on the DMT the broader circulating miRNA profile more closely resembles that of a healthy or remitting patient in those responding to treatment. Altogether these findings underscore the huge potential for the use of miRNAs as biomarkers not just in diagnosis but also in success of treatment, and as therapeutic targets in MS.

1.4 Research plan and specific aims

A specific miRNA, miR-145-5p, is expressed at relatively high levels in human OPCs but is downregulated by ~90% as they transition to differentiating OLs (134). Interestingly, miR-145-5p is over-represented in progressive MS lesions relative to both healthy white matter and relapsing-remitting MS lesions, setting it apart as a discriminating factor of the progressive lesion microenvironment (137). Additionally, miR-145-5p is upregulated in peripheral blood mononuclear cells (PBMCs), serum and plasma from treatment-naïve RRMS and early SPMS patients (151, 152). This miRNA is predicted to target factors with known importance in OL differentiation such as MYRF, Sox2 and Tcf7l2/Tcf4, as well as inflammatory factors through both direct and indirect mechanisms such as Nurr1 and interferon β (153-156).

Based on its differential regulation in OLs and myelin disease-related dysregulation, we hypothesized that miR-145-5p contributes to the pathogenesis of demyelinating disease. To
assess the validity of our hypothesis, our objectives were to investigate the importance of
the differential regulation of miR-145-5p expression in OPCs and OLs using a primary cell
culture model in vitro, and to investigate how loss of this miRNA affects disease progression
and remyelination following both toxic and inflammatory demyelination in vivo. The
following aims will address these objectives:

   Aim 1 – Investigation of the importance of miR-145-5p differential expression in OPCs
and OLs and identification of downstream mediators of miR-145-5p expression levels.

   Aim 2 – Characterization of the course of acute and chronic cuprizone-mediated
demyelination in a miR-145 knockout mouse.

   Aim 3 – Assessment of the pathological and clinical outcomes in experimental
autoimmune encephalomyelitis in a miR-145 knockout mouse.
Chapter 2

Materials and Methods
**Cell culture**

Primary rat OPCs were isolated and cultured as per Chen et al. (157). First, mixed glial cultures (MGCs) were generated by dissecting cerebral cortices from P1-P3 Sprague-Dawley rat pups. Cortices were digested for 15 min at 37°C in 1% trypsin (Sigma-Aldrich) with 10.7 µg/mL DNase I (Sigma-Aldrich) in Hank’s Balanced Salt Solution (HBSS). Digested tissue was triturated in MGC medium made up of Dulbecco’s Modified Eagle’s Medium (DMEM; Wisent) with 20% FBS, 2 mM L-glutamine (Invitrogen), 50 units/mL penicillin and 50 µg/mL streptomycin (Thermo-Fisher). Triturated tissue suspensions were allowed to settle on ice for 10 min followed by filtration through a 70 µm strainer and were then incubated in MGC medium on poly-L-lysine (PLL)-coated plates at 37°C and 5% CO₂ for 7-10 days with 75% MGC medium changes every 2-3 days. To obtain enriched OPC cultures, MGCs were shaken for 18-20 h at 37°C and 5% CO₂. Medium containing suspended OPCs was allowed to settle on coated culture plates (Corning) for 30 min at 37°C and 5% CO₂ to remove contaminating cells by differential adhesion. OPCs were pelleted from suspension by centrifugation at 100 x g for 10 min and were then resuspended in appropriately supplemented SATO medium. SATO medium comprised 5 µg/mL insulin, 1 µg/mL bovine serum albumin (BSA), 0.05 µg/mL apo-transferrin, 0.03 µg/mL sodium selenite, 0.25 µg/mL D-biotin and 0.01 µg/mL hydrocortisone (all reagents from Sigma-Aldrich) in DMEM (Wisent) with 2 mM L-glutamine (Invitrogen) and 50 units/mL penicillin and 50 µg/mL streptomycin (Thermo-Fisher). OPCs were plated in SATO medium supplemented with 0.01 µg/mL basic fibroblast growth factor (FGF2; Millipore) and 0.01 µg/mL platelet-derived growth factor AA (PDGFAA; Millipore), and 50% media changes were done every 2 days.
OPCs being differentiated into OLs were plated in SATO medium supplemented with 15 nM triiodothyronine (T3; Sigma-Aldrich), 0.01 µg/mL ciliary neurotrophic factor (CNTF; Peprotech) and 5 ng/mL N-acetyl L-cysteine (Sigma-Aldrich). To transition OPCs to OLs after plating, a 50% media change was done using 2X differentiation medium.

Primary mouse OPCs were isolated and cultured as per O’Meara et al. (158). First, MGCs were generated from P0-P2 miR-145+/+ and miR-145−/− mouse pups. Cortices were dissociated with papain, and the resulting suspension was plated in Dulbecco’s modified Eagle medium (DMEM; Wisent) containing 10% FBS, 1% Glutamax (Gibco), 5 µg/mL insulin (Sigma) and 0.33% penicillin/streptomycin (Gibco). These cells were plated in poly-L-lysine-coated filter cap flasks for 6 days and maintained at 37°C and 8.5% CO2. The medium was then supplemented with 5 µg/mL insulin (Sigma), and cultures were moved to 5% CO2 for an additional 3-4 days. Cultures were then shaken overnight at 220 rpm, and medium containing suspended OPCs was removed and further enriched by separating out contaminating glial cells by differential adhesion as above. The OPCs were then plated in SATO medium with 0.5% fetal bovine serum, 1% Glutamax, 5 µg/mL insulin, 50 µg/mL holo-transferrin (Sigma), 50 ng/mL ciliary neurotrophic factor (CNTF; Serotec) and 0.33% penicillin/streptomycin on coverslips dual-coated with poly-L-lysine and kept at 37°C and 5% CO2 until collection.

HEK293T cells were cultured in DMEM with 10% FBS, 1 mM L-glutamine (Invitrogen), 33 units/mL penicillin and 33 µg/mL streptomycin (Thermo-Fisher) at 37°C and 5% CO2 in flasks coated with 1 mg/mL PLL (Sigma-Aldrich). Cells were split as required by incubating in 0.05% trypsin-EDTA (Millipore) for 1-2 min at 37°C and 5% CO2.
**Lentiviral transduction**

Primary rat OPCs were plated in OPC medium as above at a density of $7 \times 10^3$ cells/well in a 24-well plate or $7 \times 10^4$ cells/well in a 6-well plate, and were allowed to recover for 48 h. After 48 h, cells were infected with Lenti-GFP-miR virus (Applied Biological Materials) to overexpress rat miR-145 (rno-miR-145) or scrambled non-targeting miRNA, or with Lenti-GFP-miR-off virus (Applied Biological Materials) to inhibit rno-miR-145-5p or scrambled non-targeting miRNA at a MOI ~5 in proliferation medium supplemented with 0.5 ug/mL polybrene (Sigma-Aldrich). After 24 h, cells were washed by performing 3 consecutive 60% media changes with fresh OPC medium. Following an additional 24 h, cells were either fixed or lysed for analysis of OPCs, or underwent a 50% media change to 2X differentiation medium for transition to OLs. Differentiating OLs were then fixed or lysed at differentiation day 2.5 (DD2.5) and DD5 for further analysis.

**Small RNA transfection**

MiR-145-5p mimic, scrambled miRNA mimic, miR-145-5p inhibitor, and scrambled miRNA inhibitor were all used at a concentration of 30 nM. MiR-145-5p mimic sequence is identical to mature miR-145-5p based on Sanger mirbase (mirbase.org). Inhibitor sequences are proprietary. siRNA against MYRF and scrambled siRNA control were also used at a concentration of 30 nM. All small RNAs for transfection were obtained from Applied Biological Materials (ABMgood). The siRNA duplex sequences for siMYRF are as follows: forward strand 5'-GCUGCCUAUGCUACCUCUATT-3', reverse strand 5'-AUAGGUGAGCAUAGGCAGCTT-3'.
Cells were transfected using INTERFERin siRNA transfection reagent (Polyplus) according to the manufacturer’s protocol. In brief, 30 pmol miRNA mimic or siRNA were prepared in 50 µl Opti-Mem serum-free media (Thermo-Fisher) and vortexed. To this, 1.5 µl INTERFERin was added, the mix was again vortexed, and lipid:RNA complexes were allowed to form for 10 min at room temperature. This transfection mix was added at a ratio of 50 µl for every 1 mL of cell media. For OPCs, transfected cells were assayed 24 h post transfection; for OLs, transfected cells were assayed 5 days post transfection.

RNA isolation and qRT-PCR

For all samples, total RNA was extracted using the RNeasy Mini Kit, including proprietary buffers “RLT”, “RW1” and “RPE” (Qiagen), according to the manufacturer’s protocol. For primary culture, cells grown in a 6-well plate were lysed using 350 µl RLT lysis buffer per well. Well bottoms were scraped with a spatula, lysate was placed in a Qiashredder column (Qiagen), and spun at maximal speed for 2 min. For tissues, brain, spinal column, spleen and thymus were flash frozen immediately following collection. A maximum of 30 mg tissue was used per sample. For RNA isolation, 350 µl or 700 µl of RLT buffer were added directly to frozen tissues, which were then homogenized. Continued processing after this initial step was the same for both cells and tissue. Lysate was then mixed 1:1 with 70% ethanol in RNase-free H₂O, transferred to the RNeasy spin column, and centrifuged at maximal speed for 25 s. Eluate was discarded, and the column was washed with 700 µl RW1 buffer by spinning at maximal speed for 25 s. Eluate was again discarded, and the column was washed with 500 µl RPE buffer at maximal speed for 25 s. The columns
were transferred to a clean collection tube and washed a second time with 500 µl RPE for 2 min. Columns were then placed in RNase-free microfuge tubes, and RNA was eluted in 20-30 µl RNase-free H₂O by spinning at maximal speed for 1 min.

Reverse transcription of mature miR-145-5p and snU6 was done according to Biggar et al. (159) with some modifications. In short, 300 ng total RNA was incubated with 5 µL 250 nM stem-loop primer in a total volume of 10 µL. This annealing reaction was carried out at 95°C for 5 min, followed by 60°C for 5 min. Samples were immediately centrifuged and held on ice for 1 min. Reverse transcription was done using 1 µL M-MLV Reverse Transcriptase (Invitrogen), 4 µL 5x First Strand Buffer (Invitrogen), 2 µL 100 mM dithiothreitol (Invitrogen) and 1 µL premixed dNTPs (final concentration 25 µM each). Each reaction was brought to a 25 µL total volume using RNase-free water (Qiagen). The following protocol was used for reverse transcription: 16°C for 30 min, 60 cycles of 20°C for 30 s, 42°C for 30 s and 50°C for 1 s, followed by 85°C for 5 min, using an Eppendorf Mastercycler. Primer sequences for miRNA qRT-PCR are as follows: miR-145-5p stem-loop primer 5’-CTCACAGTACGTGTTATCGATGATGCTGATGCAATATTGTACTGTGAGAGGATTTC-3’, miR-145-5p forward 5’- ACACTCCAGCTGGGGTCCAGTTTTCCCAGG-3’, snU6 stem-loop primer 5’- CTCACAGTACGTTGTATCCTTGTGATGATGCTGATGGAATTTTCCAGG-3’, snU6 forward 5’- ACACTCCAGCTGGGGTCCAGTTTTCCCAGG-3’, universal reverse 5’- CTCACAGTACGTTGTATCCTTGTGATGATGCTGATGAGAAAAATATGGAACGCTT-3’, snU6 forward 5’- ACACTCCAGCTGGGGTCCAGTTTTCCCAGG-3’, universal reverse 5’- CTCACAGTACGTTGTATCCTTGTGATGATGCTGATGAGAAAAATATGGAACGCTT-3’, snU6 forward 5’- ACACTCCAGCTGGGGTCCAGTTTTCCCAGG-3’, universal reverse 5’- CTCACAGTACGTTGTATCCTTGTGATGATGCTGATGAGAAAAATATGGAACGCTT-3’. Universal reverse primer was used for both miR-145-5p and snU6 amplifications.

Amplification of miR-145-5p and snU6 cDNA was done using specific forward primers and a universal reverse primer complementary to the stem-loop portion of the
cDNA. Each qRT-PCR reaction contained 12.5 μL 2x SsoFast EvaGreen Supermix (Bio-Rad), 0.8 μL each of 25 μM forward and universal primer, and 4 μL cDNA, filled to 25 μL total with RNase-free water (Qiagen). Samples were amplified using the following protocol: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, using a Bio-Rad CFX Connect. All samples were run in technical triplicate. Primer validation was done by standard curve efficiency analysis, melt curve analysis, and electrophoresis of qPCR products on a 5% agarose gel to verify product size. Primers for miRNA qRT-PCR were obtained from AlphaDNA. All stemloop, forward and universal primer sequences can be found in Supplementary Material A Table A2. Relative expression analysis was conducted using the CFX Manager™ software or CFX Maestro software using the ΔΔCt method, as previously described (160).

For mRNA analysis, total cDNA was constructed using the RT² First Strand Kit (Qiagen) as per the manufacturer’s protocol. Briefly, 2 μL Buffer GE (Qiagen) was used to eliminate genomic DNA from 150-200 ng total RNA in RNase-free water in a total volume of 10 μL per sample. Samples were incubated at 42°C for 5 min and then held on ice for at least 1 min. Reverse transcription was performed by adding 4 μL 5x Buffer BC3 (Qiagen), 1 μL Control P2 (Qiagen), 2 μL RE3 Reverse Transcriptase Mix (Qiagen) and 3 μL RNase-free water (Qiagen), followed by incubation at 42°C for 15 min and 95°C for 5 min. Samples were diluted with 91 μL RNase-free water (Qiagen) and stored at -20°C until use.

For mRNA qRT-PCR, PrimePCR pre-optimized primers (Bio-Rad) were used according to the manufacturer’s protocol. Briefly, reactions contained 4 ul total cDNA, 10 μl SsoFast EvaGreen master mix (Bio-Rad), and 1 ul forward/reverse primer mix in a total volume of 20
ul. Amplification was performed at 95°C for 5 min, followed by 40 cycles of 95°C for 5 s then 60°C for 30 s in a Bio-Rad CFX Connect. Relative expression analysis was conducted using the CFX Manager™ software or CFX Maestro software using the ΔΔCt method.

For spinal cord samples from EAE, lumbar spinal cords were collected and divided in half longitudinally. RNA was extracted as described above for tissue using the RNeasy Mini Kit (Qiagen). Gene expression was assessed for 84 genes using the Multiple Sclerosis RT² Profiler PCR Array (Qiagen). Using the RT² First Strand Kit (Qiagen) as above, 500 ng of RNA was used to generate total cDNA. Master mix was prepared for the array as per the manufacturer’s protocol. Briefly, each reaction contained 1 μL of reverse transcription products, 12.5 μL 2x RT² SYBR Green Mastermix (Qiagen) and 11.5 μL RNase-free water (Qiagen). Amplification was carried out at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min using a Bio-Rad CFX Connect. Relative expression was calculated using the RT² Profiler PCR Data Analysis software version 3.5, using the ΔΔCt method with significance determined by adjusted Student’s t-test.

**Immunofluorescence**

Cells were fixed in 3% paraformaldehyde (PFA) for 15 min, washed 2 x with PBS, followed by permeabilization in 0.1% Triton-X in PBS for 5 min. After washing again 3 x with PBS, cells were incubated in blocking medium (10% goat serum in PBS) for ~1 h at 4°C. Incubation with primary antibody was done in 10% goat serum overnight at 4 C, followed by 3 x washes with 1x PBS and 2-3 h incubation with secondary antibody at 4 C. F-actin was visualized using rhodamine-conjugated phalloidin (Invitrogen) 1:50 in blocking serum for ~2
h at 4°C. Nuclear staining was achieved using Hoechst 1:1000 in PBS for ~5 min at room temperature. Coverslips were mounted in Dako Fluorescence Mounting Medium. Primary antibodies were diluted in blocking serum at the following concentrations: rabbit anti-Olig2 1:500 (EMD Millipore); rat anti-MBP 1:100 (AbD Serotec), mouse anti-MAG 1:50 (EMD Millipore), mouse anti-alpha tubulin 1:1000 (Chemicon), rabbit anti-cleaved caspase 3 1:200 (Asp175; Cell Signalling Technology). Appropriate AlexaFluor secondary antibodies (Invitrogen) were diluted 1:200 in blocking serum.

**Proliferation Assay**

Proliferation in transduced/transfected OPCs was assayed using the Click-iT® Plus AlexaFluor 647 Imaging Kit (Invitrogen) as per the manufacturer’s instructions. EdU was added to the OPC medium for 6 h prior to fixation at an optimized concentration of 1 µM. Following 3% PFA fixation and 0.1% Triton-X permeabilization as outlined above, coverslips were washed 3 x with 3% BSA in PBS. Coverslips were then incubated in ~300 µl Click-iT® Plus Reaction Cocktail (264 ul 1x Click-iT® reaction buffer, 6 µl copper protectant, 0.72 µl AlexaFluor 647 picolyl azide, 30 µl reaction buffer additive) for 30 min at room temperature in the dark. Coverslips were washed 1 x with 3% BSA in PBS, 1 x with PBS alone, and were then blocked with 10% GS in PBS for 1 h to prepare for immunofluorescent staining as described above.
Identification of OL-enriched miR-145-5p putative targets

Putative target lists for miR-145-5p were generated using TargetScan Mouse version 6.1 (56, 84), using an algorithm based on miRNA seed region:3’ UTR complementarity and thermodynamic stability of target binding. Gene enrichment lists for OL-enriched factors relative to OPCs and for OL-enriched factors relative to all neural cell types were generated using the Brain RNAseq database (39). These three lists were compared pair-wise in the following manner: miR-145-5p targets versus OL-enriched factors relative to OPCs, miR-145-5p targets versus OL-enriched factors relative to all neural cell types, and OL-enriched factors relative to OPCs versus OL-enriched factors relative to all neural cell types. Targets common to all three comparisons were then assayed for expression following knock-down of miR-145-5p in OPCs, as outlined above. Targets exhibiting upregulation following miR-145-5p knock-down were selected for further investigation.

Dual luciferase assay

MiR-145 targeting of the MYRF 3’UTR was assessed by dual luciferase assay using the pEZX-MT01 vector containing both firefly and Renilla luciferase sequences (Genecopoeia). Briefly, the MYRF 3’UTR sequence was cloned downstream of the firefly luciferase gene in its wild-type form (NM_001033481.1), as well as with the following modifications in the putative miR-145-5p seed region binding sites: CUG -> GAC at positions 132-134 (mut 1) and ACU -> UGA at positions 1537-1539 (mut 2) individually as well as together in the same clone (mut 1+2). Plasmids containing wild-type and modified MYRF 3’UTR or target clone control vector were co-transfected into HEK293T cells with vector
pEZX-MR04 (Genecopoeia) carrying either miR-145 precursor or scrambled miRNA control. HEK293T cells were plated at a density of $6 \times 10^4$ cells/well in 24-well plates 24 h prior to transfection. Each well was then treated using 200 ng luciferase plasmid, 300 ng miRNA plasmid and 3 µl/mL Lipofectamine 2000 transfection reagent (Thermo-Fisher) in Opti-Mem serum-free media (Thermo-Fisher) according to the manufacturer’s protocol. Briefly, Lipofectamine 2000 was added to Opti-Mem and incubated at room temperature for 5 min. Plasmids were then added to this mix, and lipid-DNA complexes were allowed to form for 20 min at room temperature. Cells were washed with PBS, and Lipofectamine:DNA was added to each well along with HEK293T media as described above minus penicillin/streptomycin. Luciferase activity was assayed 24 h post transfection using the Luc-Pair miR Luciferase Assay Kit (Genecopoeia) and a Turner Biosystems 20/20 Luminometer.

**Animals**

Sprague-Dawley rats were obtained from Charles River. Animals were sacrificed at P1-P3 for primary cell culture. MiR-145$^{-/-}$ mice were generously provided by Dr. Eric Olson, and were generated on the C57BL/6 background as described in Xin et al. (161). Animals were sacrificed at P0-P2 for primary cell culture, at P0, P9, P15, P30 and P60 for developmental in vivo analyses, and at various time points following treatment with cuprizone and induction of EAE for in vivo analyses. All animals were provided water and chow ad libitum, and maintained on a 12:12 h light cycle.
The University of Ottawa Animal Care Committee approved all experimental protocols involving animals. The protocols conformed to or exceeded those defined in the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals, and the Animals for Research Act.

**Western blotting**

Protein was isolated by gently homogenizing tissue in 1x RIPA lysis buffer (Sigma) on ice. The lysate was centrifuged at 4°C at high speed to remove insoluble material. Samples were separated by SDS-PAGE in a 13% gel. Membranes were incubated in 1:1000 CNPase (Abcam), 1:1000 MOG (Abcam), 1:1000 MBP (AbD Serotec) and 1:50,000 alpha-tubulin (Cell Signaling) primary antibodies overnight at 4°C in Odyssey blocking buffer (Li-Cor Biosciences). Membranes were washed in 1X TBS for 3 X 5 min, then incubated with secondary antibody (IRDye 680RD and 800CW; Li-Cor Biosciences) at 1:20,000 in Odyssey blocking buffer for 1 h at room temperature, and finally washed again in 1X TBS for 3 X 5 min. Membranes were visualized and bands were quantified using the Li-Cor Odyssey CLx Infrared Imaging System.

**Cuprizone**

Chronic demyelination of the corpus callosum was achieved in miR-145+/+ and miR-145−/− animals by using the copper chelating agent cuprizone (oxalic bis-(cyclohexylidenehydrazide)). Cuprizone was milled into normal chow at a final concentration of 0.2% w/w (Envigo), and was fed *ad libitum* starting at P60 to both female
and male animals. For acute demyelination, animals were maintained on the cuprizone diet for 6 weeks and were then returned to normal chow for an additional 5 weeks. To achieve chronic demyelination, animals were maintained on the cuprizone diet for 12 weeks, as described previously, and then returned to normal chow for an additional 5 weeks (48). During cuprizone treatment, fresh food was provided and old food was removed daily. Control baseline animals were age-matched animals fed with normal chow. Behavioural analyses were performed at P60, P60 + 6 weeks cuprizone, P60 + 6 weeks cuprizone + 5 weeks recovery, P60 + 12 weeks cuprizone, and P60 + 12 weeks cuprizone + 5 weeks recovery. Animals weights were recorded and tissues were collected for biochemical, histological and transmission electron microscopy analysis at these same time points.

**Transmission Electron Microscopy (TEM)**

Mice were anesthetized via intraperitoneal injection of tribromoethanol (Avertin) and perfused transcardially with 5 ml of PBS followed by 10 ml-20 ml of Karnovsky’s fixative (4% paraformaldehyde (PFA), 2% glutaraldehyde and 0.1 M sodium cacodylate in PBS, pH 7.4). Whole brains and lumbar spinal cords were extracted and fixed overnight (or until processed) at 4°C in the same fixative. After fixation, corpus callosa were dissected and cut on either side of the midline under a stereomicroscope into straight segments of 1 mm of length. Specimens were subsequently washed twice in 0.1 M sodium cacodylate buffer for 1 h and once for overnight at room temperature. Segments were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature, and were then washed in distilled water three times for 5 min. Specimens were dehydrated
twice for 20 min for each step in a graded series of ethanol from water through 30%-50%-70%-85%-95% ethanol and 2 x 30 min in 100% ethanol, followed 2 x 15 min in 50% ethanol/50% acetone and 2 x 15 min in 100% acetone. Segments were then infiltrated in 30% Spurr resin/acetone for 20 min and once for 15 h (overnight), then in 50% Spurr resin/acetone for 6 h and in fresh 100% Spurr resin for overnight. Spurr resin was changed twice a day for three days at room temperature. All infiltration steps were performed on a rotator at low speed. Finally, specimens were embedded in fresh liquid Spurr resin and oriented inside the molds and then polymerized overnight at 70°C. Ultrathin sections (80 nm) were collected onto 200-mesh copper grids and stained with 2% aqueous uranyl acetate and with Reynold’s lead citrate.

**Immunohistochemistry and histology**

Mice were anaesthetized and perfused transcardially with 5 ml of PBS followed by 10-20 ml of 4% PFA. Brains and lumbar spinal cords were dissected and kept in the same fixative for 48 h and then transferred in 70% ethanol in water. Fixed brain and lumbar spinal cord were embedded in paraffin and sectioned by microtome in 20 µm intervals. Whole brain and lumbar spinal cord samples were processed at the Louise Pelletier Histology Core Facility, Department of Pathology and Lab Medicine University of Ottawa, where they were first embedded in paraffin wax with a LOGOS microwave hybrid tissue processor. Paraffin-embedded samples were cut by microtome at a thickness of 20 µm and mounted on slides.
Sections were deparaffinized and rehydrated prior to immunohistochemistry and staining in the following manner: slides were incubated at 59°C for 30-60 mins, and were then incubated in 100% Hemo-D (Histo-Clear, National Diagnostics) for 3 x 5 min, 50% Hemo-D/50% ethanol for 2 x 3 min, 100% ethanol for 2 x 3 min, 95% ethanol for 3 min, 70% ethanol for 3 min, 50% ethanol for 3 min, and then rinsed twice in water. Antigen retrieval was performed for Iba1 immunostaining using Tris-EDTA buffer at pH 9 for 20 min at 95-100°C using a vegetable steamer. For all antibodies, slides were dried either following antigen retrieval or directly after rehydration, and then rinsed 3 X 5 min in PBS, permeabilized in 0.5% Triton-X for 20 min, and then again rinsed 3 X 5 min in PBS. Sections were then blocked for 1 h in blocking solution containing 1% bovine serum albumin (BSA), 10% goat serum, 0.2% Triton-X in PBS, followed by primary antibody solution containing 2% BSA, 1% goat serum, 0.2% Triton-X and applicable antibodies in PBS at 4°C overnight. Antibodies were used at the following dilutions: MBP 1:100, GFAP 1:1000, Iba1 1:100. Following primary antibody incubation, sections were washed 3 X with PBS, then incubated for 1 h with AlexaFluor secondary antibodies in the same solution as primary antibody. Sections were then washed 1 X with PBS, counterstained with Dapi at 1:1000 in PBS for 5 min and finally washed 3 X 5 min PBS. Dako fluorescent mounting medium was applied sparsely directly to the section, and a coverslip was applied.

For histology, sections were deparaffinized and stained with either Cresyl violet (coronal brain and lumbar spinal cord cross-section) or hematoxylin and eosin (lumbar spinal cord cross section) using a Leica ST5010 Autostainer XL combined with Leica CV5030 Glass Coverslipper.
Behavioural analyses

Behavioural analyses were performed in cuprizone and baseline animals at the time points indicated above. For all behavioural tests, mice were acclimated to the testing room for at least 30 min prior to the start of testing.

A Rotarod was used to assess motor coordination. Mice were placed on a rotating, textured rod (IITC Life Science), programmed with the following protocol: starting speed 1 rpm, ramping up to a maximum of 45 rpm over a one minute time span, and then maintaining 45 rpm for an additional minute. Time to falling off the rod (latency to fall) was automatically recorded by magnetic signaling as animals dropped from the rods. Testing was conducted over two consecutive days, with four trials performed per day for a total of 8 trials. Inter-trial intervals were 10 min in duration, when the animals were returned to the home cage. Testing was done in a room lit at 300 lux.

The elevated plus maze (EPM) was used to assess anxiogenic responses. The maze comprised two arms, measuring 6 cm wide and 75 cm long, that crossed perpendicularly in a plus sign shape. One arm is enclosed by 20 cm high walls. The other arm along with the area where the two arms met were unenclosed platforms. The maze was raised 74 cm off of the floor. Animals were placed singly in the centre of the maze and allowed to explore freely for 10 min while monitored by overhead camera. Movements of each animal were recorded and tracked using Ethovision (Noldus).
RNA sequencing and gene ontology enrichment analysis

For RNA sequencing (RNAseq), primary rat OPCs were plated and transduced with lentivirus to overexpress miR-145 or scrambled non-targeting miRNA, as described above. RNAseq was performed on N=2 per condition. Quality control and sequencing were performed by StemCore Laboratories, Ottawa Hospital Research Institute. Samples were examined with the Qubit HS RNA Assay (ThermoFisher) and Fragment Analyzer HS NGS assay (Agilent) to determine sample concentration and RNA quality, respectively. Sequencing libraries were constructed using 500 ng RNA from each sample. Library construction was performed using the TruSeq Stranded mRNA Library Prep (Illumina) and sequenced with a NextSeq 500 High Output 75 cycle kit (Illumina).

Bioinformatics analysis of RNAseq raw data was performed by the Ottawa Bioinformatic Core Facility, Ottawa Hospital Research Institute. Reads generated from RNAseq were assigned to transcripts from GENCODE Rnor6.0 using Salmon v0.12.0 (162). Transcript quantifications were then loaded into R using txtimport library for analysis with DESeq2 (163). Following filtration to exclude transcripts with fewer than 5 reads in two or more samples, DESeq2 was used to calculate principle component analysis (PCA), hierarchical clustering, and fold change. PCA was performed using the DESeq2 plotPCA function and rlog-transformed count data. To evaluate hierarchical clustering, a heat map was generated using Pearson correlation between means of CPM normalized expression for each replicate group. Expression differences were calculated using the lfcShrink function (164). Genes were identified as significantly changed genes using a qvalue (Benjamini-Hochberg corrected p-value) of < 0.05.
Gene ontology (GO) biological process functional enrichment analysis of the significantly changed genes was performed using g:Profiler (165). Genes were separated into two sets defined as significantly upregulated and significantly downregulated with a fold change of 1.5 or greater, and were assessed by ordered query weighted by significance. GO terms used to identify targets for validation by qRT-PCR were significantly enriched and identified based on known relevance to OL biology.

**EAE**

The Hooke Laboratories kit comprised of MOG\textsubscript{35-55}/CFA emulsion plus pertussis toxin was used to induce EAE according to the manufacturer’s protocol. In brief, female mice aged 11-12 weeks were injected in two separate areas with 100 µl prepared MOG\textsubscript{35-55}/CFA emulsion subcutaneously plus 100 ng pertussis toxin in filtered PBS intraperitoneally on day 0, following by a second intraperitoneal injection of 100 ng pertussis toxin on day 1. Mice were left undisturbed following the injections until day 7 post-induction, at which point animals were scored daily and weighed on alternate days until day 30. Scores are defined as follows, based on the manufacturer’s guidelines: 0 = no change in motor function; 0.5 = tail tip paralysis; 1.0 = full tail paralysis; 1.5 = limp tail and hind leg inhibition; 2.0 = limp tail and hind limb weakness; 2.5 = limp tail and dragging of one or both hind feet; 3.0 = limp tail and dragging of hind legs (hind limb paralysis); 3.5 = limp tail and hind limb paralysis plus inability to right or loss of lower quadrant tone indicated by flattening of lower quadrant; 4.0 = limp tail, hind limb paralysis and partial front limb paralysis; 4.5 = limp tail, hind limb paralysis and severe front limb paralysis; 5.0 = limb paralysis with
uncontrolled rolling, or death. No animals in this study exceeded a score of 3.5. Animals were sacrificed for tissue collection at onset and disease peak, as determined by clinical score, as well as at remission which was arbitrarily set at day 30. Control animals were non-induced, naïve female mice 11-13 weeks old.

**Imaging and quantification**

Fluorescence images were taken using an Axio Imager M1 microscope with an AxioCamHR HRm Rev.2 camera and Axiovision 4.8.2 software. Confocal images were obtained using a Zeiss LSM 510 Meta DuoScan microscope and Zen 8.0 software. All electron micrographs of the corpus callosum were taken with a transmission electron microscope (Hitachi 7100) at x 4000 and x 10 000 magnifications. Both immunohistochemistry and histology tissue samples were imaged using a Zeiss AxioScan slide scanner with a Colibri 7 camera and Zen 2.6 slidescan software.

All images were analysed using ImageJ, and treatment conditions were blinded for all imaging analyses. For primary culture, two coverslips were analysed for each n. For images taken at 20X, 20-25 images were quantified per n; for those taken at 10X, 5-10 images were quantified per n. Sholl analysis was used to quantify branching complexity by overlaying concentric circles at equal increments from the cell body, and branch intersections were counted at each ring for 30 cells per n. Total membrane area was measured by tracing MBP⁺ cell outline and then calculating area for 20-30 cells per n. From TEM, G-ratios were calculated by measuring axon and axon + myelin areas, converting to diameter, and dividing axon diameter/axon + myelin diameter. For heavily myelinated
samples, ~300 axons were measured per n; for sparsely myelinated samples, all myelinated axons were measured in at least 90 images taken at 10 000 X. For myelinated axons/area, all myelinated axons were counted in 60-90 images taken at 10 000 X.

For histology and immunohistochemistry, corpus callosum was sectioned coronally from the genu to into the rostral areas of the thalamus and hypothalamus. To measure thickness, three linear measurements were taken of myelinated corpus callosum based on MBP⁺ staining, at the midline and adjacent to the midline on either side from 8 sections taken at 40 µm intervals. For lumbar spinal cord, cross sections were taken at the proximal end, mid-lumbar spine, and at the distal end. To calculate percent infiltrated area and percent myelinated area, the total spinal cord area and nucleus-dense area based on H&E staining or myelinated area based on MBP⁺ staining, respectively, were measured using ImageJ every third 20 µm section in 2-3 sections from each of the three sampled areas for a total of 6-9 sections per animal. For MBP area, all images were thresholded to the same value and the “measure particles” plugin was used in ImageJ to produce MBP traces. For GFAP⁺ cell quantifications, individual cells were quantified over the area of the entire section. For Iba1⁺ cell quantifications, individual cells were quantified in the parenchyma, excluding areas of dense Iba1⁺ cell infiltration.

**Statistical analysis**

Statistical analyses were performed using Prism 6 GraphPad software except for qRT-PCR analyses, which was calculated as indicated above. Pair-wise comparisons were done by two-tailed Student’s t-test using n=3-6, as indicated. Comparisons for >2 conditions
were done using one-way or two-way analysis of variance (ANOVA) followed by appropriate post-hoc testing using n=3-6, as indicated. Linear regression analyses were conducted for g-ratio assessments. Kaplan-Meier curves were analysed using the Mantel-Cox test. Error on mean values represent ± SEM, unless otherwise indicated. P-values of <0.05 were considered significant; asterisks delineate significance values as follows: * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.
Chapter 3
Aim 1: Investigating the importance of miR-145-5p differential expression in OPCs and OLs and identification of miR-145-5p downstream targets

MiR-145-5p regulates oligodendrocyte progenitor cell differentiation and regulates expression of critical myelin gene transcription factor MYRF

Contributing authors: Samantha F. Kornfeld, Sarah E. Cummings, Samaneh Fathi, Sawyer R. Bonin, Rashmi Kothary

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3.1 Abstract

The roles of specific miRNAs in oligodendrocyte (OL) differentiation have been studied in depth. However, miRNAs in OL precursors, oligodendrocyte progenitor cells (OPCs), have been less extensively investigated. MiR-145-5p is highly expressed in OPCs relative to differentiating OLs, suggesting this miRNA may serve a function specifically in OPCs. We thus knocked down miR-145-5p in primary OPCs, and found that this leads to spontaneous differentiation of these cells, as evidenced by an increased proportion of MAG$^+$ cells, increased transition from morphologically simple to more ramified cells, and upregulation of multiple myelin genes including MYRF, TPPP and MAG, and cell-cycle exit gene Cdkn1c. Supporting this transition to a differentiating state, proliferation was reduced in miR-145-5p knockdown OPCs. Interestingly, knockdown of miR-145-5p in differentiating OLs showed enhanced differentiation, increased branching and membrane production and additionally upregulating myelin genes. We identified several OL-specific genes targeted by miR-145-5p that exhibited upregulation with miR-145-5p knockdown, including MYRF, that could be regulating these processes. Due to its known importance in OL differentiation and myelination, we focused first on MYRF and found that spontaneous differentiation was rescued by concurrent knockdown of MYRF. Further, expression of the other identified miR-145-5p targets was normalized. Proliferation rate was only partially rescued with MYRF knockdown, however, and overexpression of miR-145-5p in OPCs increased proliferation rate without affecting expression of already lowly expressed differentiation genes. Taken together, these data suggest that in OPCs miR-145-5p both prevents differentiation at least in part by preventing expression of MYRF and promotes proliferation via as-yet-
unidentified mechanisms. These findings clarify the need for differential regulation of miR-145-5p between OPCs and OLs, and may have further implications in demyelinating disease such as multiple sclerosis where miR-145-5p is dysregulated.

Author contributions: SFK performed and analysed all experiments, with assistance from SEC (Figures 3.4, 3.5), SF (Figures 3.1 B, 3.3 B/D, 3.6 C/D, Supplementary Figure 4.2 B), and SRB (Figures 3.6 F/G, 3.7 A/E, Supplementary Figure 4.2). Study design was done by SFK with supervision and support from RK. Manuscript was written by SFK and critically edited by SEC and RK.
3.2 Results

Rodent primary OLs recapitulate the human miR-145-5p differential expression profile

The differential expression of miR-145-5p between OPCs and differentiating OLs has been established in human cell models. Since rodent models were used here to investigate the potential roles of miR-145-5p in myelination and in remyelination in MS, we first validated that the differential expression mirrored that observed in human OPCs and OLs. Using primary OPCs isolated from wild-type rat neonates, miR-145-5p expression was assessed by qRT-PCR in OPCs and across a time course of OL differentiation. We found that OPCs expressed relatively high levels of miR-145-5p and that a strong downregulation of ~90% occurred shortly after OPCs were induced to differentiation into OLs, and this downregulation was maintained throughout their differentiation (Figure 3.1).
Figure 3.1 MiR-145-5p is differentially expressed in rodent OPCs and OLs. A. Fluorescence micrographs of wild-type primary rat OPCs stained for F-actin (red) and Olig2 (green), counterstained with Hoechst (blue). White arrowheads indicate contaminating cells. B. Quantification of the proportion of Olig2⁺ cells, indicating OPC culture purity. C. Fluorescence micrographs of wild-type primary rat OPCs and differentiating OLs at 4 hours (4 h), 10 h, 20 h, 2 days (2 d), 3 d and 5 d. D. Relative expression (RE) analysis of miR-145-5p in OPCs and OLs over a time course of differentiation, as indicated; analysed by ΔΔCt, normalized to snU6. N=4, ***=p<0.001, one-way ANOVA with Tukey’s post hoc test.
**Knockdown of miR-145-5p in OPCs in vitro promotes spontaneous differentiation**

We next investigated the importance of miR-145-5p expression in OPCs. Knockdown of miR-145-5p was achieved by lentiviral transduction of a miR-145-5p inhibitor (anti-145) or non-targeting scrambled RNA (anti-ctl), using a method which achieves ~80% transduction efficiency and strong reduction in miR-145-5p expression (Supplementary Material, Figure 3.1 A, C, E). Forty-eight h post transduction, cultures were fixed and stained to assess expression of intermediate myelin marker MAG. A significantly larger proportion of anti-145 versus anti-ctl cells were found to express MAG at this time point, with ~10-fold increase from 0.6% of anti-ctl cells contrasted with more than 5% of anti-145 OPCs exhibiting MAG expression (Figure 3.2 A, B). These effects were observed despite the fact that cultures were maintained in OPC media without any pro-differentiation factors. Further, morphological analysis quantifying OPCs (bi/tri-polar, simple cells), early OLs (≥ 4 primary branches without myelin membrane) and late OLs (complex cells with myelin membrane) revealed significant shifts away from OPC morphology towards early-late OL morphology in anti-145 cells (Figure 3.2 C). A more thorough molecular analysis was also undertaken by assaying expression of differentiation genes in these cells. Interestingly, anti-145 OPCs exhibited increased relative expression of a host of OL-specific genes including the putative miR-145-5p target *MYRF*, plus OL-specific cell cycle exit marker *Cdkn1c*, and early-intermediate OL-markers *CC1, TPPP* and *MAG* with concurrent downregulation of OPC marker *NG2* (Figure 3.2 D). Late OL markers *PLP* and *MBP* also non-significantly trended towards upregulation. Again, note that this was in the absence of transition into
Figure 3.2 MiR-145-5p knockdown in OPCs leads to spontaneous differentiation. A.

Fluorescent micrographs of lentivirus-transduced OPCs 48 h post-infection. Left – anti-ctl; right – anti-145. Yellow boxes indicate zoomed area in bottom panels. GFP (green) indicates positive transduction; additional staining for myelin-associated glycoprotein (MAG; magenta), Olig2 (red) and counterstained with Hoechst (blue). Scale bars = 100 um. B.

Quantification of % MAG⁺/GFP⁺ and Olig2⁺ cells. N=4; **=p<0.01, Student’s t-test. C.

Quantification of cells binned by branching complexity and myelin membrane: OPC = bi- or tri-polar, representative cells indicated by white arrows in A; early OL = multi-arborized membrane-negative, representative cells indicated by orange arrows in A; late OL = multi-
arborized membrane-positive, representative cells indicated by red arrows in A. N=4; ***=p<0.001, Student’s t-test. D. Relative expression (RE) analysis of OPC and OL markers by qRT-PCR in anti-ctl and anti-145 OPCs 48 h post lentiviral transduction; analysed by ΔΔCt, normalized to actb and Ppia. N=3; *=p<0.05, Student’s t-test adjusted for multiple propagations. All values represent mean ± SEM.
differentiation medium, suggesting that anti-145 OPCs underwent spontaneous
differentiation driven solely by knockdown of miR-145-5p. This is further supported by
proliferation assays in which anti-145 OPCs were pulsed with the thymidine analog EdU
over a 6 h period. Compared to anti-ctl OPCs which demonstrated a 19.5% incorporation
rate, reduced proliferation was observed in anti-145 as indicated by a significantly
decreased EdU incorporation of only 8.7% over the same time frame (Figure 3.3 A, B). This
coincides with the observation of increased differentiation in these cells, as they must exit
the cell cycle into order to proceed to differentiation. Interestingly however, when the
reciprocal experiment was conducted in which OPCs were transduced to overexpress miR-
145, a significant increase in proliferation was observed with an incorporation rate of 28.6%
in miR-145 OPCs compared to 17.9% in miR-ctl OPCs, suggesting a secondary link to
proliferation beyond simply the prevention of differentiation (Figure 3.3 A, C, D).
Figure 3.3 Altering miR-145 expression in OPCs affects proliferation rate. A/C.

Fluorescence micrographs of lentivirus-transduced OPCs 48 h post infection with 6 h EdU pulse in anti-ctl/anti-145 OPCs (A) and miR-ctl/miR-145 OPCs (C). EdU was visualized by direct labeling (magenta), GFP (green) indicates positive transduction; additional staining for Olig2 (red), counterstained with Hoechst (blue). Scale bar = 200 um. B/D. Quantification of % EdU+/GFP+ and Olig2+ cells in miR-145 knockdown OPCs (B) and miR-145 overexpressing OPCs (D). N=4; *=p<0.05, **=p<0.01, Student’s t-test. All values represent mean ± SEM.
miR-145-5p knockdown in OLs enhances differentiation

Based on the finding that miR-145-5p knockdown was sufficient to promote differentiation of OPCs to OLs, we wished to further pursue the concept of anti-miR-145-5p as a pro-differentiation factor. Excitingly, this pro-differentiation effect was indeed observed when anti-145 cells were differentiated into OLs. Enhanced molecular differentiation was apparent at both an intermediate time point, differentiation day 2.5 (DD2.5), and at a late time point, DD5. There were small but significant increases in the proportion of anti-145 OLs expressing MBP on DD2.5, from 71.0% in anti-ctl to 81.7% in anti-145, and on DD5, from 75.2% in anti-ctl to 88.6% in anti-145 (Figure 3.4 A-D). Analysis of gene expression revealed significant increases in differentiation markers MYRF and MYRF downstream targets TPPP/p25, MAG, PLP1, and MBP (Figure 3.4 E). This shift towards a more mature OL transcription profile was not accompanied by any changes in PDGFRα or NG2 expression (Figure 3.4 E), suggesting that OLs exhibit enhanced differentiation and not an increase in the number of OPCs transitioning to OLs at these time points. Even more striking than the molecular changes in OLs with miR-145-5p knockdown were the improvements in morphological maturity. As revealed by Sholl analysis, branching of DD2.5 OLs was more complex particularly closer to the cell body (Figure 3.5 A-D). Myelin-producing anti-145 OLs on both DD2.5 and DD5 produced significantly more MBP+ compact membrane when compared to anti-ctl OLs, with anti-145 cells covering 53.4% more area at DD2.5 and 46.1% more area at DD5 than their respective anti-ctl counterparts (Figure 3.5 E, F). Production of myelin membrane in vitro is often a surrogate measure for myelination.
Figure 3.4 Knockdown of miR-145 enhances molecular OL differentiation. A/B.

Fluorescent micrographs of lentivirus-transduced OLs on differentiation day 2.5 (DD2.5; A) and differentiation day 5 (DD5; B). Left panels – anti-ctl; right panels – anti-145. GFP (green)
indicates transduced cells; additional staining for myelin basic protein (MBP; magenta), Olig2 (red), counterstained with Hoechst (blue). Scale bar = 100 um. C/D. Quantification of % MBP+/GFP+ and Olig2+ cells at DD2.5 (C) and DD5 (D). N=3; *=p<0.05, Student’s t-test.

E. Relative expression analysis of OPC and OL markers in anti-ctl and anti-145 DD2.5 OLs by qRT-PCR; analysed by ΔΔCt; mRNA normalized to actb and Ppia; miRNA normalized to snU6. N=4; *=p<0.05, Student’s t-test adjusted for multiple propagations. All values represent mean ± SEM.
Figure 3.5 Knockdown of miR-145 enhances morphological OL differentiation. A/B.

Confocal micrographs of single OLs on differentiation day 2.5 (DD2.5; A) showing GFP expression and stained for Olig2, and DD5 (B) stained for MBP. Left panels – anti-ctl; right panels – anti-145. Scale bar = 50 um. C. Sholl analysis of anti-ctl and anti-145 DD2.5 OLs. N=3; *=p<0.05, **=p<0.01, two-way ANOVA with Sidak post hoc test. D. Quantification of total branch intersections from Sholl analysis of anti-ctl and anti-145 DD2.5 OLs. N=3; *=p<0.05, Student’s t-test. E/F. Quantification of total membrane area of anti-ctl and anti-145 OLs on DD2.5 (E) and DD5 (F). N=4; *=p<0.05, **=p<0.01, Student’s t-test. All values represent mean ± SEM.
capacity in vivo, revealing potential for the downregulation of miR-145-5p as a pro-myelination target.

MiR-145-5p directly targets MYRF, and preventing MYRF upregulation in anti-145 OPCs abrogates spontaneous differentiation

In order to identify potential mechanisms underpinning the spontaneous differentiation prompted by miR-145-5p knockdown, we employed the algorithmic miRNA targeting database TargetScan (153) in combination with the Brain RNAseq database (166). Three gene lists were compiled: miR-145-5p targets generated, factors enriched in OLs relative to OPCs and factors enriched in OLs relative to all other neural cell types, using TargetScan and Brain RNAseq, respectively. Pair-wise comparisons were then made between all gene sets to identify common factors representing putative miR-145-5p targets also specific to OLs, to limit our investigation to factors most likely to be involved specifically in the process of OL differentiation and/or myelin production. A total of 14 genes were represented in all three assessed conditions, including known myelin gene MYRF as well as others with unknown roles in OLs (Figure 3.6 A, B). While MYRF was already found to be upregulated in anti-145 OPCs and OLs in our initial screen of OL markers (Figure 3.2 D, Figure 3.4 E), we additionally assayed the remaining genes identified as OL-specific putative miR-145-5p targets. In addition to MYRF, qRT-PCR analysis revealed expression of Otud7b, Zdhhc9, Onecut2, Efnb3 and Nudt4 was increased with miR-145-5p knockdown in OPCs (Figure 3.6 C; unidentified genes C030030A07Rik/5031439G07Rik were not assessed, Jph1 was not detectable). Enrichment of these genes in OLs relative to OPCs
was validated in our primary cell model (Figure 3.6 D). Currently, amongst the identified putative targets, only MYRF has a known role in OLs and importantly its overexpression is sufficient to prompt differentiation of OPCs into OLs, similar to our observations in anti-145 OPCs (68, 167). Thus, we next determined whether MYRF expression is indeed directly regulated by miR-145-5p, to determine if its reduction acts to promote differentiation of OPCs in a manner similar to direct MYRF overexpression. First, general targeting of the 3’ UTR of MYRF was assessed using a dual luciferase assay. The 3’ UTR of MYRF contains two putative binding sites for miR-145-5p, which are well conserved across vertebrate species including human, mouse and rat (Figure 3.6 E). HEK293T cells were co-transfected with a luciferase:MYRF 3’ UTR plasmid and a plasmid encoding pre-miR-145. These cells showed significantly reduced luciferase activity relative to those co-transfected with the MYRF 3’ luciferase plasmid and a scrambled miRNA control (Figure 3.6 F). As mentioned, two potential binding sites for miR-145-5p are found in the 3’ UTR of MYRF. Thus, to further elucidate the roles of each of the two putative binding sites, the nucleotides corresponding to the positions 2-4 of the seed region of miR-145-5p were mutated singly and concurrently in luciferase:MYRF 3’ UTR
Figure 3.6 *MYRF* is highly enriched in OLs and is directly targeted by miR-145-5p at two distinct binding sites. A. Fourteen putative miR-145-5p OL-specific targets were identified using TargetScan and the Brain RNAseq databases. B. Gene names and fold enrichment in OLs relative to OPCs and in OLs relative to all other CNS cell types. C. Relative expression of *MYRF* in OLs vs OPCs and in OLs vs non-OL CNS cells. D. Change in expression levels of indicated miR-145 targets over time in OLs. E. Sequence alignment of miR-145-5p binding sites in *MYRF* human, rat, and mouse 3' UTRs. F. Luciferase reporter assay showing repression of luciferase activity by miR-145-5p in HeLa cells. G. Effects of mutations on luciferase activity.
(RE) analysis of detectable genes identified in A/B in lentivirus-transduced anti-ctl and anti-145 OPCs. D. Validation of OL/OPC enrichment in targets upregulated in anti-145 OPCs in primary rat OPCs and OLs over a time course of differentiation, as indicated; C/D. Analysed by ΔΔCt, normalized to actb, N=4. E. Schematic depicting miR-145-5p targeting of wild-type and mutated MYRF 3’UTR; two binding sites are predicted, and this is conserved in human, rat and mouse, amongst others. F/G. Dual luciferase assays conducted in HEK293T cells co-transfected with wild-type or mutated MYRF 3’UTR and miR-145 or scrambled control (miR-ctl) as indicated. E. Relative luminescence of wild-type MYRF 3’UTR co-transfected with miR-ctl and miR-145. N=6; ****p<0.001, Student’s t-test. F. Relative luminescence of wild-type MYRF 3’UTR, MYRF 3’UTR with mutation CUG->GAC in the first putative binding site (mut 1), MYRF 3’UTR with mutation ACU-> UGA in the second putative binding site (mut 2), and MYRF 3’UTR with both mut 1 + mut 2, all co-transfected with miR-145. N=6; ****p<0.001, one-way ANOVA with Tukey’s post hoc test.
plasmid (Figure 3.6 E). Luciferase activity was equally abrogated in the wild-type MYRF 3’ UTR, mutated site 1 (mut 1) MYRF 3’ UTR and mutated site 2 (mut 2) MYRF 3’ UTR; however, when both sites were mutated (mut 1+2), the luciferase activity was restored to significantly higher levels (Figure 3.6 G). This strategy of seed region mutations confirms that miR-145-5p directly binds at both predicted binding sites.

Based on the master regulation of MYRF over OL differentiation and having established a direct relationship between miR-145-5p and MYRF expression, we then used a dual transfection system of miRNA inhibitors and siRNA to determine whether blocking MYRF expression concurrent with miR-145-5p knockdown was sufficient to prevent the observed spontaneous differentiation of anti-145 OPCs as illustrated above. Efficient knockdown of miR-145-5p and MYRF was achieved with >75% reduced expression of each target relative to non-targeting controls (Supplementary Material, Figure 3.2, 3). The proportion of MAG⁺ cells was again quantified. While anti-145 treatment alone resulted in the expected increase in MAG⁺ cells, simultaneous treatment with siMYRF returned this number to control levels (Figure 3.7 A, B). The proportions of cells showing OPC, early OL and late OL morphology were similarly normalized in cells treated with both anti-145 and siMYRF (Figure 3.7 A, C).

The transcription profile assaying OPC and OL markers was also recapitulated, revealing that simultaneous treatment of OPCs with anti-145 mimic and siMYRF returned relative expression of Cdkn1c, CC1, TPPP/p25, MAG, PLP1 and MBP to normal levels (Figure 3.7 D). Interestingly, MYRF knockdown in this context also normalized expression of Otud7b, Zdhhc9, Efnb3 and Nudt4 (Figure 3.7 D). Finally, proliferation assays comprised of a
6 h EdU pulse were also conducted in OPCs in which both miR-145-5p and MYRF were knocked down. In contrast to the complete normalization of measures of differentiation, proliferation rate was only partially rescued by knockdown of MYRF in anti-145 OPCs, exhibiting a significant increase from 15.6% in anti-145 cells to 20.2% in anti-145/siMYRF cells, but not a return to control levels of ~30%. This suggests that the link between miR-145-5p and proliferation is likely not due to its regulation of MYRF but acts via another mechanism (Figure 3.7 E).
Figure 3.7 Knockdown of MYRF prevents spontaneous differentiation in miR-145-5p knockdown OPCs. A. Fluorescence micrographs of OPCs 24 h post transfection with 30 nM siRNAs and 30 nM miRNA inhibitors, as indicated. Cells stained for MAG (magenta), f-actin (green), and Olig2/Hoechst (blue).
(green), Olig2 (red), counterstained with Hoechst (blue). Top panels – MAG only; bottom panels – merge. Scale bar = 100 um. B. Quantification of % MAG+/Olig2+ cells 24 h post transfection. C. Quantification of cells binned by morphology: OPC = bi- or tri-polar; early OL = multi-branched/membrane-negative; late OL = multi-branched/membrane-positive. B/C. N=3; ns = not significant, *=p<0.05, **=p<0.001, ***=p<0.0001, one-way ANOVA with Tukey’s post hoc test. D. Relative expression analysis of OPC and OL markers by qRT-PCR in OPCs 24 h post transfection with 30 nM siRNAs and 30 nM miRNA inhibitors, as indicated. Analysed by ΔΔCt, normalized to actb and Ppia. N=4; *p<0.05, one-way ANOVA with Tukey’s post hoc test. E. Cells 24 h post transfection with 6 h EdU pulse, stained for EdU (magenta), α-tubulin (green), Olig2 (red), counterstained with Hoechst (blue). Top panels – EdU only; bottom panels – merge. Scale bar = 200 um. F. Quantification of % EdU+/Olig2+ cells. N=3, *=p<0.05, **=p<0.001, ***=p<0.0001, one-way ANOVA with Tukey’s post hoc test. All values represent mean ± SEM.
3.3 Discussion

The progression of proliferating, migrating OPC to mature, myelinating OL is highly complex, requiring a multitude of differentially expressed intrinsic and extrinsic factors. The roles of pro-differentiation miRNAs have been the subject of broad exploration to date; miR-219, miR-338, miR-297c-5p and miR-138 are strong examples. Conversely, despite their identification, less investigation has been done to characterize OPC-specific miRNAs. MiR-145-5p was initially identified by microarray as highly expressed in OPCs but strongly downregulated as they transitioned to OLs, using human embryonic stem cells induced to differentiate through the stages of the OL lineage (134). Employing a method of primary cell culture, we recapitulated this finding in a rodent model (Figure 3.1) which we subsequently used to investigate the role played by miR-145-5p in OPCs.

Our initial finding that knockdown of miR-145-5p is sufficient to promote a shift in population from proliferating OPC towards differentiating OLs suggests that this miRNA is required for maintenance of the OPC identity as a morphologically simple, proliferating cell type (Figure 3.2). Based on our findings, miR-145-5p appears to play a dual role in this context. First, it has a clear function in repressing expression of MYRF, which is critical for OPCs since forced MYRF expression promotes spontaneous differentiation and MYRF-driven expression of myelin genes (68). This strongly parallels our results when miR-145-5p was knocked down in OPCs, where we saw an upregulation of MYRF and downstream targets as well as a reduction in proliferation rate (Figure 3.2, Figure 3.3 A). The concept that miR-145-5p acts upstream of MYRF and that these two represent a regulatory axis is additionally supported by the fact that the promotion of myelin gene expression and
increased branching in OPCs could be halted by blocking anti-145-driven MYRF upregulation (Figure 3.7 A-D). These results, taken together with the confirmation that miR-145-5p directly binds the MYRF 3’ UTR (Figure 3.6 E, F), suggest that miR-145 is a strong post-transcriptional regulator of MYRF expression. In this way, downregulation of miR-145-5p is highlighted as having pro-differentiation potential by way of increasing expression of MYRF, bolstered by the enhanced differentiation and increased MYRF expression observed when even the relatively low expression of miR-145-5p found in differentiating OLs is knocked down further still (Figures 3.4, 3.5). This control of MYRF may have implications in demyelinating diseases such as progressive MS, where at the lesion site MYRF+ OLs are severely depleted and OL differentiation fails despite the presence of OPCs and early MYRF− OLs, resulting in a lack of remyelination (104, 105). Of note, lesions in progressive MS show an overabundance of miR-145-5p when compared to healthy white matter (137); this begs the question of how interconnected these findings are, and suggests that further study is strongly warranted to characterize the effects of miR-145-5p overabundance on OL differentiation and whether knocking down miR-145-5p in the context of demyelinating disease can promote remyelination.

Of course, it is unlikely that the role of miR-145 differential expression in the progression of OL differentiation is solely to regulate the expression of MYRF. Fourteen OL-specific putative targets were identified for potential involvement in miR-145-mediated derepression as OPCs transition to OLs (Figure 3.6 A, B). Of these, 5 – including MYRF, Otud7b, Zdhhc9, Efnb3 and Nudt4 – were indeed found to be upregulated in miR-145-5p knockdown OPCs (Figure 3.6 C). Aside from MYRF, none of the additionally identified
targets have known roles in OL differentiation or myelin formation – though Otud7b, Zdhhc9, and Efnb3 have been characterized to some degree in neurons (168-170). This certainly does not preclude them from having undiscovered roles in OLs; however, expression of all four were normalized concurrent with MYRF knockdown in anti-145 OPCs. Since they are typically enriched as OLs differentiate (Figure 3.6 D; (166)), these findings suggest that their upregulation was more likely the result of general initiation of the OL differentiation program and not upregulation due to loss of miR-145-5p specifically (Figure 3.7 D). Despite this, other targets that may not be OL-specific are clearly also at play when it comes to miR-145-5p expression. This is illustrated in our study of OPC proliferation with miR-145-5p knockdown, which led to a reduction in proliferation that was only partially rescued by concurrent MYRF knockdown, indicating that reduced proliferation cannot be attributed solely to cell cycle exit caused by increased differentiation (Figure 3.3 A, Figure 3.7 E, F). The opposite effect was also found when miR-145 overexpression in OPCs led to increased proliferation potential (Figure 3.3 B) without altering already low levels of differentiation markers (data not shown), further indicating that one or more additional targets are being regulated by miR-145 specifically in OPCs to promote proliferation and not simply to prevent differentiation. This requires additional study to clarify the targets that translate the pro-proliferation influence of miR-145. Interestingly, this is in contrast to the much more studied role of miR-145 outside of the CNS, where it is known to be a potent tumor suppressor and is staunchly pro-differentiation/anti-proliferation; its loss through mutation is a common theme among various cancer types including but not limited to prostate, gallbladder, pancreas, breast, bladder and lung, and restoration of miR-145
expression is sufficient to dampen cancer cell proliferation and migration (171-174). It also acts as a pro-differentiation factor in cells such as myofibroblasts, vascular smooth muscle cells following injury, and odontoblasts, through the common mechanism of targeting fundamental pluripotency factors (132, 161, 175-178). This apparent dichotomy between inside the CNS and outside the CNS suggests that the role of miR-145 may have evolved differently based on the segregation of these two compartments, and that continued study of miR-145 function specifically in the CNS is merited.

MiRNA roles in OPCs in general require additional study. Confoundingly, complete loss of miRNAs in the OL lineage in animal models appears to have little effect on OPCs but thoroughly derails OL differentiation (131, 133); this suggests that expression of multiple miRNAs may temper each other in OPCs such that their mutual loss negates their collective presence, or that the complexities of the CNS in vivo compensate for miRNA loss in OPCs in a manner that is lost in vitro. Though the emphasis in current research is on pushing differentiation for purposes such as promoting remyelination in disease, the ability of OPCs to proliferate and migrate normally must not be forgotten – it is not useful to produce a mass of OLs if the OPC pool cannot be replenished or if OPCs cannot get to where they are needed for remyelination. Thus, continued study of the role of miR-145-5p in OPCs and in OLs is needed to continue teasing out its potential as a target for remyelination.

To conclude, we have demonstrated that miR-145-5p is a negative regulator of OL differentiation. Mechanistically it controls this process at least in part by targeting the critical myelination factor MYRF in OPCs to prevent differentiation, while conversely its downregulation allows expression of MYRF and subsequent progression through the
differentiation program (Figure 3.8). Further, miR-145-5p appears to play the dual role of both maintaining OPCs in the progenitor stage and potentiating proliferation as distinct functions, the latter working through an as-yet-unidentified target or targets. Altering the miR-145-5p:MYRF axis by inhibiting miR-145-5p promotes upregulation of MYRF and MYRF downstream targets, leading to spontaneous differentiation of OPCs and enhanced differentiation of OLs. This may have implications in promoting remyelination in diseases such as MS, and may be particularly relevant in progressive forms of MS where miR-145-5p is found to be overabundant in lesions. Further investigation must be undertaken to understand if miR-145-5p downregulation is necessary for OL differentiation, and if it is a viable therapeutic target to promote remyelination in demyelinating disease.
Figure 3.8 In oligodendrocyte progenitor cells, miR-145-5p expression prevents differentiation by targeting MYRF. A working model of one of the roles of miR-145-5p in OPCs and differentiating OLs. Left panel: High expression in OPCs prevents expression of MYRF, which in turn prevents expression of critical differentiation and myelin genes governed upstream by MYRF. This allows OPCs to remain proliferative and morphologically simple. Right panel: Downregulation of miR-145-5p allows expression of MYRF and its downstream targets, promoting the transition of OPCs to differentiating OLs and progression through the differentiation program from an increasingly ramified morphology to finally contacting and wrapping an axon and producing a myelin internode.
3.4 Supplementary Materials

Supplementary Figure 3.1 High efficiency lentiviral transduction protocol allows overexpression and knockdown of miR-145-5p in primary OPCs and OLs in vitro. A. Schematic of the lentiviral transduction paradigm. B. Fluorescence micrographs of primary rat OLs on differentiation day 3 (DD3) following transduction with scrambled miRNA/GFP lentivirus; transduced cells express GFP (green), and are additionally stained with MAG...
(red) and MBP (white). C. Quantification of the proportion of GFP⁺ OLs following infection with scrambled miRNA/GFP lentivirus according the paradigm outlined in panel A. D-G. Relative expression of miR-145-5p in OPCs (D/E) and DD3 OLs (F/G) transduced to overexpress (D/F) or knockdown (E/G) miR-145-5p by qRT-PCR using the ΔΔCt method and normalized to snU6. N=3-4, *p<0.05, Student’s t-test adjusted for multiple propagations.
Supplementary Figure 3.2 Validation of miR inhibitors and anti-MYRF siRNA. A. Relative expression of miR-145-5p after 24 h miRNA inhibitor transfection in OPCs treated with 30 nM scrambled inhibitor (anti-ctl) and miR-145-5p inhibitor (anti-145). B. Relative expression of MYRF after 24 h transfection in OPCs treated with 30 nM siRNA control (sictl) or siRNA against MYRF. A. Normalized to snU6. B. Normalized to actb. A/B. Analysis by qRT-PCR using ΔΔCt method.
Chapter 4
Aim 2: Characterization of the course of acute and chronic cuprizone-mediated demyelination in a miR-145 knockout mouse

Loss of miR-145 promotes remyelination and functional recovery in a model of chronic central demyelination

Contributing authors: Samantha F. Kornfeld, Yves De Repentigny, Sarah E. Cummings, Sabrina Gagnon, Samaneh Fathi, Rebecca Yaworski, Rashmi Kothary
4.1 Abstract

MiR-145-5p was previously identified as a regulator of the transition from proliferating oligodendrocyte progenitor cell (OPC) to differentiating oligodendrocyte (OL). Its knockdown revealed potential as having pro-differentiation effects in OLs. To investigate this in the context of remyelination, we employed a miR-145 knockout mouse in which demyelination was induced by both acute and chronic cuprizone exposure. Loss of miR-145 led to a subtle acceleration of differentiation in primary OLs when assessed \textit{in vitro}, evidenced by increased numbers of MAG$^+$ and MBP$^+$ OLs, but this could not be detected during development \textit{in vivo}. Following acute cuprizone exposure, where animals are already able to remyelinate with high efficiency, again no advantage to miR-145 loss could be detected. However, in animals in which chronic demyelination was assessed, miR-145 loss demonstrated a striking impact by allowing remyelination to occur where in wild-type animals remyelination occurred with paucity. The number of myelinated axons in the corpus callosum was strongly increased in miR-145 deficient animals, and the corpus callosum thickness was improved relative to wild-type. Further, behavioural assessments demonstrated that miR-145 loss prompted a return of normal anxiogenic responses and a partial recovery of motor coordination following chronic demyelination. The improved response of miR-145 knockout animals coincided with a pathological upregulation of miR-145-5p in wild-type animals with chronic cuprizone exposure, which was not observed in acutely treated animals. The effects of miR-145-5p upregulation on OL differentiation and myelin production was specifically assessed, and revealed that miR-145 overexpression in these cells severely stunts their differentiation and negatively impacts their survival, and
their transcriptome is widely altered to downregulate major pathways involved in both OL differentiation and myelination. As a whole, our data suggest that pathological upregulation of miR-145-5p is a unique feature of chronic demyelination and is strongly implicated in the failure of OL differentiation and remyelination, since its removal allowed extensive remyelination and neurologic recovery in that context. This is mirrored in chronic multiple sclerosis (MS) lesions, and thus miR-145-5p may serve as a relevant therapeutic target in progressive forms of MS.

Author contributions: SFK performed and analysed all experiments, with assistance from YDR (Figures 4.4 B, 4.5 B/C, 4.6 B/C,), SEC (Figures 4.4 A, 4.6 F-I, 4.7 A/B, 4.11 G/H), SF (Figures 4.1 A), SG and RY. Study design was done by SFK with supervision and support from RK. Manuscript was written by SFK and critically edited by SEC and RK.
4.2 Results

Loss of miR-145 accelerates developmental OL differentiation in vitro but not in vivo

Excitement generated by the pro-differentiation effects of miR-145-5p knockdown on OLs prompted expansion of our studies into a miR-145 knockout mouse. Unlike rat and human genomes, the mouse genome encodes a second putative miR-145 sequence – miR-145b. However, expression of the putative miR-145b gene has never been demonstrated. Transgenic mice were kindly provided by Dr. Eric Olson (University of Texas Southwestern). Complete loss of miR-145 expression was confirmed in CNS tissues by qRT-PCR in miR-145−/− animals, with the expected Mendelian expression of ~50% in miR-145+/− animals relative to miR-145+/+ (Figure 4.1 A). We additionally confirmed that miR-145b was not detectable in our model (Figure 4.1 A). MiR-145−/− mice do not exhibit any overt phenotypes. Survival up to one year is normal, as are total body and brain growth during development (Figure 4.1 B-D).

In order to observe the effects of miR-145 loss selectively during OL differentiation, primary OPCs were isolated from miR-145+/+ and miR145a−/− neonates and cultured over a time course up to differentiation day 6 (DD6). Myelin marker expression as well as mature morphology were assessed on DD1-6 every 24 h. The proportion of MAG+ OLs was significantly increased on DD2-4 and of MBP+ OLs was increased on DD1-4 in miR-145−/− cultures (Figure 4.2 A-C). By DD5 and DD6 maximal maturity rates were achieved and miR-145+/+ OLs caught up to their knockout counterparts.
**Figure 4.1 miR-145<sup>-/-</sup> mice exhibit normal survival and growth.** A. Relative expression analysis of miR-145-5p and miR-145b-5p in miR-145<sup>+/+</sup>, miR-145<sup>+/−</sup> and miR-145<sup>-/-</sup> CNS tissue by qRT-PCR. Analysed by ΔΔCt, normalized to snU6. N=3. UD = undetectable. B. Kaplan-Meier survival curve for miR-145<sup>+/+</sup> and miR-145<sup>-/-</sup> mice. N=10, Mantel-Cox test. C/D. Body weights (C) and whole brain weights (D) of miR-145<sup>+/+</sup> and miR-145<sup>-/-</sup> mice from neonate to adult. No significant differences based on multiple t-tests of two-way ANOVA using Holm-Sidak method. All values represent mean ± SEM. C. N=5-10. D. N=5-7.
Figure 4.2 Loss of miR-145 accelerates OL differentiation in vitro. A. Fluorescence micrographs of primary miR-145+/+ (top panels) and miR-145−/− (bottom panels) OLs at differentiation day 1 (DD1), DD2, DD3, DD4 DD5 and DD6. Cells labeled for Olig2 (red), MAG (green), MBP (magenta) and counterstained with Hoechst. Scale bar = 100 um. B/C/D. Quantifications of % MAG+/Olig2+ (B), % MBP+/Olig2+ (C) and % full myelin sheet/Olig2+ cells at DD1-DD6. N=3-4, * = p<0.05, ** = p<0.01, *** = p<0.001, multiple t-tests of two-way ANOVA using Holm-Sidak method. All values represent mean ± SEM.
Similarly, OLs achieving formation of full compact membrane sheets were more numerous in miR145a\(^{-/-}\) OLs on DD3-DD5, with miR-145\(^{+/+}\) OLs again matching miR-145\(^{-/-}\) by DD6 (Figure 4.2 A, D).

We next measured myelin protein expression in CNS tissues from neonates (postnatal day 0; P0), juveniles (P9 and P15), early adults (P30) and mature adults (P60). CNS tissues were subdivided into forebrain and midbrain (FB/MB), cerebellum and brainstem (C/BS) and spinal cord (SC) to assess their myelin content separately. Myelin proteins assessed included CNP, MBP and MOG, associated with intermediate myelin formation, late myelin formation, and final internode assembly, respectively. At P0, only CNP was detectable, and no differences were found between miR145a\(^{+/+}\) and miR-145\(^{-/-}\) in any CNS tissues (Figure 4.3 A-D). By P9 and onward, MBP and MOG were also expressed at measurable levels; however, no differences were observed at P9, P15, P30 or P60 in any myelin markers assessed in any tissue (Figure 4.3 E-T).

Since production of myelin protein is a gross measure of myelin production and does not necessarily measure correct myelination, we also assessed morphology and myelination of corpus callosum as an example of a major white matter structure in juvenile, early and mature adult animals to determine if any differences in myelination were present between miR-145\(^{+/+}\) and miR-145\(^{-/-}\) animals.

Finally, corpus callosum myelination was investigated with greater depth by transmission electron microscopy again at P15, P30 and P60. When assessing corpus callosum in cross section from miR-145\(^{+/+}\) and miR-145\(^{-/-}\) brains, no differences were found in the number of myelinated axons at any time point, and both acquired increases in
myelinated axon numbers at a similar rate as they developed from juvenile to adult (Figure 4.4 A, B).
Figure 4.3 Changes in CNS myelin protein expression were not detected with loss of miR-145 during development. A-T. Representative western blots (A, E, I, M, Q) and quantifications (B-D, F-H, J-L, N-P, R-T) for intermediate myelin marker CNP and late myelin markers MBP and MOG in forebrain and midbrain (FB/MB), cerebellum and brainstem (C/BS) and spinal cord (SC) from miR-145+/+ and miR-145−/− neonate (P0), juvenile (P9, P15), early adult (P30) and mature adult (P60) animals. N=5, Student’s t-test. All data represent mean ± SEM.
Figure 4.4 Ultrastructure and number of myelinated axons in corpus callosum is unchanged by loss of miR-145 during development. A. Fluorescence micrographs of coronal brain in juvenile (P15) and early adult (P30) miR-145+/+ and miR-145−/− animals. Sections stained for MBP (green), counterstained with Dapi (white). Scale bar = 500 um. B. TEM micrographs of sagittally sectioned corpus callosum from miR-145+/+ and miR-145−/− P15, P30 and P60 animals. Scale bar = 2 um. C. Quantifications of the numbers of myelinated axons per 100 um² in corpus callosum from P15, P30 and P60 miR-145+/+ and miR-145−/− animals. D. Mean g-ratios of myelinated axons at P60. E. Linear regression analyses of myelin thickness at P60. C/D. N=3-4, ns = not significant by Student’s t-test for, values represent means ± SEM.
**Loss of miR-145 promotes remyelination and functional recovery following chronic, but not acute, toxic demyelination**

Though no differences could be detected during developmental myelination, perceiving enhancements in this context may be difficult when comparing to an already healthy system as they are likely transient, as was observed in our *in vitro* study of primary miR-145\(^{+/+}\) and miR-145\(^{-/-}\) OLs. Thus, we proceeded to determine whether loss of miR-145 might impact remyelination following both acute and chronic demyelination, in this case driven by the copper-chelating agent cuprizone. Cuprizone exposure leads to death of mature OLs and subsequent demyelination primarily in the corpus callosum as well as in adjacent structures such as the cortices and hippocampus. With acute exposure of 6 weeks, remyelination occurs efficiently, with substantial remyelination expected an additional 5-6 weeks after withdrawal from cuprizone treatment. Chronic exposure of 12 weeks typically results in little to no remyelination even after cuprizone withdrawal.

Using transmission electron microscopy (TEM), we first evaluated myelination in the corpus callosum following 6 weeks cuprizone exposure followed by 5 weeks of recovery (Figure 4.5 A). Demyelination at 6 weeks was extensive in both miR-145\(^{+/+}\) and miR-145\(^{-/-}\) animals, with significant loss of ~90% of myelinated axon numbers (miR-145\(^{+/+}\) 12.6 ± 8.9, miR-145\(^{-/-}\) 4.5 ± 2.9; Figure 4.5 B, D). After 5 weeks of recovery, remyelination was apparent in corpus callosum from both 145a\(^{+/+}\) and miR-145\(^{-/-}\) animals with return to ~50% the number of myelinated axons relative to age-matched controls, with no detectable difference between genotypes (miR-145\(^{+/+}\) 44.7 ± 8.1, miR-145\(^{-/-}\) 49.7 ± 11.2; Figure 4.5 C, E).
Figure 4.5 Loss of miR-145 does not affect remyelination following acute cuprizone-mediated demyelination. A. Schematic of 0.2% cuprizone (cup) treatment timeline and return to normal (norm) diet. B/C. TEM micrographs of sagittally sectioned corpus callosum from miR-145+/+ and miR-145−/− animals after 6 weeks (P60 + 6 wks; B) on normal diet (Normal) or cuprizone diet (Cuprizone) and after 6 weeks + 5 weeks (P60 + 6 + 5 wks; C).
normal diet (Normal) or 6 weeks cuprizone diet + 5 weeks normal diet (Cuprizone). Scale bar = 2 um. D/E. Quantifications of the numbers of myelinated axons per 100 um² in normal and cuprizone corpus callosum at P60 + 6 wks (D) and P60 + 6 + 5 wks (E). N=3-4, two-way ANOVA with Tukey’s post hoc test. D-G. *p<0.05, **p<0.001. Values represent mean ± SEM.
We next assessed corpus callosum following 12 weeks cuprizone exposure followed by 5 weeks of recovery (Figure 4.6 A). Similar to our findings at 6 weeks cuprizone, at 12 weeks cuprizone exposure miR-145\(^{+/+}\) animals demonstrated ~90% loss of myelinated axons (9.3 ± 4.9) relative to healthy age-matched controls (Figure 4.6 D). By exciting contrast, corpora callosa from miR-145\(^{-/-}\) animals were replete with myelinated axons showing ~50% the number found in controls and significantly more than miR-145\(^{+/+}\) cuprizone-treated animals (miR-145\(^{+/+}\) 9.3 ± 4.9, miR-145\(^{-/-}\) 52.1 ± 1.2; Figure 4.6 D). This was mirrored after 5 weeks recovery, where miR-145\(^{+/+}\) appeared to gain a small proportion of myelinated axons with ~80% reduction compared to controls, while miR-145\(^{-/-}\) once again showed a significantly larger proportion than cuprizone-treated wild-type counterparts at ~60% of controls (miR-145\(^{+/+}\) 16.8 ± 3.9, miR-145\(^{-/-}\) 57.6 ± 6.3; Figure 4.6 E). Since myelin produced as a result of remyelination is canonically thinner than developmentally-derived myelin, we validated the findings in miR-145\(^{-/-}\) animals by measuring g-ratios at both 12 weeks cuprizone and 5 weeks recovery. At both time points, miR-145\(^{-/-}\) g-ratios were significantly increased relative to control with values calculated at 0.80 ± 0.008 at 12 weeks and 0.80 ± 0.001 after 5 weeks recovery compared to controls which remained steady at 0.77, indicating that miR-145\(^{-/-}\) myelin in cuprizone-treated animals was indeed thinner (Figure 4.6 F, G). This is further demonstrated by alterations in the linear regression analyses when plotting myelin thickness by axon diameter from miR-145\(^{-/-}\) in comparison to healthy controls, particularly in larger axons, which typically are preferentially the first to be remyelinated (Figure 4.6 H, I). G-ratios from cuprizone-treat miR-145\(^{+/+}\) corpus callosa were 0.77 ± 0.003 and 0.79 ± 0.005 at 12 weeks cuprizone and 5 weeks recovery, respectively, suggesting that the small
increase in myelinated axon number observed between these two time points in miR-145+/+ animals may be due to a minor amount of remyelination. Finally, gross structural analysis of corpus callosum was undertaken after 5 weeks recovery to determine if remyelination detected by TEM could be detected at a more macroscopic level. Tissue staining for MBP revealed observable recovery of corpus callosum thickness in cuprizone-treated miR-145−/− animals, which upon measurement was significantly thicker than miR-145+/+ cuprizone-treated animals though, similar to our TEM findings, was not returned to levels of healthy controls at this time point (Figure 4.7).
A

Acute demyelination
6 wks cup diet → 5 wks norm diet
P60: cup diet begins

12 wks cup diet → 5 wks norm diet
Chronic demyelination

B

P60 + 12 wks

<table>
<thead>
<tr>
<th>miR-145a+/+</th>
<th>miR-145a-/-</th>
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<tr>
<td>Normal</td>
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<tr>
<td>Cupprzone</td>
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C

P60 + 12 + 5 wks

<table>
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<tr>
<th>miR-145a+/+</th>
<th>miR-145a-/-</th>
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<tr>
<td>Normal</td>
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<tr>
<td>Cupprzone</td>
<td>Cupprzone</td>
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D

Myelinated axons/100 um²

E

Myelinated axons/100 um²

F

Mean g-ratio

G

Mean g-ratio

H

Myelin thickness (um)

I

Myelin thickness (um)

Axon Diameter (um)

Axon Diameter (um)

- Norm miR-145a +/+  
- Cup miR-145a +/+  
- Norm miR-145a -/-  
- Cup miR-145a -/-
Figure 4.6 Loss of miR-145 promotes remyelination with chronic cuprizone exposure. A. Schematic of 0.2% treatment timeline and return to normal diet. B/C. TEM micrographs of sagittally sectioned corpus callosum from miR-145+/+ and miR-145−/− animals after 12 weeks (P60 + 12 wks; B) on normal diet (Normal) or cuprizone diet (Cuprizone) and after 12 weeks + 5 weeks (P60 + 12 + 5 wks; C) normal diet (Normal) or 12 weeks cuprizone diet + 5 weeks normal diet (Cuprizone). Scale bar = 2 um. D/E. Quantifications of the numbers of myelinated axons per 100 um² in normal and cuprizone corpus callosum at P60 + 12 wks (D) and P60 + 12 + 5 wks (E). N=3-4, two-way ANOVA with Tukey’s post hoc test. F/G. Mean g-ratios of myelinated axons at 12 wks (F) and 12 + 5 wks (G). N=3-4, one-way ANOVA with Tukey’s post hoc test. D-G. *=p<0.05, **=p<0.01, ***=p<0.001, ****p=<0.0001. Values represent mean ± SEM. H/I. Linear regression analyses of myelin thickness at P60 + 12 wks (H) and P60 + 12 + 5 wks (I) plotted by axon diameter. Legend at I also applies to H.
**Figure 4.7 Loss of miR-145 promotes partial corpus callosum thickness recovery following chronic cuprizone exposure.** A/B. Fluorescence micrographs of coronally sectioned corpus callosum in miR-145\(^{+/+}\) (left panels) and miR-145\(^{-/-}\) (right panels) animals on normal diet after 12 weeks + 5 weeks normal diet (Normal) or 12 weeks cuprizone diet + 5 weeks normal diet (Cuprizone). Images in (B) are magnified from (A). Tissue was stained for MBP (green) and counterstained with Dapi (white). Scale bars = 500 um. C. Measurements of corpus callosum thickness at the midline in miR-145\(^{+/+}\) and miR-145\(^{-/-}\) animals on normal diet after 12 weeks + 5 weeks normal diet (Norm) or 12 weeks cuprizone diet + 5 weeks normal diet (Cup). N=8-16, *=p<0.05, ****=p<0.0001, one-way ANOVA with Tukey’s post hoc. Data represent mean ± SEM.
Finally, we wished to determine if the remyelination observed in miR-145\(^{-/-}\) animals following chronic cuprizone exposure was sufficient to result in functional recovery. The neurological consequences of cuprizone-mediated demyelination are measurable as altered anxiety behaviours and motor coordination deficits. To test these in our acute and chronic cuprizone models, we utilized the elevated plus maze (EPM) and rotarod to measure changes in anxiety and motor coordination, respectively. Anxiogenic responses are altered in cuprizone-treated animals, usually resulting in reduced preference to remain in dark, enclosed spaces and increased desire to explore when exposed to unfamiliar environments. No inherent differences in anxiety between miR-145\(^{+/+}\) and miR-145\(^{-/-}\) animals were observed at the start of cuprizone treatment at P60, or in age-matched healthy controls during the time courses of acute or chronic cuprizone treatment (Figure 4.8 A-O). After 6 weeks cuprizone exposure, reduced anxiogenic responses were detectable in both miR-145\(^{+/+}\) and miR-145\(^{-/-}\) animals to similar degrees, as both spent increased time in the open arms of the EPM (Figure 4.8 D). Further, cuprizone-treated animals spent more time exploring the maze, as evidenced by greater distance traveled (but no change in velocity) during the timed 10-min trial when compared to age-matched controls (Figure 4.8 F, H). These alterations in acutely treated cuprizone animals were returned to normal after 5 weeks recovery in both miR-145\(^{+/+}\) and miR-145\(^{-/-}\) animals, consistent with the remyelination observed in the corpus callosum (Figure 4.8 E, G, I). Increased time in the EPM open arms and greater distance traveled were maintained in both miR-145\(^{+/+}\) and miR-145\(^{-/-}\) animals after chronic cuprizone exposure (Figure 4.8 J, L).
Figure 4.8 Loss of miR-145 restores normal anxiogenic response after recovery from chronic cuprizone exposure. Elevated plus maze (EPM) was used to assess anxiety behaviours in miR-145\(^{+/+}\) and miR-145\(^{-/-}\) animals on normal (Norm) or cuprizone (Cup) diet at P60 (A-C), with acute cuprizone exposure (D-I) and with chronic cuprizone exposure (J-O). A/D/E/J/K. Time spent in open arms of the EPM. B/F/G/L/M. Distance traveled throughout the trial in the EPM. C/H/I/N/O. Average velocity throughout the trial in the EPM. N=9-44, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, one-way ANOVA with Tukey’s post hoc. All data represent mean ± SEM. Legend applies to all panels.
This suggests that in miR-145−/− animals, the extent of remyelination observed above was not sufficient to negate the neurological deficits incurred at this stage with respect to anxiety. However, after 5 weeks recovery from chronic treatment, miR-145−/− animals reverted to healthy control amounts both of time spent in the EPM open arms and distance traveled while miR-145+/+ animals retained their acquired abnormal anxiogenic responses (Figure 4.8 K, M, O). These results at 5 weeks recovery from chronic treatment are consistent with the remyelination and lack thereof observed in miR-145−/− and miR-145+/+ animals, respectively. With respect to motor coordination, animals were assessed by measuring time taken to fall from a rotating rod programmed to increase rotations per minute (rpm) at a steady state over time. Again, no inherent differences were observed at healthy miR-145+/+ and miR-145−/− at any time point assessed (Figure 4.9 A-D). Further, no motor deficits were detectable using this measurement even after 6 weeks cuprizone exposure (Figure 4.9 B); thus, acutely-treated animals were not assessed at recovery using this measure. After 12 weeks cuprizone treatment, motor coordination deficits were detectable and both miR-145+/+ and miR-145−/− exhibited similar reduced latency to fall (Figure 4.9 C); this again suggests that the remyelination observed in miR-145−/− animals at this time point was insufficient to correct motor coordination deficits caused by chronic cuprizone exposure. After 5 weeks recovery, however, miR-145−/− animals did exhibit partial recovery of motor coordination with latency to fall significantly increased over miR-145+/+ cuprizone animals, though still significantly reduced relative to age-matched healthy controls (Figure 4.9 D).
Figure 4.9 Motor coordination deficit is partially restored with miR-145 loss following recovery from chronic cuprizone exposure. A-D. Rotarod testing was used to assess motor coordination in miR-145<sup>+/+</sup> and miR-145<sup>-/-</sup> animals on normal (Norm) or cuprizone (Cup) diet at P60 (A), P60 + 6 wks (B) normal or cuprizone diet, P60 + 12 wks (C) normal or cuprizone diet, and P60 + 12 + 5 wks (D) normal diet or 12 wks cuprizone diet followed by 5 wks normal diet. Latency to fall was measured for 4 trials on 2 consecutive days. N=6-13, *=p<0.05, **=p<0.01, one-way ANOVA with Tukey’s post hoc.
Overexpression of miR-145-5p is a pathological feature of chronic demyelination, and leads to severe OL differentiation deficits and OL death

Based on the ability of miR-145−/− to remyelinate following chronic cuprizone exposure but no detectable enhancement of remyelination following acute cuprizone exposure, clearly differences exist in the pathology of these two models that make miR-145 loss useful in one but not in the other. Previous work has revealed that the chronic cuprizone model leads to accumulation of CSPGs in demyelinated areas while the acute model does not, analogous to the same accumulation observed in chronic MS lesions that is absent from RRMS lesions (48). Based on this, we assessed expression levels of miR-145-5p in miR-145+/+ CNS tissues to determine whether miR-145-5p is differentially expressed with cuprizone treatment, since like CSPGs it is also upregulated in chronic MS lesions (137). Expression of miR-145-5p was not altered in forebrain/midbrain tissues at 6 weeks cuprizone treatment or after 5 weeks recovery relative to age-matched controls (Figure 4.10 A). Conversely, miR-145-5p was upregulated relative to age-matched controls following 12 weeks cuprizone exposure, and this differential upregulation is maintained even after 5 weeks recovery (Figure 4.10 B). This suggests that increased miR-145 expression is a pathological feature of chronic but not acute demyelination, and that this upregulation contributes to the inhibition of remyelination since its loss promotes remyelination in a context where it normally does not occur.
Figure 4.10 Chronic but not acute cuprizone exposure results in miR-145 upregulation.

A. Relative expression (RE) analysis of miR-145 in forebrain/midbrain tissue from miR-145+/+ animals after 6 wks cuprizone exposure and after 5 wks return to normal diet.

B. Relative expression analysis of miR-145 in forebrain/midbrain tissue from miR-145+/+ animals after 12 wks cuprizone exposure and after 5 wks return to normal diet. A/B.

Analysed by ΔΔCt method, normalized to snU6. N=3, ns = not significant, ***=p<0.001, Student’s t-test adjusted for multiple propagations. All values represent mean ± SEM.
Recalling that intrinsic regulation of miR-145-5p expression in OPCs exhibits a strong downregulation upon their transition to differentiating OLs (Kornfeld et al., in revision), we questioned how the pathological upregulation of miR-145-5p with chronic demyelination might specifically affect OL maturation. We thus employed lentiviral transduction in primary rat OLs to overexpress miR-145 (see Supplementary Material Figure 3.1) and assessed both molecular and morphological differentiation of OLs at intermediate differentiation time point DD2.5 and late differentiation time point DD5. Both aspects of OL differentiation suffered deficits with miR-145 overexpression. The proportion of MBP+ OLs was significantly decreased on DD2.5 from 73.3% in scrambled control OLs (miR-ctl) to 6.6% in miR-145 overexpressing OLs (miR-145), and on DD5 from 77.3% in miR-ctl to 40.2% in miR-145 OLs (Figure 4.11 A-D). Morphologically, miR-145 OLs were severely stunted. Branching quantification by Sholl analysis of DD2.5 OLs showed few branches with very limited complexity in miR-145 cells when compared to miR-ctl (Figure 4.11 E, G, H). The number of membrane positive OLs at DD2.5 was reduced to a degree that they were not sufficient to conduct meaningful assessments in miR-145 OLs. By DD5, sufficient OLs reached the milestone of MBP expression to assess membrane area, but of those miR-145 OLs able to produce MBP their membrane area was reduced by half that of miR-ctl OLs (Figure 4.11 F, I). Finally, the generally unhealthy appearance of miR-145 OLs prompted us to conduct an evaluation of cell death. Based on cleaved-caspase 3 (CC3) staining, caspase-mediated apoptosis occurred at a similar rate in miR-ctl and miR-145 OLs at DD2.5 (Figure 4.12 A, C); however, by DD5, the proportion of CC3+ OLs was more than double in miR-145 cells when compared to miR-ctl cells (Figure 4.12 B, D).
Figure 4.11 Overexpression of miR-145 limits myelin protein expression and severely stunts morphological differentiation of OLs. A/B. Fluorescent micrographs of lentivirus transduced OLs on differentiation day 2.5 (DD2.5; A) and differentiation day 5 (DD5; B). Left panels – miR-ctl; right panels – miR-145. GFP (green) indicates transduced cells; additional staining for myelin basic protein (MBP; magenta), Olig2 (red), counterstained with Hoechst (blue). Scale bar = 100 um. C/D. Quantification of % MBP+/GFP+ and Olig2+ cells at DD2.5 (C).
and DD5 (D). N=4; **=p<0.01, ****=p<0.0001, Student’s t-test. E/F. Confocal micrographs of single OLs on DD2.5 (E) showing GFP expression and stained for Olig2, and DD5 (F) stained for MBP. Left panels – miR-ctl; right panels – miR-145. Scale bars = 50 um. G. Sholl analysis of miR-ctl and miR-145 DD2.5 OLs. N=4; ***=p<0.001, ****=p<0.0001, two-way ANOVA with Sidak post hoc test. H. Quantification of total branch intersections from Sholl analysis of miR-ctl and miR-145 DD2.5 OLs. N=4; ***=p<0.001, Student’s t-test. I. Quantification of total membrane area of miR-ctl and miR-145 DD2.5 OLs. N=4; ***=p<0.001, Student’s t-test. All values represent mean ± SEM.
Figure 4.12 miR-145 overexpression eventually leads to caspase-mediated apoptosis in OLs. A/B. Fluorescence micrographs of DD2.5 (A) and DD5 (B) OLs. Left panels – cleaved-caspase 3 (CC3; grey); right panels – merge with GFP and Olig2, counterstained with Hoechst. Scale bar = 100 um. White arrows indicate CC3+/GFP+/Olig2+ cells. C/D. Quantification of % CC3+/GFP+ and Olig2+ OLs on DD2.5 (C) and DD5 (D). N=4; ns = not significant, *=p<0.05, Student’s t-test.
Thus, overabundance of miR-145-5p not only rigorously limits OL differentiation ability, prolonged exposure promotes OL death.

To obtain a broader understanding of the molecular underpinnings of the stunted differentiation observed in miR-145 OLs, we performed RNAseq to assess the transcriptomic response to overexpression of miR-145. Sequencing was performed in two replicates for each of miR-145 and miR-ctl, treated with miR-145-5p mimic or scrambled miRNA, respectively, on DD5 (Supplementary Figure 4.1). Treatment with miR-145-5p mimics as opposed to lentiviral transduction resulted in significant miR-145-5p upregulation and the same differentiation deficits as described above (Figure 4.13 A, Supplementary Figure 4.1). Reads processed by RNAseq exceeded 40 million in all samples, with ~80% mapping coverage for each (Supplementary Table 4.1). Raw data variance was assessed by principal component analysis (PCA) and hierarchical clustering; differences between miR-ctl and miR-145 samples explained 56% of the total variance, while intra-sample variance explained 36% of the total variance (Figure 4.13 B). Despite the intra-sample variance, replicates clearly clustered together based on condition (Figure 4.13 C). Of 16,129 genes identified by mapping, 1086 of these showed significant differential expression in miR-145 OLs relative to miR-ctl (Figure 4.13 D). Significantly altered genes were divided into two sets based on upregulation and downregulation, and these two data sets were each subjected to gene ontology enrichment analysis. Since the direct impact of overexpressing any miRNA is downregulation of its targets, we focused primarily on the downregulated gene set in miR-145 OLs. The top three significantly enriched gene ontology categories arising from
downregulated factors were oligodendrocyte differentiation, negative regulation of
canonical Wnt signaling, and nervous system development – all systems that promote OL
differentiation. In fact, multiple significantly enriched additional categories kept with these
themes, resulting in 12 categories generally representing OLs/myelin formation, 5
representing regulation of Wnt signaling, and 10 representing CNS development (Figure
4.13 E, Supplementary Table 4.1). Specific genes from these enriched pathways included
some of those previously found to be upregulated with miR-145-5p knockdown (see Figure
3.2 D, Figure 3.4 E), such as MYRF, MAG, MBP, and PLP, and expanded upon those to
include others known to be critical to OL differentiation such as ZFP488, ZFP365, SOX10,
DUSP15, Tcfl72, and Prickle1, amongst others (Figure 4.13 F). Of the genes identified by
gene ontology enrichment, six were confirmed or putative targets of miR-145-5p,
suggesting initiating points for the dramatic downstream effects observed with miR-145-5p
overexpression (Figure 4.13 G, bold genes/red bars). Upregulated genes were broadly
categorized as being involved with damage response, extracellular matrix organization and
ion homeostasis, likely to be compensatory due to the massive dysregulation of
differentiation genes (Supplementary Figure 4.2, Supplementary Table 4.2). Selected genes
identified by gene ontology enrichment, including the miR-145-5p targets, were validated
by qRT-PCR, confirming downregulation of a host of critical differentiation factors as well as
upregulation of several genes with known roles in differentiation in OLs overexpressing
miR-145 (Figure 4.13 G).
Figure 4.13 RNAseq reveals downregulation of multiple factors in critical OL differentiation and myelination pathways with overexpression of miR-145. A.

Fluorescence micrographs of primary differentiating OLs transfected with scrambled control (miR-ctl; left panel) or miR-145 mimic (miR-145; right panel) on differentiation day 5. Cells are stained for f-actin (red), Olig2 (green) and MBP (magenta) and counterstained with Hoechst.
with Hoechst. B. Principal component analysis (PCA) of all mapped transcripts for all samples. Plot shows two-dimensional comparison of N=2 for miR-ctl and miR-145 calculated using the DESeq2 plotPCA function and rlog-transformed count data. C. Heat map displaying clustering analysis for N=2 for miR-ctl and miR-145. D. Volcano plot of log2 fold change versus log10 adjusted p-value for all mapped transcripts after filtering for miR-145 relative to miR-ctl. Fold change was calculated using DESeq2, p-value was adjusted using the Benjamini-Hochberg method. E. Significantly enriched gene ontology (GO) terms from genes differentially downregulated in miR-145 OLs relative to miR-ctl, sorted by overarching category. F. All differentially downregulated genes identified by GO term analysis overarching categories. G. Validation of selected significantly differentially expressed genes by qRT-PCR. N=3, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, Student’s t-test adjusted for multiple propagations. Analysed by ΔΔCt, expressed as log2 fold change relative to miR-ctl. Genes in bold/red bars indicate confirmed/putative targets of miR-145-5p.
4.3 Discussion

A host of dysregulated miRNAs have been identified in lesion tissue taken from both RRMS and progressive MS brains, but the potential roles of these miRNAs in disease pathogenesis remain poorly characterized. We previously identified miR-145-5p as an anti-differentiation/pro-proliferation gatekeeper in oligodendrocytes, whose downregulation alone is sufficient to promote OL differentiation at least in part due to targeting of the critical myelin transcription factor MYRF (Kornfeld et al., in revision). Here we employed a miR-145 knockout model to characterize the effects of miR-145 loss both in developmental myelination and in remyelination using both the acute and chronic cuprizone demyelination paradigms.

When primary OPCs were isolated from wild-type and miR-145 deficient animals and differentiated into OLs in vitro, we demonstrated a subtle acceleration of the appearance of myelin markers MAG and MBP, as well as of the formation of compact myelin membrane sheets (Figure 4.2 A-D). Despite this, we were unable to detect evidence of accelerated myelination during development in vivo (Figure 4.3, Figure 4.4). Other factors may be at play which prevent the untimely differentiation of OPCs in vivo, since spontaneous differentiation is not necessarily beneficial to the developing CNS. OPCs are required to migrate extensively and often proliferate in situ to meet the myelination demands of specific compartments and under specific circumstances, such as in the event of demyelination – premature differentiation would prevent fulfillment of these functions. Additionally, OL differentiation and myelination are achieved in a drastically shorter timeframe in vivo than in vitro, the former likely being achieved in a matter of hours while
the latter requires days (179). This raises the possibility that OL differentiation is indeed accelerated with loss of miR-145 *in vivo*, but that the window of difference is miniscule and may not be biologically relevant. Further, any difference is clearly transient, since in corpus callosum from mature adults from P60 and several time points up to and including P179 (normal animals at P60 + 12 + 5 wks) showed similar numbers of myelinated axons at every assessed stage (Figures 4.4-4.6). Regulation of myelin thickness is also normal with loss of miR-145, as g-ratios were also similar at those same assessed time points (Figures 4.4-4.6). Upon induction of demyelination following an acute course of 0.2% cuprizone administration, remyelination speed appeared similarly unaffected as after 5 weeks of recovery on normal diet numbers of myelinated axons in the corpus callosum were not different between miR-145+/+ and miR-145−/− animals and functional recovery was equally efficient in both (Figure 4.5, Figure 4.8). Again, this does not preclude the presence of differences that may have occurred at an earlier time point, but by the same token any changes to the course of remyelination were transient.

In contrast with the acute demyelination paradigm, chronic demyelination revealed striking differences between wild-type and miR-145 deficient animals. Remyelinated axons were apparent following recovery from 12 weeks of cuprizone exposure (Figure 4.6, Figure 4.7), as well as at the end of the cuprizone course prior to their return to normal diet, suggesting that remyelination occurred even as the animals were maintained on the cuprizone diet (Figure 4.6). While this was initially perplexing in comparison with the acute treatment which showed no impact on remyelination, we further discovered in wild-type animals that while miR-145-5p expression is unchanged with acute cuprizone
administration, it is significantly upregulated both at 12 weeks cuprizone and after recovery relative to untreated animals (Figure 4.10). These findings suggest that whilst loss of normal physiological levels of miR-145-5p in vivo does not seem to enhance remyelination under circumstances when remyelination already proceeds normally, its loss when it is pathologically upregulated can allow remyelination to proceed when it otherwise cannot. This in turn implies that upregulation of miR-145-5p is in itself an inhibitor to remyelination, supported by our findings in primary OLs where overexpression of miR-145-5p severely stunts differentiation and even leads to OL death (Figure 4.11, Figure 4.12), as well as by the fact that during the normal course of OL differentiation, miR-145-5p is strongly downregulated as OPCs transition to OLs (Kornfeld et al., in revision; (134)). These effects on OL differentiation and survival when miR-145-5p is overexpressed are driven by reduced downstream expression of multiple known OL differentiation factors such as MYRF and its downstream targets MAG, PLP, MBP and Dusp15, other pro-differentiation factors Zfp488, Zfp365, Gpr17, Tcf7l2, Sox2 and negative regulators of Wnt signaling Prickle1 and Nkd2, amongst others (Figure 4.13). Several of these are targets of miR-145-5p, including Sox2, Tcf7l2 and MYRF (Kornfeld et al., in revision; (67, 180, 181)). Intriguingly, Tcf7l2 is upstream of MYRF, and Sox2 is in turn upstream of Tcf7l2, suggesting that miR-145-5p may exert multi-level control over critical regulatory networks involved in OL differentiation (67, 180).

When considering genes upregulated in response to miR-145-5p overexpression, most categorized as a response to damage or wounding and organization of the extracellular matrix – all consistent with cells undergoing stress, as OLs induced to differentiate whilst overexpressing miR-145 clearly are. Some interesting factors which may be upregulated in
an attempt to compensate for stalled differentiation pathways include validated genes
*Mertk, Hmox1, Yap1* and *Plek* (Figure 4.13 G). Mertk and Hmox1 are typically upregulated
in OLs as a protective response to inflammatory stress, while Yap1 is involved in promoting
branch extension in the face of mechanical stress (182-184). In addition, mutations in *Mertk*
and *Plek* are associated with increased MS risk, and may thus represent a reduced ability of
OLs to respond to stress in that context, making them more vulnerable to inflammatory
attack (185, 186).

The upregulation of miR-145-5p is an important distinction between the acute and
chronic demyelination paradigms. The chronic cuprizone model of demyelination also
results in the build-up of CSPGs and FGF2, which are both inhibitory to remyelination via
prevention of OL differentiation (48, 187). Upregulation of these factors along with miR-
145-5p is mirrored in progressive MS lesions, also reticent to remyelination (137). Models
such as this, that recapitulate accumulation of inhibitory factors found in progressive
lesions, are critical to the study of remyelination therapies in ways we are only coming to
appreciate now. Several pro-myelinating factors have been identified in recent years such
as clemastine, benztropine, quetiapine, miconazole and clobetasol, all of which promote
OPC differentiation *in vitro* and promote myelination *in vivo* under healthy conditions (109,
188, 189). While these initially generated considerable excitement as potential therapeutics
for progressive MS, they have thus far failed to promote OL differentiation or myelin
production when faced with even a single inhibitory challenge from amongst the plethora
of those found in a progressive MS lesion. This was thoroughly illustrated in a study by
Keough *et al.*, in which these small molecules could not push OLs to overcome
differentiation deficits to any degree when cultured with CSPGs in vitro; the only path to success in this context was to enzymatically remove CSPGs (110). This highlights the need not only to further our understanding of the mechanisms behind how these factors inhibit OL differentiation, but also how to promote OL differentiation specifically in the face of these inhibitors. Only then can truly relevant therapeutic targets for progressive MS be identified, since the promotion of positive cues without a concurrent focus on the removal of inhibitory cues is obviously not sufficient to promote OL differentiation and remyelination in this context.

When considering the progressive MS lesion, the hallmark that sets it apart from a RRMS lesion is its inability to regenerate lost myelin. Remyelination requires recruitment of OPCs to the lesion site, and their subsequent differentiation and remyelination of denuded axons in a manner that largely recapitulates the process of developmental myelination. The population of OPCs and OLs particularly at chronic lesions exhibits some heterogeneity, and includes both OPCs and early differentiating OLs (104-106). Importantly the latter are largely characterized as OLs that have entered the differentiation program but are arrested prior to achieving MYRF positivity (105). An overexpression of miR-145 may have two possible roles in this case – first, our previous work showing the ability of miR-145 to potentiate proliferation suggests that it may play a role in OPC proliferation at the chronic lesion site (Kornfeld et al., in revision). Secondly, based on our findings here, an overabundance at the lesion site may contribute to the arrest of early OLs during the differentiation program by blocking the expression of MYRF and its upstream mediators Sox2 and Tcf7l2 as well as dysregulating Wnt signaling such that they cannot mature to
completion. In this sense, miR-145-5p may contribute to remyelination failure in two ways – by preventing both the transition of OPCs to OLs, as well as the transition from differentiating OL to myelinating OL.

While the source of miR-145-5p at the site of chronic demyelination remains unclear, it may be OPC-intrinsic, -extrinsic, or both. Abnormal intrinsic expression may be driven by p53, which is upregulated in OPCs and OLs in active MS lesions and drives miR-145 expression via direct interaction with the miR-145 promoter (190, 191). Uptake of exogenous miR-145 may also occur, as its expression is common to other cell types present in the lesion, which may secrete miRNA-laden exosomes subsequently taken up by OPCs incoming to the demyelinated area (Supplementary Figure 4.3; (192, 193)). In this sense, though OLs are strongly implicated in the effects of overexpression of miR-145-5p at the lesion site, other cell types may also be at play. Both reduced inflammation and neuroprotection are possible outcomes with the loss of miR-145; though miR-145 knockout did not attenuate demyelination, as both wild-type and miR-145 deficient animals were equally demyelinated by six weeks exposure to cuprizone (Figure 4.5), altered responses to chronic demyelination may be present in astrocytes, neurons and/or microglia due to miR-145 loss. Not unlike in OLs, miR-145 overexpression has been shown to stunt branching in astrocytes and neurite extension in neurons, and also promotes pro-inflammatory functions in microglia (194-196); additional work must therefore be undertaken to understand other cell-type specific implications of the pathological upregulation of miR-145-5p with chronic demyelination and how negating this upregulation aids in overcoming remyelination failure.
In conclusion, when taken collectively our work has demonstrated that miR-145 loss is inadequate as a strategy to enhance myelination or remyelination under circumstances where its expression remains at normal levels, but is sufficient to promote remyelination and functional neurological recovery following chronic demyelination where we report that miR-145-5p expression is pathologically upregulated. Like the overabundance of CSPGs and FGF2 with chronic demyelination, the dysregulation of miR-145-5p is established as an important factor that distinguishes remyelination-reticent lesions from those that can be remyelinated. From an OL perspective, we show that excessive miR-145-5p severely limits OL differentiation and contributes to caspase-mediated apoptosis of OLs, underpinned by reduced expression of critical differentiation and myelination pathways and despite the upregulation of multiple protective genes. These are important findings as miR-145-5p is also upregulated in chronic lesions in progressive MS. This work begins to unravel not only how miR-145-5p may contribute to remyelination failure in this disease, but also highlights it as a potential therapeutic target to alleviate the OL differentiation block observed in the progression MS lesion microenvironment.
4.4 Supplementary Material

**Supplementary Figure 4.1 Validation of miR-145 mimic.** Relative expression of miR-145-5p after 5 days in OLs transfected with 30 nM miRNA mimic (miR-ctl) or miR-145 mimic. A/C. Analysis by qRT-PCR using ΔΔCt method, normalized to snU6.
Supplementary Figure 4.2 Significantly enriched GO terms for genes upregulated in miR-145 OLs. Genes differentially upregulated in miR-145 OLs relative to miR-ctl OLs were assessed for enriched gene ontology terms.
Supplementary Figure 4.3 Conditioned media from miR-145-transduced OLs results in increased miR-145-5p in untreated OLs. Conditioned media was collected from miR-ctl and miR-145 lentivirus transduced OLs on differentiation day 2.5 (DD2.5). Untreated OLs were cultured in the conditioned media and assessed for miR-145-5p expression by qRT-PCR after 24 h. N=3, normalized to snU6, analysed using ΔΔCt. **=p<0.01.
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**Supplementary Table 4.1** All significantly enriched gene ontology terms for genes downregulated with miR-145-5p overexpression in OLs. Assessment based on weighted query performed using gprofiler.
Supplementary Table 4.2 All significantly enriched gene ontology terms for genes upregulated with miR-145-5p overexpression in OLs. Assessment based on weighted query performed using goProfiler.
Chapter 5
Aim 3: Assessment of the pathological and clinical outcomes in experimental autoimmune encephalomyelitis in a miR-145-5p knockout mouse

MiR-145 deficiency improves clinical outcomes and alters neuroinflammatory responses in murine experimental autoimmune encephalomyelitis

Contributing authors: Samantha F. Kornfeld, Sarah E. Cummings, Yves De Repentigny, Rebecca Yaworski, Sabrina Gagnon, Ariane Beauvais, Rashmi Kothary
5.1 Abstract

Previously, miR-145-5p has been implicated as an inhibitory factor to remyelination following chronic demyelination, where it is pathologically upregulated in CNS tissue in both an animal model of chronic toxic demyelination and in progressive multiple sclerosis (MS). While this miRNA is not known to be dysregulated in CNS tissue in acute or inflammatory instances of demyelination, it is dysregulated in serum and circulating mononuclear blood cells taken from relapsing-remitting MS (RRMS) patients, suggesting it may also play a role in autoimmune-mediated demyelination. To elucidate the role of miR-145-5p in inflammatory demyelination, we induced miR-145 deficient animals with experimental autoimmune encephalomyelitis (EAE), a murine model of relapsing-remitting MS (RRMS) driven by MOG\textsubscript{35-55} antigen autoimmunity. Interestingly, loss of miR-145 in this model resulted in reduced clinical severity over the course of EAE, characterized by delayed onset, reduced instances and duration of hind limb paralysis and reduced clinical scores throughout disease. MiR-145 deficient animals exhibited improved myelin retention in lumbar spinal cord at onset, peak and during chronic stage. Infiltration of immune cells into the lumbar spinal cord was reduced at both onset and during chronic stage with miR-145 loss, but this was not accompanied by major alterations in inflammatory marker expression in the thymus or spleen. However, significant alterations in neuroinflammation driven by changes in both astrocytic and microglial reactivity in miR-145 knockout lumbar spinal cords were detectable, suggesting that the reduced severity of EAE may be underpinned by changes in the neuroinflammatory response in these animals. Expression of cytokines and chemokines in the lumbar spinal cord showed widespread changes, particularly at disease
onset. As a whole, these results suggest that miR-145 plays a role in the development of the CNS neuroinflammatory milieu following autoimmune mediated demyelination, and may represent a therapeutic target worthy of investigation in inflammatory demyelinating disease such as RRMS. Future work is aimed at identifying miR-145-driven mechanisms underlying these changes, and characterizing any alterations in de/remyelination and neurodegeneration over the course of EAE in miR-145 deficient animals.

Author contributions: SFK conducted and analysed the experiments with assistance from SEC (Figures 5.5, 5.8), YDR (Figures 5.5, 5.8, 5.9), RY (Figures 5.1-5.3), SG and AB. SFK conceived and designed the study, supervised and supported by RK. Manuscript was written by SFK, and critically edited by RK.
5.2 Results

Loss of miR-145 results in reduced clinical severity over the course of experimental autoimmune encephalomyelitis (EAE)

Improved recovery from toxic demyelination has previously been shown using the miR-145 knockout mouse model (Kornfeld et al., unpublished). Inflammation in that model is restricted to the CNS and is elicited specifically in response to OL death and subsequent demyelination, and thus it is not an appropriate model to assess autoimmune mediated demyelination or neuroinflammation. Since miR-145-5p is dysregulated in RRMS and is ubiquitously expressed both inside and outside the CNS, we employed EAE, the murine model of RRMS, to assess whether loss of this miRNA altered aspects of autoimmune driven demyelination. MiR-145^{+/+} and miR-145^{-/-} mice were challenged with MOG_{35-55} antigen induced EAE, and were assessed for clinical score and weight over a 30 day course of disease (Figure 5.1 A). All induced animals developed EAE regardless of genotype. Overall, the severity of disease was lessened in miR-145^{-/-} animals, as indicated by reduced clinical scores and improved weight retention (Figure 5.1 B, C). Onset of disease as indicated by the first instance of a scorable clinical symptom was slightly delayed in miR-145^{-/-} animals, with the first perceptible symptoms arising ~1.5 days earlier in miR-145^{+/+} animals than in miR-145^{-/-} (Figure 5.2 B). Mean clinical score at onset was also reduced in miR-145^{-/-} mice (Figure 5.2 C). Time to the first instance of paralysis was also delayed in miR-145^{-/-} animals relative to time of EAE induction (i.e., day 0); however, time from symptom onset (score > 0) to paralysis respectively in miR-145^{+/+} and miR-145^{-/-} animals was the same (Figure 5.3 A, B). At the peak of disease, the average
Figure 5.1 The overall clinical course of EAE is altered when miR-145 is lost. A. Schematic of treatment and assessment paradigm for EAE. Myelin oligodendrocyte antigen (MOG ant.) is injected with complete Freund’s adjuvant on day 0 along with pertussis toxin (PTX), followed by a second dose of PTX on day 1. Animals were monitored daily from days 7-30. B. Mean clinical scores by day of EAE disease course. C. Mean weights expressed as percent of starting for EAE disease course. B/C. miR-145+/+ N=20, miR-145−/− N=16, *=p<0.05, **=p<0.01, ***=p<0.001, multiple T-tests of two-way ANOVA. Values represent mean ± SEM.
Figure 5.2 Onset is slightly delayed and clinical severity is reduced at onset with miR-145 loss. A. Kaplan Meier curve illustrating time to onset. p<0.0001 by log-rank test. B. Mean onset day. C. Mean score at onset. A-C. miR-145+/+ N=41, miR-145−/− N=31. B/C. *=p<0.05, ****=p<0.0001, Student’s t-test. Values represent mean ± SEM.
Figure 5.3 Clinical severity is reduced with loss of miR-145. A. Kaplan Meier curve illustrating time to onset of first instance of paralysis. miR-145<sup>+/+</sup> N=38, miR-145<sup>-/-</sup> N=28, p<0.0001 by log-rank test. B. Mean time from onset to paralysis. C. Mean maximum clinical score. D. Mean total days paralysed. E. Proportions of all EAE-induced animals monitored for full disease course divided by those exhibiting no paralysis, 1 instance of paralysis, or >1 instance of paralysis during the 30-day course of disease. F. Mean score during chronic stage at study end, day 30. B-F. miR-145<sup>+/+</sup> N=20, miR-145<sup>-/-</sup> N=16. B-D, F. **=p<0.01, ****=p<0.0001, Student’s t-test. Values represent mean ± SEM.
maximum clinical score attained was lessened in miR-145−/− animals (Figure 5.3 C). Further, amongst animals that reached a stage of hind limb paralysis (score ≥ 3), the duration of paralysis was much shorter in miR-145−/− mice (Figure 5.3 D). Incidences of paralysis were generally reduced in miR-145−/− mice; when considering the entire cohort of EAE animals, the proportion of those that experienced relapse as defined by more than one instance of paralysis was 52.7% in miR-145+/+ compared to 17.7% in miR-145−/-, and only 6.3% of miR-145+/+ animals did not reach a state of paralysis compared to 23.1% of miR-145−/− animals (Figure 5.3 E). At the end of the disease course, most animals achieve partial recovery indicated by regained use of hind limbs, but retain a chronically altered gait and a flaccid tail. At this chronic stage, assessed at day 30 in our study, miR-145−/− animals exhibited a reduced clinical score compared to miR-145+/+ animals (Figure 5.3 F).

Myelin retention is improved and cellular infiltration is reduced in miR-145 deficient spinal cord

To assess pathological changes underlying the altered clinical course of EAE with loss of miR-145, lumbar spinal cord was assessed for myelin content in miR-145+/+ and miR-145−/− in naïve animals, and at onset, peak and chronic stage in EAE. For this and all subsequent experiments, onset samples were collected on the day the first clinical symptom was observed, peak samples were collected on the first day animals achieved a score of 3.0 or higher (i.e., all peak animals were experiencing hind limb paralysis at time of sample collection), and chronic samples were arbitrarily collected on day 30 after induction. To measure myelin content specifically, cross sections of lumbar spinal cord were
immunostained for MBP, and MBP⁺ area was calculated as a proportion of total spinal cord section area. No differences were observed between naïve miR-145⁺/+ and miR-145⁻/⁻ animals (Figure 5.4 A, B, I). In accordance with reduced clinical symptoms at both onset and at chronic stage, miR-145⁻/⁻ animals exhibited increased myelinated area over miR-145⁺/+ animals (Figure 5.4 C, D, G, H, I). Similar myelin loss was observed at peak between miR-145⁺/+ and miR-145⁻/⁻ animals (Figure 5.4 E, F, I), though note that all animals at peak were selectively collected when experiencing hind limb paralysis. Over time, miR-145⁺/+ animals displayed a progressive loss of myelinated area, while miR-145⁻/⁻ myelinated area was relatively stable between naïve and EAE onset, and between peak and chronic stage (Figure 5.4 I).

Immune cell infiltration in lumbar spinal cord was next assessed by histological H & E staining. As expected, naïve animals were clear of any subpial or parenchymal dense nuclei accumulations (Figure 5.5 A, B). At onset, miR-145⁺/+ animals exhibited several areas of infiltration indicated by accumulation of dense nuclei, primarily in the ventral horn as well as at the dorsal root-spinal cord junction, whereas miR-145⁻/⁻ showed very little infiltration and was largely limited to the ventral horn (Figure 5.5 C, D, J). Infiltration was extensive in both miR-145⁺/+ and miR-145⁻/⁻ animals at peak of EAE (Figure 5.5 E, F, I, K). Finally, at chronic stage, infiltration of miR-145⁺/+ spinal cords remained extensive while miR-145⁻/⁻ spinal cords showed reduced infiltration (Figure 5.5 G, H, L). Quantification of the infiltrated area of the spinal cord at both onset and chronic was significantly reduced in miR-145⁻/⁻ animals, but was similar at peak of disease (Figure 5.5 J-L). These findings
correspond with the improved myelination status in miR-145^{-/-} animals at both onset and chronic stage of EAE.
Figure 5.4 MBP loss is reduced in the lumbar spinal cord at onset and during the chronic stage of EAE in miR-145 deficient animals. A-H. Fluorescence micrographs and MBP area traces in lumbar spinal cord in naïve (A/B) animals and at onset (C/D), peak (E/F) and during chronic stage (G/H) of EAE in miR-145+/+ (left panels) and miR-145−/− (right panels). Spinal
cords stained for MBP (green), counterstained with Dapi (blue). Scale bar = 200 um. I.

Quantifications of MBP area as a percentage of total spinal cord area in naïve animals, and at onset, peak and during chronic stage of EAE. N=3, *=p<0.05, **=p<0.01, ***=p<0.0001, two-way ANOVA with Tukey’s post hoc. Values represent mean ± SEM.
Figure 5.5 Immune cell infiltration of lumbar spinal cord is reduced at onset and during chronic stage of EAE with miR-145 loss. A-H. Hematoxylin and eosin stained lumbar spinal cord in naïve (A/B) animals and at onset (C/D), peak (E/F) and during chronic stage (G/H) of EAE in miR-145+/+ (left panels) and miR-145−/− (right panels). Scale bar = 200 um. I.

Representative image of submeningeal and parenchymal infiltration (red arrows) taken from (E). Scale bar = 200 um. J-L. Quantifications of infiltrated area as a percentage of total spinal cord area at onset (J), peak (K) and during chronic stage (L) of EAE. N=3, **=p<0.01, ***=p<0.001, Student’s t-test. Values represent mean ± SEM.
**Loss of miR-145 leads to minor alterations in cytokine/chemokine expression in peripheral immune organs during EAE**

MiR-145-5p has been implicated in peripheral immune responses, is expressed in thymus and spleen, and is differentially expressed to some degree at onset and during chronic stage EAE in spleen and thymus, respectively (Figure 5.6 A, H). Thus, we next questioned whether the reduced infiltration of immune cells into miR-145\(^{-/-}\) EAE spinal cords at onset and chronic stage was due to alterations in cytokine and chemokine expression, which may in turn affect T-cell activation in EAE. Thymus and spleen were assessed for cytokines *I\(l\)1b, Ifng, I\(l\)6* and *Tnfa* and chemokines *Cxcl1* and *Ccl5*, all factors with known roles in the development of EAE in the periphery. Some modest but statistically significant downregulations were detected in thymus, including reduced expression of *Tnfa* and *Cxcl1* at disease onset and reduced expression of *Ccl5* in naïve miR-145\(^{-/-}\) animals (Figure 5.6 E, F, G). Conversely, significantly upregulated factors were observed in miR-145\(^{-/-}\) spleen, including *Ifng* and *Cxcl1* at peak of disease and chronic stage, respectively (Figure 5.6 J, M). No other significant changes to gene expression were found in any assessed factor at any time point in either thymus or spleen, suggesting that while singular alterations to cytokine and/or chemokine expression may yet affect disease development and progression, the clinical and histological findings observed thus far with miR-145 loss are not due to widespread overhaul of peripheral immune organ response to EAE induction.
Figure 5.6 Cytokine and chemokine expression shows few alterations in miR-145 deficient thymus and spleen over the course of EAE. A/H. Relative expression of miR-145-5p from miR-145+/+ thymus (A) and spleen (H) in naïve animals, and at onset, peak and during chronic stage of EAE. B-G/I-N. Relative expression of cytokines Il1β, IFN-γ, Il6, and TNFα and chemokines Cxcl1 and Ccl5 in thymus (B-G) and spleen (I-N) from miR-145+/+ and miR-145−/− naïve animals, and at onset, peak and during chronic stage of EAE. A-N. N=3, *=p<0.05, **=p<0.01, ****=p<0.0001, Student’s t-test at each time point corrected for multiple propagations. A/H. Normalized to snU6. B-G/I-N. Normalized to actb and Ppia. Analysed by ΔΔCt. Values represent mean ± SEM. Legend at J applies to all panels B-G/I-N.
**Neuroinflammatory responses are altered in miR-145 deficient animals**

With no evidence to support pervasive changes to the inflammatory profile in peripheral immune organs with miR-145 loss, we next assessed cytokine and chemokine expression in spinal cord. The expression of miR-145-5p was again assessed in miR-145\(^{++}\) animals over the course of EAE, revealing a significant upregulation at onset and modest but significant downregulation at peak and during chronic stage in lumbar spinal cord (Figure 5.7 A). Similar to thymus and spleen, expression of cytokines \(IL1b\), \(IFNG\), \(IL6\) and \(TNFA\) and chemokines \(CXCL1\) and \(CCL5\) was assessed in lumbar spinal cord at all stages of EAE. All factors were differentially regulated at disease onset in miR-145\(^{-/-}\) animals, with significant downregulation of \(IL1b\), \(IFNG\), \(IL6\), \(TNFA\) and \(CCL5\), and upregulation of \(CXCL1\) (Figure 5.7 B-G). Further changes in gene expression were found at peak with upregulated \(IL1b\), and during chronic stage with downregulated \(IL6\) and \(CCL5\) (Figure 5.7 D, G). In the EAE-induced CNS, cytokine/chemokine expression is reflective not just of peripheral immune cell activity but also that of resident immune cells, microglia and astrocytes. We thus evaluated activation of microglia and astrocytes to determine if changes in neuroinflammatory EAE responses might coincide with the improved clinical and differing pathological findings observed in miR-145\(^{-/-}\) animals. Lumbar spinal cord from miR-145\(^{++}\) and miR-145\(^{-/-}\) was stained for Iba1 and GFAP to visualize microglia and astrocytes, respectively, in naïve animals, and in EAE at onset, peak and chronic stage. Total microglia numbers were quantified in the parenchyma, excluding submeningeal areas of dense cellular infiltration. Alterations in microglial activation were observed at all stages of EAE in miR-145\(^{-/-}\) animals relative to miR-145\(^{++}\), with microglial numbers reduced by ~50% at
Figure 5.7 Cytokine and chemokine expression is altered in spinal cord particularly at EAE onset with miR-145 loss. A. Relative expression of miR-145-5p from miR-145+/+ lumbar spinal cord in naïve animals, and at onset, peak and during chronic stage of EAE. B-G. Relative expression of cytokines Il1β, IFN-γ, Il6, and TNFα and chemokines Cxcl1 and Ccl5 in lumbar spinal cord from miR-145+/+ and miR-145−/− naïve animals, and at onset, peak and during chronic stage of EAE. A-N. N=3, *p<0.05, ****=p<0.0001, Student’s t-test at each time point corrected for multiple propagations. A. Normalized to snU6. B-G. Normalized to actb and Ppia. Analysed by ΔΔCt. Values represent mean ± SEM. Legend at C applies to all panels B-G.
Figure 5.8 MiR-145 loss results in differentially altered microglial activation over the course of EAE. A-H. Fluorescence micrographs of whole lumbar spinal cord cross section from naïve (A/B), EAE onset (C/D), EAE peak (E/F) and during chronic stage EAE (G/H) in miR-145<sup>+/+</sup> (left panels) and miR-145<sup>−/−</sup> animals (right panels) stained for Iba1 (white) and counterstained with Dapi (blue). Scale bar = 200 um. I. Quantification of the number of Iba<sup>+</sup> cells/mm<sup>2</sup> at different stages of EAE in miR-145<sup>+/+</sup> and miR-145<sup>−/−</sup> animals. J-L. Relative expression levels of Ym1, I Nos, Nurr1 at different stages of EAE in miR-145<sup>+/+</sup> and miR-145<sup>−/−</sup> animals.
cells per mm$^2$ in the parenchyma, excluding areas of dense cellular infiltration. N=3, *=p<0.05, **=p<0.01, ****=p<0.0001, two-way ANOVA with Tukey’s post hoc. J-L. Relative expression of Ym1 (J), iNos (K) and Nurr1 (L) in lumbar spinal cord from miR-145+/+ and miR-145−/− naïve animals, and at onset, peak and during chronic stage of EAE. N=3, *=p<0.05, **=p<0.01, ****=p<0.0001, Student’s t-test at each time point corrected for multiple propagations. Analysed by ΔΔCt, normalized to actb and Ppia. Values represent mean ± SEM.
onset and ~30% during chronic stage EAE but increased at peak by ~30% (Figure 5.8 A-I). We further assessed expression of microglia-specific Ym1, a marker of anti-inflammatory M2 polarization, iNos, a marker of pro-inflammatory M1 polarization, and Nurr1, a miR-145-regulated anti-inflammatory microglial factor. Interestingly, expression changes were observed at all EAE stages in Ym1 and iNos; while both were downregulated at onset and peak in miR-145−/− animals, correspondent with reduced overall numbers of microglia at these same time points, iNos was upregulated at peak while Ym1 remained downregulated, suggesting a possible shift in activated microglia polarization between miR-145+/+ and miR-145−/− animals when animals are experiencing hind limb paralysis (Figure 5.8 J, K).

Expression of Nurr1, a negative regulator of TNFa, was upregulated at onset (Figure 5.8 L).

To evaluate astrocyte activation during EAE, both total numbers of GFAP+ astrocytes and the number of hypertrophic reactive astrocytes as defined by GFAPhigh signal and thickened processes were also quantified at all stages. Naïve miR-145−/− animals showed a non-significant trend towards higher total astrocyte numbers, but not reactive astrocytes (Figure 5.9 A, B, I). Interestingly, at both onset and peak, miR-145−/− exhibited higher total astrocyte and reactive astrocyte numbers (Figure 5.9 C-F, J, K). Further, reactive astrocytes at onset were found diffusely distributed throughout the parenchyma with few located submeningeally in miR-145+/+ spinal cords, while reactive astrocytes in miR-145−/− were primarily localized to submeningeal areas (Figure 5.9 C′-D′). Contrasting the increased numbers and reactivity of astrocytes at onset and peak, miR-145−/− animals showed fewer total astrocytes and fewer reactive astrocytes at chronic stage (Figure 5.9 G, H, L), altogether suggesting that astrocyte-driven inflammation arises more quickly and with
greater potency but abates earlier in miR-145⁻/⁻ animals when compared to their wild-type counterparts. Both GFAP and aquaporin 4 (AQP4), a component of astrocytic end feet that critically contributes to blood brain barrier integrity, have been reported as targets downregulated by miR-145-5p when it is overexpressed (194, 197). We assessed relative expression of these factors in spinal cord in naïve animals as well as over the course of EAE to determine whether absence of miR-145 in our model resulted in altered expression of either or both targets, which might drive the increased presence and reactivity of astrocytes observed in miR-145⁻/⁻ animals. However, naïve animals exhibited no change in GFAP or AQP4 (Supplementary Figure 5.1, A-E). Altered expression of GFAP was observed over the course of EAE that correlated with changes in the observed hypertrophy of astrocytes at these timepoints, while AQP4 showed no changes in expression in EAE animals at any time point (Supplementary Figure 5.1 D, E). When taken together, these findings suggest broad modifications to the CNS resident immune response with loss of miR-145, though we have yet to elucidate clear underlying mechanisms for these striking observations.
Figure 5.9 MiR-145 loss differentially affects astrocyte distribution and reactivity over the course of EAE. A-H. Fluorescence micrographs of whole lumbar spinal cord cross section from naïve (A/B), EAE onset (C/D), EAE peak (E/F) and during chronic stage EAE (G/H) in miR-145^{+/+} (left panels) and miR-145^{-/-} animals (right panels). A’-H’. Magnified regions from indicated regions in A-H. A-H’. Sections stained for GFAP (white), counterstained with Dapi (blue). Scale bars = 200 um. I-L. Quantifications of total astrocytes (GFAP^+) cells per spinal cord section and total number of hypertrophic astrocytes (GFAP^{high}/thickened processes) cells per section in naïve animals (I), at onset (J), peak (K) and during chronic stage EAE (L) in miR-145^{+/+} and miR-145^{-/-} animals. N=3, *=p<0.05, two-way ANOVA with Tukey’s post hoc. Values represent mean ± SEM.
5.3 Discussion

In RRMS, miR-145-5p was previously identified as a circulating biomarker – both for diagnosis, as its overrepresentation can distinguish RRMS samples from healthy controls as well as SPMS, and for treatment efficacy, as its dysregulation is normalized in RRMS patients treated successfully with interferon-β (151, 155, 198). How it may contribute to disease onset and progression, however, has not been previously investigated. Here, we employed a miR-145 knockout mouse coupled with the inflammatory demyelinating RRMS model, EAE, to address these points. Clinically, loss of miR-145 led to important improvements over the course of EAE (Figure 5.1-5.3). Time from induction to onset was slightly delayed, and symptoms at onset were milder in miR-145 deficient animals. Even more striking, fewer animals reached the milestone of hindlimb paralysis with loss of miR-145, and those that did regained hindlimb use significantly faster. Further, during the chronic stage of EAE, miR-145−/− animals maintained a greater degree of recovery illustrated by reduced clinical scores over the latter course of disease. These findings suggest that miR-145-5p does indeed contribute to the pathophysiology of EAE, and that its loss is beneficial in this context.

The initiation and progression of EAE is complex and multi-faceted, involving both peripheral immune and neuroimmune activation to culminate in demyelination and consequent functional deficits. In our case, differences with miR-145 loss were observed at onset, peak (as defined as loss of hindlimb use) and during chronic stage EAE, necessitating characterization of underlying pathologies at all stages of disease. Both cellular infiltration and demyelination were altered with miR-145 loss, with corresponding reductions both at
onset and during chronic stage of disease. Animals selectively assessed at peak of disease displayed equal infiltration and myelin loss; however, it is worth noting that had animals been arbitrarily assessed at a specific “peak” time post induction differences would likely have been detectable, as animals escaping paralysis were excluded from our assessment.

MiR-145-5p has been implicated in both pro- and anti-inflammatory capacities in different contexts in the periphery (199-201). In EAE, both spleen and thymus showed differential expression of miR-145-5p at onset and during chronic stage, respectively, suggesting this miRNA might serve some role at specific points during the course of EAE in peripheral lymphoid organs (Figure 5.6). This seemed logical based on the dysregulation reported in circulation both in PBMCs and in serum/plasma in RRMS, as well as the fact that relative expression is greatly increased over spinal cord in both tissues, with miR-145-5p expression in spleen ~25 times more and in thymus ~625 times more than in lumbar spinal cord in naïve wild-type animals (Supplementary Figure 5.2). Despite this, however, minimal changes were observed when assessing EAE-related inflammatory cytokine and chemokine expression in spleen and thymus (Figure 5.6). This does not preclude other alterations in the activation of EAE induction in the periphery or of T-cell proliferation upon activation in the CNS; additional investigation is required to better understand what effects, if any, loss of miR-145 has on the initiation and progression of the adaptive immune response in EAE.

Conversely, altered neuroinflammatory responses with miR-145 loss were quite clear, with changes in both microglia and astrocyte activation relative to wild-type animals at all stages of EAE (Figures 5.8, 5.9). Microglia activation precedes the onset of EAE symptoms and even the infiltration of immune cells into the CNS (202). This has significant
implications for the BBB, as activated microglia can alter expression of factors critical for
tight junction maintenance amongst endothelial cells lining the neurovasculature,
facilitating immune cell entry into the CNS (203). Thus, in our model the reduced microglial
activation observed at onset and during chronic stage may contribute to the reduction in
cellular infiltration observed at these same time points by limiting loss and/or allowing
reconstitution of BBB integrity (Figures 5.5, 5.8). Mir-145-5p has been specifically
implicated in negative regulation of tight junction maintenance by targeting critical junction
proteins, suggesting that the upregulation observed in spinal cord at onset may contribute
to BBB breakdown in wild-type animals and adding to the hypothesis that integrity may be
improved with miR-145 loss (204, 205).

Reduced microglial activation/cellular infiltration further coincided with reduced
expression of several proinflammatory cytokines and chemokines, including IFN-γ, TNFα,
Il1β, Il6 and Ccl5 at onset, and Il6 and Ccl5 during chronic stage (Figure 5.7). These factors
are secreted by infiltrating T-cells and monocyte/macrophages, and by activated microglia,
and their reduced expression corresponds with reduced populations of these cells with
miR-145 loss. The links between alterations in microglia behaviour and loss of miR-145
remain somewhat unclear, as few miR-145 regulated pathways have been identified in
microglia to date. Two known targets of miR-145-5p in microglia are Twist2 and Nurr1 (154,
196). Downregulation of both via miR-145-5p promotes microglial inflammation, Twist2 by
its positive regulation of anti-inflammatory factor c-Maf, and Nurr1 by its negative
regulation of pro-inflammatory TNFα. Though we did not assess Twist2 expression in our
model, c-Maf expression was unaltered in miR-145−/− spinal cord during EAE
(Supplementary Figure 5.3). However, *Nurr1* expression was upregulated particularly at EAE onset – concurrent with *TNFα* downregulation and coincident with miR-145-5p upregulation in wild-type mice – suggesting, that this pathway may be implicated in our model at least at this time point (Figures 5.7, 5.8).

MiR-145-5p has also been connected with the polarization of activated microglia, with increases pushing polarization to favour an anti-inflammatory M2 phenotype over pro-inflammatory M1, as demonstrated in a model of ischemia (206). Since the loss of miR-145 might consequently lead to more inflammatory microglia activation, we assessed expression of M1 marker *iNos* and M2 marker *Ym1* over the course of EAE (Figure 5.8). Both markers were reduced at onset and chronic stage and is likely reflective of overall reduced numbers of activated microglia at these time points, though proportions of pro-inflammatory relative to anti-inflammatory microglia cannot be hypothesized at these time points based on this assessment. However, at peak of disease, where microglial numbers were increased in miR-145<sup>-/-</sup> animals, M1 marker *iNos* was upregulated while M2 marker *Ym1* remained downregulated. This may indeed indicate that microglial activation at disease peak favoured an inflammatory phenotype; since microglial inflammation is believed to detrimentally contribute to EAE progression and demyelination, the quick recovery of miR-145<sup>-/-</sup> from this point in disease is somewhat confounding from this perspective. However, recent studies based on single cell RNAseq highlight that activated microglia may represent a much more diverse population than simply an M1/M2 dichotomy, and thus attempting to interpret microglial activation based on this paradigm
may oversimplify a more complicated state of heterogeneity, particularly following demyelination (207).

In addition to differences in activated microglia, astrocyte activation was also modified with miR-145 loss compared to wild-type over the course of EAE. Similar to microglial activation, astrocyte activation was increased at peak and reduced at chronic stage. At onset in miR-145⁻/⁻ animals, astrocyte activation was increased in contrast to decreased microglial activation relative to wild-type animals. This in itself is unusual, as astrocyte activation is generally a trigger for microglial activation, suggesting that loss of miR-145 may influence crosstalk between these two neuroimmune cells (208, 209).

Interestingly, previous work in EAE characterizing the role of NFκB revealed that inhibition of this pathway specifically in astrocytes results in delayed onset and improved clinical severity, underpinned by reduced inflammatory cytokine expression and reduced leukocyte infiltration over the course of disease (210, 211). Moreover, in a model of atherosclerosis, miR-145-5p was shown to promote inflammation by activating NFκB signaling, driving expression of TNFα and IL1β, amongst others (201). Taken together, this suggests that loss of miR-145 in our model may lead to reduced activation of the NFκB pathway in astrocytes, resulting in reduced inflammation. Interestingly, expression of adhesion molecules associated with reduced BBB integrity and of neuronal markers associated with improved neuroprotection was also detected with astrocyte-specific NFκB loss, indicating these factors may also play a role in our model and are worthy of additional investigation.

Finally, remyelination may occur with greater efficiency during EAE in miR-145 deficient animals. We previously demonstrated that pathological upregulation of miR-145-
5p inhibits OL differentiation, and that miR-145 loss in the context of pathological upregulation is sufficient to allow remyelination in a chronic model of demyelination (Kornfeld et al., unpublished). The improvements in myelinated area with miR-145 loss in EAE may represent both reduced demyelination, resultant from reduced inflammatory attack, concurrent with more efficient remyelination. This could be driven by several factors, particularly at onset. In lumbar spinal cord, miR-145-5p was found to be upregulated uniquely at EAE onset in wild-type animals (Figure 5.7 A), suggesting that this may contribute to the hindrance of initial remyelination efforts early in disease.

Interestingly, the chemokine Cxcl1 was also upregulated in miR-145−/− spinal cord, a factor secreted in the CNS by astrocytes that promotes recruitment of OPCs. Taken together, this suggests that in our model, the demyelinated environment both promotes influx of OPCs to the site of myelin damage with increased chemoattractants and removes a barrier to their differentiation with loss of miR-145. Reduced neuroinflammation in general, as is observed during chronic stage EAE in miR-145−/− animals, is also more conducive to remyelination. Interestingly, for astrocytes in particular, early and strong activation is beneficial while early inactivation exacerbates EAE outcome; chronic stage exhibits the opposite response, where high levels of activation are detrimental while reduced activation improves clinical outcome (212-214). Loss of astrocyte activation in these studies early in EAE resulted in loss of BBB integrity, increased leukocyte infiltration and more severe demyelination, but in chronic stage resulted in improved remyelination. Thus, loss of miR-145 appears to promote the most favourable sequence of astrocyte activation over the course of EAE, likely providing a
strong contribution to the ameliorations in clinical outcome and underlying pathologies we observed in our model.

In conclusion, we show herein that loss of miR-145 results in improved clinical outcome in murine EAE at multiple levels and leads to widespread changes in neuroinflammation at all stages of disease. The CNS cellular landscape in EAE is complex, and glial responses are extensively intertwined. Future work to continue elucidating the mechanisms of EAE improvements will include characterization of OPC recruitment and remyelination efficiency, assessment of BBB integrity, exploration of NFκB signaling in spinal cord, and investigation of neuronal survival and regenerative capacity with miR-145 loss. Together with the findings presented here, these results will serve to clarify the undoubtedly multi-cellular importance of miR-145-5p in EAE pathogenesis, and determine its viability as a potential therapeutic target in RRMS.
5.4 Supplementary Figures

Supplementary Figure 5.1 Inherent changes are not present in GFAP or AQP4 with miR-145 loss. A. Representative fluorescence immunoblots for GFAP and AQP4 in naïve miR-145<sup>+/+</sup> and miR-145<sup>-/-</sup> spinal cord. B/C. Quantification of GFAP and AQP4 protein content in naïve miR-145<sup>+/+</sup> and miR-145<sup>-/-</sup> spinal cord. N=5, Student’s t-test. D/E. Relative expression of GFAP (D) and AQP4 (E) in spinal cord in naïve animals and at EAE onset, peak and during chronic stage in miR-145<sup>+/+</sup> and miR-145<sup>-/-</sup> animals. N=3, *=p<0.05, ****=p<0.0001, Student’s t-test at each time point corrected for multiple propagations. Analysed by ΔΔCt, normalized to actb and Ppia. Values represent mean ± SEM.
Supplementary Figure 5.2 MiR-145-5p is differentially expression in lumbar spinal cord, spleen and thymus. Relative expression of miR-145-5p in CNS and lymphoid organ tissues from naïve miR-145+/+ animals. N=3, **=p<0.01, ****=p<0.0001, Student’s t-test at each time point corrected for multiple propagations. Analysed by ΔΔCt, normalized snU6. Values represent mean ± SEM.
Supplementary Figure 5.3 Expression of c-Maf is not altered over the course of EAE with miR-145 loss. Relative expression of c-Maf in spinal cord in naïve animals and at EAE onset, peak and during chronic stage in miR-145^{+/+} and miR-145^{-/-} animals. N=3, Student’s t-test at each time point corrected for multiple propagations. Analysed by ΔΔCt, normalized to actb and Ppia. Values represent mean ± SEM.
Chapter 6

General Discussion
6.1 MiR-145-5p in MS and models of demyelination

At the initiation of this project, our primary goal was to characterize the importance of miR-145-5p in OPC and OL biology, followed by extension of our studies into demyelinating models based on the previously reported dysregulation of miR-145-5p in different forms of MS. Despite clear miR-145-5p-mediated regulation of OPC and OL differentiation *in vitro* via MYRF targeting (Chapter 3, Chapter 4 Figure 4.2), enhanced differentiation was not detectable during developmental myelination in the miR-145 deficient CNS (Chapter 4, Figures 4.3, 4.4). As discussed previously, this may highlight the caveats of extrapolating expectations into a whole animal from the artificial context of cell culture. Or, more likely, our results may simply be confounded by the protracted and thus more measurable speed at which OL differentiation occurs *in vitro* versus the relative quickness of the OPC transition to fully myelinating OL *in vivo* – a matter of only a few hours in zebrafish spinal cord, for example, versus ~16 days for those same zebrafish OLs in culture (179, 215).

Based on these findings, we were thus unsurprised to detect no enhancements in remyelination following acute cuprizone exposure with loss of miR-145, after which even wild-type animals regenerate myelin with efficiency (Chapter 4, Figure 4.5). The striking remyelination and behavioural deficit recovery in miR-145 knockout animals after chronic demyelination therefore elicited excitement but a degree of perplexity (Chapter 4, Figures 4.6-4.9); this was resolved, of course, by the discovery of pathological upregulation of miR-145-5p in wild-type animals uniquely following chronic demyelination, and by our
characterization of the detrimental effects overabundance of miR-145-5p has specifically on OL differentiation and myelin production (Chapter 4, Figure 4.10-4.13).

In connection with our first findings with miR-145-5p knockdown in OPCs and OLs, MYRF along with a host of other critical myelin genes were altered with miR-145 overabundance (Chapter 3, Figures 3.2, 3.4; Chapter 4, Figure 4.13). These findings relate importantly to the progressive MS lesion microenvironment where miR-145-5p is also upregulated, supporting the hypothesis that in that environment too, miR-145-5p overabundance may contribute to remyelination failure and its removal may benefit remyelination (137). Furthermore, chronic cuprizone exposure is strengthened as a valid means to study the potential of remyelinating therapies for progressive MS, as upregulated miR-145-5p represents an additionally and previously unknown disease factor recapitulated in this model.

Extension of our studies into the inflammatory demyelinating model EAE revealed yet more benefits to miR-145 loss, where clinical outcomes were greatly improved in EAE animals deficient in miR-145 and myelin damage and peripheral immune cell infiltration were lessened (Chapter 5, Figures 5.1-5.5). Characterization of miR-145 loss in this model necessitated a broader approach, as opposed to the OL/myelin focus employed in our molecular and cuprizone investigations, as the study of remyelination in this context is made difficult by multiple factors. MiR-145-5p dysregulation was previously identified in circulation in RRMS/early SPMS, but not in RRMS lesions nor in EAE lesions (137, 151, 198, 216). However, our assessments of miR-145-5p expression in whole spinal cord tissue identified upregulation at onset – prior to widespread lesion development – as well as
modest but significant downregulations at peak of disease and during chronic stage when lesioned white matter is extensive, indicating that dysregulation does occur in CNS tissue in EAE though perhaps is not isolated to the lesion microenvironment (Figure 5.7). Interestingly, these findings suggest that CNS dysregulation of miR-145-5p may also be present in RRMS, but has not been detected because of methodologies used to investigate microRNA changes in that context.

Based on discovery of miR-145-5p dysregulation in our wild-type EAE model coupled with the lack of widespread changes in lymphoid organ inflammatory marker expression with miR-145 loss (Chapter 5, Figure 5.6), we focused on the activation of neuroinflammatory glia: microglia and astrocytes. Here, we discovered broad changes in both microglia and astrocytes across the course of EAE disease, correspondent with both cytokine and chemokine expression differences in spinal cord with miR-145 loss. We have yet to clearly elucidate the underlying mechanisms linking miR-145 loss with these neuroinflammatory differences, and how they in turn improve the severity of EAE pathogenesis. However, together with our findings from chronic cuprizone, we show that miR-145-5p is clearly involved in the pathophysiology of both chronic and inflammation-driven demyelination. Moreover, loss of its expression influences multiple CNS cell types implicated in both progressive MS and RRMS, ultimately improving outcomes in both models of demyelination.
6.2 MiR-145-5p dysregulation in CNS disease – a recurring theme?

Interestingly, amongst CNS disorders MS is not singular in its dysregulation of miR-145-5p. Other CNS injuries and diseases in which miR-145-5p is specifically upregulated include traumatic brain injury (TBI), spinal cord injury (SCI), ischemic stroke (IS), and autism spectrum disorder (ASD) (Figure 6.1). Overabundance of miR-145-5p is detectable in circulation following TBI in rodents/humans, in circulation shortly after SCI in humans and in CNS tissue at the site of trauma in rodents, in circulation in humans/rodents and in CNS tissue in rodents following IS, and in circulation in humans with ASD (217-222). Best studied to date in IS, miR-145-5p antagonism was in fact beneficial in an IS rodent model, leading to significantly reduced infarct size (220). Polymorphisms in the promoter of miR-145 and its genetic partner miR-143 leading to reduced expression specifically of miR-145-5p have in fact been linked to reduced risk of IS in humans, suggesting that dysregulation may not only be a negative downstream effector, but a primary disease driver in itself (223).

Despite vastly different underlying causes, MS, IS, TBI and SCI share the common pathologies of extensive myelin and neuron damage, highlighting miR-145-5p upregulation as a potentially general feature of CNS tissue damage. ASD might seem an outlier in this collection of CNS disorders; however, recent work in that field has revealed myelin abnormalities in that myelin sheaths in autistic brains are typically thinner than in healthy controls (224). Building on that study, very recent work in an ASD mouse model also discovered issues with myelination, underpinned by reductions in the number of mature OLs and loss of an array of myelin-related genes. Interestingly, these findings were
dependent on loss of Tcf4 expression – a target of miR-145-5p and a downregulated factor in our model of miR-145 overexpression in OLs (225).

Though common pathways are not apparent when comparing data from IS and from our own models of demyelination, it seems intuitive that additional similarities in the molecular sources of detriment with pathological miR-145-5p overexpression must exist and are worth identifying. Many examples exist of the investigation of very specific roles for miR-145-5p in CNS biology and in specific CNS disease, our data among them. However, as highlighted above, miR-145-5p upregulation may provide a link amongst this subset of neurological disorders that has yet to be explored from a bird’s eye view. Expansion of pre-clinical studies to investigate miR-145-5p as a potential therapeutic target in TBI, SCI and ASD, as is ongoing in IS and as we have now begun in MS, might provide even more widespread benefits in neurological disease if collaborative efforts are made to identify universally affected pathways. Furthermore, several less common diseases characterized by myelin damage and loss such as transverse myelitis, acute disseminated encephalomyelitis and the rare MS variant myelinoclastic diffuse sclerosis have not been assessed at all for miRNA dysregulation, providing additional opportunity to add to our understanding of miRNA contributions to CNS myelin-related diseases.
Figure 6.1 MiR-145-5p upregulation as a pathological feature of multiple CNS disorders.

Schematic overview of CNS disorders in which miR-145-5p has been shown to be upregulated, where the upregulation has been identified, and any effects of therapeutic miR-145-5p antagonism.
6.3 MiRNAs as therapeutic targets – potential for moving miR-145-5p towards the clinic

Investigation of miRNA mimic and antagonist delivery for the treatment of disease is a booming field unto itself. For studies targeting specifically the CNS, intrathecal injection of a variety of small RNA vectors has seen success in animal models, including lentivirus, multiple types of nanoparticles, engineered stem cells and exosomes (226, 227). Intrathecal injection is a highly invasive method of delivery, however, and may not be a feasible approach in humans particularly if ongoing treatment is a requirement. Exosomes provide a promising avenue of investigation in that sense, as aerosolized intranasal delivery localizes specifically to brain, is non-invasive, and potential recipients could likely self-treat (193, 228). Finally, of particular interest in progressive MS, the possibility is beginning to arise of specifically targeting lesion tissue using methods in development for cancer treatment. Interestingly, the increased deposition of ECM components in chronic lesions contributes to alterations in their inherent level of mechanical stiffness, not dissimilar to cancerous tumours (229). In the MS lesion, this is disadvantageous as it contributes to the inhibition of remyelination, since stiffer environments result in the negative regulation of OL differentiation (230). However, this may serve as an advantage for lesion-targeted treatment with the advent of stem cells engineered not only to deliver a therapeutic compound, but to deliver it only upon interaction with tissue exhibiting heightened stiffness via exploitation of a cellular mechanosensory pathway (231). This method was designed for use in targeting cancer tumours to negate the detrimental systemic effects of many cancer therapies, but may also provide a highly relevant and exciting avenue of investigation for MS.
A major consideration with miRNA-based therapies in addition to delivery is off-target effects, and this is of critical importance for miR-145-5p. First detected in mouse heart, expression of miR-145-5p is now known to be ubiquitous across all tissue types. As discussed previously, a reoccurring theme is its role as a tumour suppressor, which when lost can single-handedly promote unchecked proliferation and metastasis of cancerous cells in humans in a variety of tissues (232, 233). With this in mind, care must be taken in expanding preclinical study with an eye towards clinical application. Though tumours do not develop in miR-145 knockout animals in our hands nor have they been reported by others, most data regarding miR-145-5p cancer roles have been generated using human cancer cell lines and human tissue. Limited data can be found regarding miR-145-5p in mouse models of cancer, and in fact existing studies suggest that loss of the mir-143/145 cluster is beneficial to the inhibition of cancer cell survival, expansion and invasion in rodents (234). This apparent dichotomy has been used to argue against miR-145 as a human tumour suppressor in a somewhat reductionist manner; however, it must instead be considered that despite its strong evolutionary conservation the role of miR-145 is not equal in all species. Of importance for our purposes, miR-145-5p dysregulation appears highly concordant between rodent models and humans in several examples emphasized above as well as in EAE and supported by our own findings with chronic demyelination, suggesting that its roles in CNS disease are likely not as disparate across species as they may be in cancer. This does not then discourage the pursuit of miR-145-5p as a therapeutic target in MS and other neurological disorders, but does strengthen the case for targeted CNS cell-type or CNS-compartmentalized approaches to limit the risks systemic treatments
might beget. In addition, steps to move miR-145 antagonism into the clinic should include studies in a mouse model with humanized CNS and/or non-human primates, where its effects are most likely to best resemble those it plays in humans.

6.4 Concluding remarks

With this body of work, we have identified miR-145-5p as an important regulator of OL differentiation and myelination, both in culture in vitro and in the context of chronic cuprizone-mediated demyelination in vivo. Further, expansion of our studies into the EAE model allowed discovery of possible roles for miR-145-5p in other CNS glial cell types during the process of inflammatory demyelination. Collectively, our data show that loss of miR-145 leads to important benefits in both chronic and inflammatory demyelinating models, evidenced by improvements not only in pathophysiological readouts but in functional/clinical outcomes as well. These studies represent useful additions to the field of pre-clinical MS research, and set the stage for future study into miR-145-5p antagonism as a therapeutic strategy for both progressive and relapsing-remitting forms of MS.
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Appendices
Cytoskeletal Linker Protein Dystonin Is Not Critical to Terminal Oligodendrocyte Differentiation or CNS Myelination

Samantha F. Kornfeld1,2, Anisha Lynch-Godrei1,2,*, Sawyer R. Bonin1, Sabrina Gibeault1, Yves De Repentigny1, Rashmi Kothary1,2,3,4*

1 Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada K1H 8L6, 2 Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5, 3 Department of Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5, 4 University of Ottawa Centre for Neuromuscular Disease, Ottawa, Ontario, Canada K1H 8M5

☯ These authors contributed equally to this work.

Abstract

Oligodendrocyte differentiation and central nervous system myelination require massive reorganization of the oligodendrocyte cytoskeleton. Loss of specific actin- and tubulin-organizing factors can lead to impaired morphological and/or molecular differentiation of oligodendrocytes, resulting in a subsequent loss of myelination. Dystonin is a cytoskeletal linker protein with both actin- and tubulin-binding domains. Loss of function of this protein results in a sensory neuropathy called Hereditary Sensory Autonomic Neuropathy VI in humans and dystonia musculorum in mice. This disease presents with severe ataxia, dystonic muscle and is ultimately fatal early in life. While loss of the neuronal isoforms of dystonin primarily leads to sensory neuron degeneration, it has also been shown that peripheral myelination is compromised due to intrinsic Schwann cell differentiation abnormalities. The role of this cytoskeletal linker in oligodendrocytes, however, remains unclear. We sought to determine the effects of the loss of neuronal dystonin on oligodendrocyte differentiation and central myelination. To address this, primary oligodendrocytes were isolated from a severe model of dystonia musculorum, Dstdt-27J, and assessed for morphological and molecular differentiation capacity. No defects could be discerned in the differentiation of Dstdt-27J oligodendrocytes relative to oligodendrocytes from wild-type littermates. Survival was also compared between Dstdt-27J and wild-type oligodendrocytes, revealing no significant difference. Using a recently developed migration assay, we further analysed the ability of primary oligodendrocyte progenitor cell motility, and found that Dstdt-27J oligodendrocyte progenitor cells were able to migrate normally. Finally, in vivo analysis of oligodendrocyte myelination was done in phenotype-stage optic nerve, cerebral cortex and spinal cord. The density of myelinated axons and g-ratios of Dstdt-27J optic nerves was normal, as was myelin basic protein expression in both cerebral cortex and spinal cord. Together these data suggest that, unlike Schwann cells, oligodendrocytes do not have an intrinsic requirement for neuronal dystonin for differentiation and myelination.
Introduction

Oligodendrocytes (OLs), the myelinating cells of the central nervous system (CNS), undergo complex morphological and molecular changes during differentiation and myelination. As oligodendrocyte progenitor cells (OPCs), they are migratory, mitotic cells with a relatively simple morphology. Upon interaction with appropriate environmental signals, OPCs exit the cell cycle and initiate differentiation to become increasingly branched, non-migratory, post-mitotic OLs. OL branches are able to contact and wrap their membrane around axons to produce the compact myelin necessary for saltatory conduction and trophic and metabolic axonal support [1–3]. Contrary to the myelinating Schwann cells of the peripheral nervous system (PNS), which myelinate axons in a 1:1 ratio, a single oligodendrocyte can contact and myelinate many axons simultaneously [4,5].

Requirements for the transition from OPC to OL are numerous and include both cell intrinsic and extrinsic factors. A major outcome of the initiation of differentiation is massive reorganization of the cytoskeleton into a progressively ramified arrangement. In OLs, this means promoting and maintaining the organization of microfilaments and microtubules, as intermediate filaments are absent from these cells [6,7]. Initial membrane protrusions are instigated by microfilament growth to form filopodia, which then expand to form thicker, microfilament- and microtubule-rich lamellipodia [7]. Multiple actin- and tubulin-organizing factors are necessary and essential to OL differentiation. A loss of OL morphological complexity is observed in the absence of actin-organizing factors Wiscott-Aldrich syndrome protein (N-WASP), WASP family verprolin-homologous protein 1 (WAVE1), focal adhesion kinase (FAK) and integrin-linked kinase (ILK) [8–11], as well as tubulin-organizing factors tubulin polymerization promoting protein (TPPP/p25), 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase), Fyn kinase and tau [12–14], amongst others. As morphological differentiation is a requirement for successful myelination to occur, it is clear that the capacity of an OL for cytoskeletal reorganization is critical to CNS function and health.

One cytoskeleton-organizing factor with an unclear role in OLs is the large cytoskeletal linker protein dystonin (DST), which is a member of the plakin family [15]. Tissue-specific isoforms of Dst are found in neural, muscle and epithelial compartments [16–19]. Dst in neural tissue can be alternatively spliced to give rise to the three neuronal transcripts, Dst-A1, -A2 and -A3. The proteins encoded by these transcripts differ only in their N-terminal regions which localize them regionally within the cell [15]. In common between the neuronal isoforms are both an actin-binding and a microtubule-binding domain, conferring their function as cytoskeletal linkers [17,20]. Loss of dystonin results in ultimately fatal peripheral neuropathies, namely hereditary sensory and autonomic neuropathy type VI (HSAN-VI) in humans and dystonia musculorum (Dsst) in mice, characterized by ataxia, dystonic muscle and sensory neuron degeneration [21,22].

While pathology of sensory neurons is the major contributor to Dsst pathogenesis, investigation has also been undertaken to assess the effects of neuronal Dst loss in myelinating glial cells. Two models of Dsst, Dsst-Tg4 (lacking Dst-A1 and -A2) and Dsst-Tg71 (lacking all neuronal isoforms), have peripheral myelination abnormalities that were attributable to intrinsic differentiation defects in Schwann cells [23]. Subsequently, CNS myelination was explored in Dsst-Tg71 animals through analysis of optic nerve and spinal cord, and it was suggested that OLs are unable to myelinate normally in the absence of Dst [24]; however, tools for comprehensive analysis of the character of these OLs were lacking at the time, leaving conclusions about the intrinsic role of neuronal Dst in OL differentiation and myelination somewhat ambiguous.

Here, we take advantage of a recently developed method for primary culture of mouse OLs [25], as well as an assay to measure OL migratory capacity [26] to characterize the morphological
and molecular differentiation of OLs from the more severe $Dst^{dh-27J}$ model of $Dst^{dh}$, which lacks all neuronal DST isoforms. While we show that both OPCs and OLs do express all three neuronal isoforms of $Dst$ endogenously, OLs isolated from $Dstdt^{dh-27J}$ animals do not show a deficiency in morphological or molecular maturity in vitro. Survival is not compromised in OLs lacking Dst, and OPC migration is unaffected. Further, contrary to previous observations made in the less severe $Dst^{dh-Tg4}$ model, we found no defects in myelin sheath thickness or MBP expression in vivo. Taken together, our in vitro and in vivo findings suggest that OLs do not possess a critical requirement for Dst in differentiation and myelination.

Materials and Methods

Animals

For qRT-PCR experiments, wild-type Sprague-Dawley rats were obtained from Charles River. The mutant mouse line $Dstdt^{dh-27J}$ was used for all other experiments. This line arose from a spontaneous mutation in the $Dst$ allele, which confers phenotype in a recessive manner. The mutant animals were first identified at the Jackson Laboratory, and were then characterized as expressing very low levels of neuronal $Dst$ transcript levels relative to wild-type animals [27]. The animals were sacrificed at P0-P1 for primary OL cell culture and at P15 for in vivo analyses. Genotypes were determined by PCR amplification of genomic tail DNA. The University of Ottawa Animal Care Committee approved all experimental protocols. The protocols conformed to or exceeded those defined in the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals, and the Animals for Research Act.

Cell culture

Primary rat OPC and OL cultures were generated from P2 cerebral cortex tissue as described previously [28]. In brief, cortices were dissociated with 0.01% trypsin (Sigma) in the presence of 10 μg/mL DNase (Sigma). Mixed glial cultures were plated in DMEM (Wisent) containing 20% fetal bovine serum, 2% Glutamax (Gibco) and 0.5% penicillin/streptomycin (Gibco) on poly-L-lysine-coated filter cap flasks for 10 days at 37°C and 5% CO2. Flasks were then shaken overnight at 220 rpm, and medium containing suspended OPCs was removed from the flasks. Contaminating glial cells were removed from suspension by differential adhesion. To obtain proliferating OPC cultures, OPCs were plated in SATO medium with 2% Glutaxam, 0.1% bovine serum albumin (Sigma), 50 μg/mL apo-transferrin (Sigma), 5 μg/mL insulin (Sigma), 30 nM sodium selenite (Sigma), 10 nM D-biotin (Sigma) and 10 nM hydrocortisone (Sigma) supplemented with 10 ng/mL PDGF-AA (Millipore) and 10 ng/mL bFGF (Millipore). To obtain differentiating OL cultures, OPCs were plated in SATO medium as above but supplemented with 5 μg/mL N-acetyl-L-cysteine (Sigma), 15 nM triiodothyronine (Sigma) and 10 ng/mL ciliary neurotrophic factor (CNTF; AbD Serotec).

Primary mouse OL cultures were generated from P0 cerebral cortex tissue as described previously [25]. Briefly, cortices were dissociated with papain, and mixed glial cultures were plated in DMEM containing 10% fetal bovine serum, 1% Glutaxam, 5 μg/mL insulin and 0.33% penicillin/streptomycin on poly-L-lysine-coated filter cap flasks for 6 days at 37°C and 8.5% CO2. Medium was further supplemented with 5 μg/mL insulin and cultures were kept at 5% CO2 for an additional 3–4 days. Cultures were then shaken overnight at 220 rpm, and enriched for OPCs by differential adhesion. The OPCs were then plated in differentiating SATO medium with 0.5% fetal bovine serum, 1% Glutaxam, 5 μg/mL insulin, 50 μg/mL holo-transferrin (Sigma), 50 ng/mL CNTF and 0.33% penicillin/streptomycin on coverslips dual-coated with poly-L-lysine and laminin and kept at 37°C and 5% CO2.
OPC migration assay

OPC aggregates were collected and plated as described previously [26]. In brief, following shaking of the mixed glial cultures, medium containing suspended OLs was filtered through 0.45 μm mesh. The mesh was then inverted, and a pipet was used to wash medium back through the mesh to dislodge isolated OPC aggregates. These were collected in a dish, and a dissection microscope was used to identify and remove single aggregates to be plated on laminin-coated coverslips. The aggregates were placed in conditioned mixed glial culture medium (containing insulin) collected from flasks prior to shaking. Cells were left to migrate for 4 and 24 hours prior to fixation. For quantification, concentric circles were overlaid placing the original OPC aggregate at the center. Rings were set at 50 μm increments from the aggregate for migration at 4 hours, and at 100 μm increments for 24 hours migration.

qRT-PCR

RNA was isolated from ~1.5 x 10⁵ primary rat OPCs and OLs using the RNeasy Mini Kit (Qiagen). Samples were collected from proliferating OPCs 4 hours post-seeding in proliferation medium, and from a mixed population of pre-myelinating and myelinating OLs on day 1.5 post-seeding in differentiation medium. Samples were reverse-transcribed with RT² First Strand cDNA synthesis kit (Qiagen) using 120 ng total RNA. Relative expression of each of the three neuronal Dst transcripts in proliferating OPCs and differentiation OLs was determined by qRT-PCR. Briefly, each 25 μL reaction contained 5–12 μL total cDNA, 12.5 μL SsoFast EvaGreen Supermix (Bio-Rad), either 0.2 μL 10 μM forward + reverse primers for dystonin-A1, 1 μL 10 μM forward + reverse primers for dystonin-A2 and–A3 transcripts or 0.2 μL 10 μM forward + reverse primers for actin transcripts, and RNase-free water. All samples were amplified using the following protocol: 3 min at 95°C, followed by 40 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 30 sec. Runs were amplified using a Bio-Rad CFX96 and analysed using qBase+ software. For each primer set, 4–6 biological replicates were analysed (n = 5–6, n = 1 animal) and samples were run in technical quadruplicates. Technical replicates were used for analysis only when Cq values differed by 0.25 or less. The following primers were used for amplification: Dst-A1 forward CTA CAT GTA CGT GGA GGA GCA (779 bp), Dst-A2 forward GAG GCC TGT GCT TCG GAT AG (741 bp), Dst-A3 forward GTGCACCAGTGGACAACAGG (741 bp), Dst-A1/A2/A3 reverse CAT CGT TTG CAC CAA TGC C, actin forward CTG CGT TGG CACCAAAG CAC C, actin reverse ACTGACC CCT CCG AAC ACT CGT. Bands for each transcript were visualized by running qRT-PCR products in a 1% agarose gel containing RedSafe™ nucleic acid staining solution.

Immunofluorescence

All samples were fixed in 3% paraformaldehyde. Cells were fixed for 15 min at room temperature, while OPC aggregates were fixed overnight at 4°C. Following fixation, coverslips were washed with PBS, permeabilized for 5 min in 0.1% Triton-X (Sigma), and blocked for 1 hour in 10% goat serum. Cells stained for F-actin were additionally blocked with 0.1% bovine serum albumin (BSA) for 30 min, and incubated with 1:50 rhodamine-phalloidin (Life Technologies) in 0.1% BSA for 45 min at room temperature. Samples were incubated with primary antibody in 10% goat serum overnight at 4°C, at the following concentrations: NG2 1:250 (EMD Millipore), MAG 1:50 (EMD Millipore), MBP 1:100 (AbD Serotec), Olig2 done 211.F1.1 1:50 (EMD Millipore), and cleaved-caspase 3 (CC3) 1:100 (Cell Signaling). Cells were again washed with PBS, and then incubated with secondary antibodies (Alexa-555, Alexa-488, Alexa-647; Invitrogen) at 1:200 in 10% goat serum for 2 hours at room temperature. All samples were counterstained with Hoechst and mounted in Dako fluorescent mounting medium. For
maturation marker and CC3 analyses, n = 3 for both WT and Dst<sup>dl</sup>-27. For quantification of cells undergoing caspase-mediated apoptosis, the proportion of CC3<sup>+</sup>/Olig2<sup>+</sup> cells out of all Olig2<sup>+</sup> cells was calculated, to avoid quantification of any contaminating cells. Quantification was done for 20–30 photographs taken at 20X magnification from at least two separate coverslips.

**Morphological analysis**

Sholl analysis was used to quantify branching complexity of DD3 OLs by overlaying concentric circles at 30 μm increments from the cell body using ImageJ. Branch intersections were counted at each ring. Cells used for Sholl analysis were co-stained for F-actin to visualize branches and Olig2 to confirm OL identity. For DD6 OLs, total membrane area was measured by tracing total cell outline using ImageJ. Cells used for quantification of membrane area were co-stained with MAG and MBP to visualize both non-compact and compact myelin membrane. For both Sholl and membrane area analyses, n = 3 for WT and Dst<sup>dl</sup>-27. Sholl analysis was conducted for 30 cells per n, and membrane area was measured for 60 cells per n. Cells were deemed membrane positive when they showed any MBP stained region in which branches were no longer discernible but instead were replaced by flattened membrane sheet of any size. Membrane negative cells showed no MBP staining and no membrane sheets. The presence or absence of membrane was quantified for n = 3 for WT and Dst<sup>dl</sup>-27. For each n, 20–30 photographs taken at 20X magnification were analysed from at least two separate coverslips.

**Western blotting**

Cerebral cortex and spinal cord samples were collected from P15 WT and Dst<sup>dl</sup>-27 animals and flash-frozen in liquid nitrogen. Tissue from one animal was considered 1 n, and a total of n = 6 was collected. Protein was isolated by gently homogenizing tissue in 1x RIPA lysis buffer (Sigma) on ice. The lysate was centrifuged at 4°C at high speed to remove insoluble material. Samples were separated by SDS-PAGE in a 15% gel. Membranes were incubated in 1:1000 CNPase (Abcam), 1:1000 MOG (Abcam), 1:1000 MBP (AbD Serotec) and 1:50,000 alpha-tubulin (Cell Signaling) primary antibodies overnight at 4°C in Odyssey blocking buffer (Li-Cor Biosciences). Membranes were washed in 1X TBS, then incubated with secondary antibody (IRDye 680RD and 800CW; Li-Cor Biosciences) at 1:10,000 in Odyssey blocking buffer for 1 hour at room temperature. Membranes were visualized and bands quantified using the Li-Cor Odyssey CLx Infrared Imaging System.

**Transmission Electron Microscopy (TEM)**

P15 WT and Dst<sup>dl</sup>-27 mice were anesthetized by CO<sub>2</sub> and transcardially perfused with 5 mL of phosphate-buffered saline (PBS) followed by 10 mL of Karnovsky’s fixative (4% paraformaldehyde, 2% glutaraldehyde and 0.1 M sodium cacodylate in phosphate-buffered saline, pH 7.4). Optic nerves were collected and fixed overnight at 4°C in the same fixative. Following fixation, 1 mm segments were cut transversely from the mid-point of each nerve. Segments were subsequently washed twice in 0.1 M sodium cacodylate buffer for 1 hour and again overnight at room temperature. Segments were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at room temperature. These were washed twice in distilled water for 5 min, and then dehydrated twice for 20 min for each step in a graded series of ethanol from water through 30%-50%-70%-85%-95% ethanol. This was followed by two 30 min washes in 100% ethanol, two 15 min washes in 50% ethanol/50% acetone, and two 15 min washes in 100% acetone. Optic nerve segments were infiltrated in 30% spurr resin/acetone for 20 min and once overnight, then in 50% spurr resin/acetone for 6 hours, and finally in 100% spurr
resin overnight. Segments remained in 100% spurr resin, which was changed twice a day for three days at room temperature. All infiltration steps were performed on a rotator. Specimens were embedded in fresh liquid spurr resin and then polymerized overnight at 70°C. Ultrathin sections (80 nm) of the optic nerve segments were collected onto 200-mesh copper grids and dried overnight prior to staining. Ultrathin sections were stained with 2% aqueous uranyl acetate and Reynold’s lead citrate. Sections were observed under a transmission electron microscope (Hitachi 7100).

Optic nerves were collected from n = 4 for both WT and Dstdt-27J. Quantification of myelinated axons was done for 12 electron micrographs per n taken at 4000X. G-ratio was measured by calculating the diameter of the axon/diameter of the axon+myelin using ImageJ. For each n, 25 g-ratios were measured for a total of 100 measurements from both WT and Dstdt-27J.

Toluidine blue staining

Semithin transverse sections (0.5 μm) of optic nerves were embedded in resin, mounted on glass slides, and stained with toluidine blue. Sections were scanned with a MIRAX MIDI (Zeiss) and viewed using Zeiss MIRAX viewer software. These samples were used for qualitative optic nerve assessment only.

Statistical analysis

All statistics were done using Prism 6 GraphPad software, with the exception of the qPCR data, which was analysed using qbase+ software (Biogazelle). All two-way comparisons were done using a two-tailed Student’s t-test. Comparisons for qPCR data were done using one-way ANOVA followed by Tukey’s post hoc test. Analysis of g-ratios by axon caliber was done by linear regression analysis. Data shown represent mean ± SEM with the exception of myelinated axon density and mean g-ratio, which represent mean ± SD.

Results

OPCs and OLs endogenously express all neuronal Dst transcripts

Expression of neuronal dystonin in CNS tissue has previously been demonstrated by both RNA in situ hybridization and RT-PCR [24,29,30]. However, we wished to confirm expression of the three transcripts specifically in OLs. Using RNA extracted from primary proliferating OPCs and differentiating OLs we performed qRT-PCR for Dst-A1, -A2, and -A3. All three transcripts are expressed in proliferating OPCs, and in differentiating OLs (Fig 1). Dst-A1 and -A2 showed similar expression levels between OPCs and differentiating OLs, while Dst-A3 showed significantly increased expression in differentiation OLs relative to proliferating OPCs.

Loss of neuronal Dst does not affect morphological maturation in vitro

It has been illustrated in many instances that loss of cytoskeleton-interacting proteins bears a negative effect on OL differentiation and myelin membrane formation. Since Dst is able to directly interact with both microfilaments and microtubules, we first sought to determine whether loss of dystonin affects the morphological complexity of differentiating OLs. We isolated mixed glial cultures from Dst-27J mice and WT littermates, and then further isolated OPCs to produce purified cultures as described previously [25]. OPCs were allowed to differentiate on a mixed PLL/Ln2 substrate for three days (DD3) or six days (DD6), representing intermediate differentiated or terminally differentiated time points, respectively. At DD3, Sholl analysis was used to assess complexity but revealed no significant differences in branch extension between Dst-27J and WT OLs (Fig 2A and 2B). OLs in the two conditions were also
equally capable of producing membrane at this stage of differentiation, as evidenced by similar proportions of membrane-positive cells (Fig 2C). This was maintained at DD6, where again similar proportions of cells were capable of producing membrane (Fig 2F). Cell complexity was further assessed at DD6 by measuring membrane area, which again revealed no differences between Dstdt-27J and WT OLs (Fig 2D and 2E).

Fig 1. Proliferating OPCs and differentiating OLs express neuronal Dst transcripts. Top. Representative RT-PCR products from Dst-Δ1, -Δ2 and -Δ3 with actb loading control in primary proliferating OPCs (left lanes) and differentiating OLs (right lanes). Bottom. qRT-PCR analysis of Dst-Δ1, -Δ2 and -Δ3 expression in primary proliferating OPCs and differentiating OLs. n = 4–6; ΔΔCt, Dst normalized to actb. * p<0.05, n.s. = p>0.05; two-tailed Student’s t-test. Data represent mean ± SEM.

Fig 2. Dstdt-27J OLs exhibit normal morphological differentiation. A. Immunofluorescence micrographs of WT and Dstdt-27J showing branching at DD3. B. Sholl analysis of DD3 OLs. Rings were set at 30 μm increments for quantification of intersections. C. Quantification of the percentage of membrane positive OLs at DD3. D. Immunofluorescence micrographs of WT and Dstdt-27J total membrane production at DD6. E. Quantification of membrane area at DD6. F. Quantification of the percentage of membrane positive OLs at DD6. B, C, E, F: n = 3; all comparisons non-significant by two-tailed Student’s t-Test. Data represent mean ± SEM. Scale bars = 50 μm.
Loss of neuronal Dst does not affect molecular OL maturation in vitro

While OL morphological maturity is associated with increased branch complexity and eventual membrane formation, molecular maturity involves decreased expression of NG2 concurrent with increased expression first of MAG followed by MBP. To determine whether Dst^−/− OLs were able to follow a normal molecular differentiation time course, we assessed proportions of NG2+ (OPC stage), MAG+/MBP− (intermediate OL stage) and MAG+/MBP+ (terminal OL stage) cells at both DD3 and DD6. No significant differences were found in the presence of any of these markers at DD3 (Fig 3A and 3B) or DD6 (Fig 3C and 3D), indicating that OLs lacking DST are able to follow a normal molecular differentiation pattern in vitro.

Apoptosis is not induced in OLs in vitro in the absence of neuronal Dst

To ensure that the apparent health of Dst-deficient OLs was not isolated to a subpopulation of surviving cells, we assessed OL cultures at both DD3 and DD6 for apoptosis. Differentiating OLs were not undergoing significantly more apoptosis at either DD3 or DD6, as assessed by the presence of apoptotic marker cleaved-caspase 3 (Fig 4).

OPCs lacking neuronal Dst migrate normally in vitro

While the effects of loss of neuronal Dst have been little investigated, loss of muscle dystonin has been found to impair migration in myoblasts [31]. Since OPCs are also highly migratory cells, we sought to determine if their motility is affected by a lack of neuronal dystonin. Using a recently established method (ROM unpublished) OPC aggregates were isolated from OPCs in suspension and plated on laminin-2 substrate. Aggregates of roughly equal size were selected and allowed to migrate for 4 or 24 hours. Migration was assessed by quantifying the proportion of total NG2+ cells found at set distances from the original aggregate after 4 and 24 hours post-seeding. Migration ability was similar between Dst^−/− OPCs and WT OPCs at both 4 hours and 24 hours (Fig 5).

CNS myelination is normal in mice lacking neuronal Dst

In vitro analyses revealed no intrinsic differentiation or migration defects in OPCs/OLs lacking DST; however, this does not preclude myelination deficiencies from occurring in vivo. Thus, we assessed myelination of the optic nerve in P15 Dst^−/− mice along with WT littermates for comparison. In this model, P15 is end-stage phenotype, and all Dst^−/− animals exhibited typical Dst^−/− symptoms of ataxia, hind-limb clamping and twisting of the trunk [15].

No obvious size difference or myelination defects could be observed on examination of toluidine blue-stained optic nerves (Fig 6A). A more detailed analysis was performed in optic nerves by transmission electron microscopy (TEM; Fig 6B). WT and Dst^−/− optic nerves contained similar numbers of myelinated axons (Fig 6C). Further assessment of myelin sheath thickness by TEM revealed no overall difference in g-ratio between WT and Dst^−/− optic nerves (Fig 6D), nor was there any shift in myelin distribution based on axon caliber (Fig 6E). Finally, assessment of myelination status was done outside of the optic nerve by quantifying CNPase, MOG and MBP levels in both cerebral cortex and spinal cord, also revealing no change in any of these markers of mature myelin between WT and Dst^−/− (Fig 6F–6M).

Discussion

The loss of functional neuronal Dst isoforms results in a severe phenotype characterized by ataxia, dystonia and death [21,22]. While the phenotype is largely a consequence of sensory neuron degeneration, previous work has implicated myelinating cells in both the PNS and
CNS as contributors to, if not instigators of, the Dstdt phenotype [23, 24]. Specifically in the CNS, it has been argued that minor myelination pathologies are in fact early signs of OL-intrinsic defects in the CNS, and that further deterioration would occur if longevity of the animal model was not so limited [24]. However, tools to investigate this hypothesis were wanting at the time. With our present study, we sought to clarify the effects of Dst loss on OLs using the severe Dstdt-27J model lacking all neuronal Dst isoforms. By employing methods to isolate and culture Dstdt-27J OPCs and OLs in vitro, we were able to divorce OLs from not only the short phenotypic lifespan of the animal model but also from the influence of Dstdt-27J neurons. This allowed us to observe OL-intrinsic behaviors in the absence of neuronal Dst.

Our results suggest that even lacking all three neuronal Dst isoforms, no intrinsic defects exist in OL differentiation at the morphological or molecular levels. This apparent regularity in OL behavior was not limited to a subpopulation of surviving healthy cells, as apoptosis was not increased in Dstdt-27J. Further, in vivo analysis revealed no deficits in CNS myelination of axons or in myelin sheath thickness in optic nerve, and no loss of CNPase, MOG or MBP in spinal cord or cerebral cortex. These findings are dissimilar to what was previously found in the less severe Dstdt-Tg4 model, which retains functional Dst-A3 [24]. While our results do not disqualify the existence of myelination defects in that model, it seems intuitive that if CNS myelination defects are indeed part of Dst pathology then the more severe model would exhibit more severe CNS myelination problems. It must also be noted that while some qualitative in vitro analyses of OLs was undertaken in the initial study (revealing no obvious morphological defects in Dstdt-Tg4 OLs) [24], the tools to isolate and culture primary mouse OLs were limited.

Fig 3. Dstdt-27J OLs exhibit normal molecular differentiation. A. Immunofluorescence micrographs of WT and Dstdt-27J showing maturation marker expression at DD3. B. Quantification of the proportion of NG2+, MAG+/MBP+, and MAG+/MBP+ OLs at DD3. C. Immunofluorescence micrographs of WT and Dstdt-27J showing maturation marker expression at DD6. D. Quantification of the proportion of NG2+, MAG+/MBP+, and MAG+/MBP+ OLs at DD6. B, D: n = 3; all comparisons non-significant by two-tailed Student’s t-test. Data represent mean ± SEM. Arrowheads: yellow = NG2+, orange = MAG+/MBP+, grey = MAG+/MBP+, white = contaminating cell. Scale bars = 50 μm.

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and thus restricted the ability for a more comprehensive analysis of intrinsic OL biology, as we have done here.

We must also acknowledge that while this study provides fairly detailed molecular and morphological analyses, these assessments were done at a single pre-myelinating time point and at a point where terminal differentiation is known to occur for these cells in vitro. Thus, it is possible that differences in morphological and/or molecular development may occur at time points...

**Fig 4. Apoptosis is not increased in Dst<sup>Δ<sub>27</sub></sup><sup>−</sup> OLs.** A. Immunofluorescence micrographs of WT and Dst<sup>Δ<sub>27</sub></sup><sup>−</sup> showing colocalization of nuclear CC3 and Olig2 in apoptotic OLs at DD3. B. Quantification of the proportion of CC3<sup>+</sup>/Olig2<sup>+</sup> relative to total Olig2<sup>+</sup> OLs at DD3. C. Immunofluorescence micrographs of WT and Dst<sup>Δ<sub>27</sub></sup><sup>−</sup> showing colocalization of nuclear CC3 and Olig2 in apoptotic OLs at DD6. D. Quantification of the proportion of CC3<sup>+</sup>/Olig2<sup>+</sup> relative to total Olig2<sup>+</sup> OLs at DD6. A, C: Arrowheads represent CC3<sup>+</sup>/Olig2<sup>+</sup> OLs. B, D: n = 3; all comparisons non-significant by two-tailed Student's t-test. Data represent mean ± SEM. Scale bars = 50 μm.

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prior to terminal differentiation, which we did not investigate. However, since terminal differentiation is achieved with equal success in both WT and Dstdt-27J OLs in vitro within the expected time line, any differences in their development prior to this point must be transient and are unlikely to affect their biological function as mature OLs. This is supported by our in vivo data, which reveal no defects in optic nerve myelination, cerebral cortex myelin protein expression or spinal cord myelin protein expression even at end-stage of the dystonia musculorum disease. Again, differences in developmental timing in OL maturation and myelination prior to this point cannot be ruled out in vivo, but if they do occur these too must be temporary as even end-stage Dstdt-27J animals exhibit myelin status and health that is indistinguishable from WT animals. While our study is primarily concerned with the biological relevance of the loss of Dst in OPCs and OLs, additional investigation would need to be undertaken to clarify whether any transient differences in differentiation dynamics arise when Dst is absent.

Interestingly, intrinsic differentiation and myelination defects do exist in Schwann cells in both the Dst-124 and Dstdt-277 models, and are more severe when all three neuronal isoforms are absent [23]. This was illustrated in in vitro primary cultures and in vivo transplant experiments in which Dst-124 and Dstdt-277 Schwann cells failed to differentiate or myelinate normally. While many similarities exist between Schwann cells and OLs—most obviously their function as producers of myelin—there are also many differences that may account for their apparent disparate requirement for neuronal Dst. First, intermediate filaments are present in Schwann cells and play a role in peripheral myelination, while OLs do not contain intermediate filaments [2,6,7,32]. Dst can bind some intermediate filaments in addition to microfilaments and microtubules, and loss of Dst leads to disorganization of intermediate filaments α-
Fig 6. Myelination occurs normally in vivo in Dst\textsuperscript{27J–27J} animals. A. Light microscope images of transected toluidine blue-stained optic nerves from P15 WT and Dst\textsuperscript{27J–27J} mice. B. Electron micrographs of transected optic nerves from P15 WT and Dst\textsuperscript{27J–27J} mice. C. Quantification of the number of myelinated axons per field of view (FOV) in optic nerves from P15 WT and Dst\textsuperscript{27J–27J} mice. D. Quantification of average g-ratio of all myelinated axons per FOV in optic nerves from P15 WT and Dst\textsuperscript{27J–27J} mice. E. g-ratios plotted by axon caliber in optic nerves from P15 WT and Dst\textsuperscript{27J–27J} mice. F, G. Fluorescence Western blot analysis of CNPase, MOG, MBP and α-tubulin (α-tub) from cerebral cortex (CC—top panels) and spinal cord (SC—bottom panels) from P15 WT and Dst\textsuperscript{27J–27J} mice. Protein sizes—CNPase: 48 kDa; MOG: 27 kDa; MBP isoforms, from top to bottom: 21.5 kDa, 18.5 kDa, 17.0 kDa, 14.0 kDa. α-tub: 52 kDa. H, I. Quantification of total CNPase normalized to α-tub (from blots pictured in F, G). J, K. Quantification of total MOG normalized to α-tub (from blots pictured in F, G). L, M. Quantification of total MBP normalized to α-tub (from blots pictured in F, G). C, D: n = 4; H-M: n = 6; all comparisons non-significant by two-tailed Student’s t-test. C, D: data represent mean ± SD. H-M: data represent mean ± SEM. E: n = 100; data represent individual measurements, comparison non-significant by linear regression analysis. A: scale bar = 50 μm. B: scale bar = 2 μm.
internexin, neurofilament and peripherin in sensory neurons [33,34]. A second key difference between OLs and Schwann cells is the production of a basement membrane. Following contact with an axon, Schwann cells surround their exterior layer with a basement membrane, the mechanical integrity of which is required for continued wrapping of the axon and subsequent myelin production [35,36]. Further, OLs and Schwann cells express a different complement of integrins, transmembrane heterodimeric complexes that convey signals between the extracellular matrix and intracellular space largely to elicit cytoskeletal reorganization [11,37,38]. It has been speculated that Dst may interact with some integrins to mediate cytoskeletal organization [23,24]. Thus it is possible that in Schwann cells, Dst is involved in any or all of intermediate filament organization, basement membrane formation and mediation of signaling of specific integrin complexes—none of which are present in OLs.

Further explanation may exist for the seemingly disposable nature of neuronal Dst in OLs. In mammals, a second member of the spectraplakin exists: microtubule-actin crosslinking factor 1 (Macf1; variably actin crosslinking family 7/Acf7). Two isoforms of Macf1 are preferentially expressed in CNS tissues with little expression in PNS tissues, and share significant homology with neuronal Dst [39,40]. Macf1 can regulate cytoskeletal dynamics, and has both actin-binding and microtubule-binding domains, similar to Dst [40–42]. This raises the possibility that neuronal Dst does serve a function in the CNS, which may be compensated for by Macf1 if the former is lost. However, this idea was previously investigated in the Dstdt-27J model; no increase in Macf1 was observed in brain or spinal cord from Dst<sup>−/−</sup> animals, as would be expected if additional Macf1 were required to fill a role normally played by Dst [40]. While this does not preclude a compensatory function for Macf1, it does suggest that—much like OLs and Schwann cells—Macf1 and neuronal Dst serve similar functions that are likely restricted to their respective nervous system compartments.

Finally, though the lack of Dst does not appear to be biologically relevant to the terminal differentiation of OLs during developmental myelination, it may be required for remyelination following a demyelinating injury. In the healthy CNS, any damage to mature myelin that may occur can be repaired by the differentiation of resident OPCs into new myelinating OLs, which subsequently ensheath the denuded areas of an axon. Though this process is believed to largely mimic the processes of developmental myelination—the so-called recapitulation hypothesis [43,44]—the final product of remyelination includes both thinner sheaths and shorter internodes than expected. This raises the possibility that different mechanisms are involved in remyelination than in developmental myelination, and this remains an important open question in OL biology. Interestingly, it has also been noted that in some instances of CNS demyelination, remyelination is carried out not by OLs but by Schwann cells [45]. These Schwann cells are not emigrated from the PNS, but surprisingly arise from CNS glial progenitors in specific circumstances when the injury results not only in demyelination but also in depletion of astrocytes at the injury cite [45,46]. The expression of dystonin has not been investigated in the context of remyelination. Future work should include the use of remyelination models applied to Dst<sup>−/−</sup> animals—such as organotypic brain slice culture—to explore the role of neuronal Dst in this important process, particularly when Schwann cells are implicated in CNS recovery from demyelination.

**Conclusions**

Here, comprehensive *in vitro* and *in vivo* analyses were performed to better understand the role of neuronal Dst in OLs using the Dstdt<sup>−/−</sup> animal model. Our results revealed that even in the absence of all three isoforms of Dst, OLs are able to differentiate normally both morphologically and molecularly. Survival of OLs lacking Dst is not compromised, nor is migration of
OPCs. *In vivo* assessment of optic nerve myelination at disease end-stage showed normal density of myelinated axons and myelin thickness in optic nerve, as well as normal expression levels of CNPase, MOG and MBP in both spinal cord and cerebral cortex in animals with *dystonia muscolorum*. While we cannot rule out the occurrence of transient differences in OL maturity or that other cellular processes may be affected in OPCs and OLs in the absence of Dst, these data suggest that neuronal Dst is not essential for proper terminal OL differentiation or functional myelin development in the CNS.

**Supporting Information**

S1 Fig. Proliferating OPCs and differentiating OLs express neuronal Dst transcripts. Whole-gel view of RT-PCR Dst-A1, -A2 and -A3 with actb loading control in primary proliferating OPCs and differentiating OLs. L = ladder; bp = base pairs. (TIF)

S2 Fig. CNPase, MOG, and MBP expression is unchanged in *Dstdt-27J* cerebral cortex and spinal cord. Whole-membrane view of all CNPase, MOG, and MBP isoforms, as well as α-tubulin (green) in cerebral cortex (CC) and spinal cord (SC) from P15 wild-type (WT) and *Dstdt-27J* mice. L = ladder; kDa = kilodaltons. (TIF)

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**Author Contributions**

Conceived and designed the experiments: SFK ALG RK. Performed the experiments: SFK ALG SRB SG YDR. Analyzed the data: SFK ALG SRB. Contributed reagents/materials/analysis tools: SFK ALG SRB SG YDR RK. Wrote the paper: SFK RK.

**References**


Exercise to Manage MS Fatigue: Counterintuitive Yet Effective

A. V. Caprariello,1,8† S. F. Kornfeld,2,3,8† K. M. Othonos,4,6† & A. Hicks5,8,*

1University of Calgary, Hotchkiss Brain Institute, Calgary, AB, Canada; 2University of Ottawa, Ottawa, ON, Canada; 3Ottawa Hospital Research Institute, Ottawa, ON, Canada; 4University of British Columbia, Vancouver, BC, Canada; 5McMaster University, Department of Kinesiology, Hamilton, ON, Canada

5Scholar Program for Researchers in Training (SPRINT) Program, MS Society of Canada

†These authors contributed equally.

*Address all correspondence to: Audrey Hicks, PhD, Ivor Wynn Centre, Room 204, McMaster University, 1280 Main Street West, Hamilton ON, Canada, L8S 4L8; hicksal@mcmaster.ca

ABSTRACT: The natural history of multiple sclerosis (MS), a disease characterized by multifocal and unpredictable inflammatory attacks, varies widely both between and within affected individuals. Despite variable and often severe neurological symptoms, the vast majority of the approximately 2.5 million people worldwide with MS describe fatigue as the single-greatest impediment to quality of life. It is unknown whether fatigue in MS is a direct consequence of disease activity or if instead is secondary to co-morbidities such as medication side effects or depression. Regardless of its cause, physical exercise is becoming increasingly recognized as a safe and efficacious therapeutic approach for managing MS-related fatigue. In fact, there now exist evidence-based physical activity guidelines designed specifically for adults with MS. It remains unclear how peripheral adaptations might ameliorate symptoms originating centrally. Resolving such a paradox requires an understanding of exercise-induced changes at multiple levels of analysis spanning the molecule to the psyche. Mounting evidence for the safety and efficacy of physical activity together with the availability and relative affordability of exercise and rehabilitation facilities places this treatment modality as a potentially potent combatant in the fight against MS fatigue.

KEY WORDS: multiple sclerosis, fatigue, exercise

I. INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). It is typified by areas of inflammation and demyelination in CNS white matter, followed by eventual neuronal degeneration. Areas of demyelination, or plaques, may be found anywhere in the CNS; however, they occur most frequently in the brain stem, optic nerve, spinal cord, and periventricular regions.¹ Loss of myelin in the CNS is manifested as both physical and cognitive disabilities in patients. Early in the disease, remyelination can occur, leading to an apparent recovery. But MS is a progressive disease, and these symptoms return and become more debilitating with disease chronicity. MS most commonly presents in young adulthood, with diagnosis occurring typically...
between the ages of 20 and 40, and affects approximately 2.5 million people globally. The etiology of MS remains poorly understood. It is widely considered to be a T-cell-dependent autoimmune disease, driven by inflammatory events. Support for this is found in the fact that risk alleles associated with MS are almost entirely immune-related genes. Additional roles for B cells, environmental factors, and viral infection also have a basis in scientific evidence.

While most symptoms will follow the ebb and flow of relapse and remission experienced by the majority of MS patients, an overwhelming symptom experienced by most is fatigue—a fatigue that can be devastating to quality of life, and which persists continuously. Past research on fatigue in MS was unable to delineate its source; is fatigue part of MS pathophysiology, perhaps a consequence of constant CNS inflammation? Or is it merely secondary to MS disease treatments? More recent investigation, spurred by the concrete realization of the often devastating nature of this symptom for patients, has started to better tease apart mechanisms which may be driving MS fatigue. Concurrently, evidence has begun to emerge which highlights a potentially surprising but clearly beneficial treatment for MS fatigue: physical exercise. Contrary as this may seem, both patient-reported results on fatigue as well as study of some underlying mechanisms that may drive MS fatigue reveal positive results after exercise. Coupled with the accessibility, affordability, and non-harmful nature of exercise, it must be recognized as an important therapeutic strategy to combat fatigue for MS patients. This review aims to highlight the value of exercise for MS fatigue by evaluating emerging concepts in the context of a vast, historical literature surrounding human muscle fatigue, and then addressing the putative cellular and molecular mechanisms that are theorized to mediate the beneficial effects of exercise on fatigue in MS populations.

II. MUSCLE FATIGUE IN HEALTHY INDIVIDUALS

Attempting to understand fatigue in MS requires an understanding of the same phenomenon in healthy subjects, and deciphering whether or not parallels can be drawn between the two. In contrast to fatigue in MS, the mechanisms of fatigue in able-bodied individuals are well elucidated. Decades of research in muscle and exercise physiology have unraveled the vast complexities of the neuromuscular system and their putative contributions to fatigue during periods of constant activity. Fatigue, or the reversible reduction of force-generating power during sustained activity, can be broadly separated into two categories: (1) peripheral, occurring at the neuromuscular junction (NMJ) and the applicable muscle(s); and (2) central, arising at the level of the spinal cord, above the spinal cord ("supraspinal"), and the relevant control centers in the brain. Comparatively more is known about the former than the latter; factors underlying muscle fatigue in peripheral domains have been previously described in vast detail.

Peripheral fatigue describes changes at or distal to the neuromuscular junction (NMJ). The NMJ, in its simplest form, consists of a motor neuron originating in the spinal cord together with the skeletal muscle fibers that its axon innervates, together comprising a motor unit. Motor units increase the force of contraction in one of two ways: (1) spatial
activation during which force generation is increased simply by increasing the number of activated motor units; or (2) temporal activation, in which the same number of motor units increases its firing frequency, producing summated force through fusion of muscle twitches. In reality, muscle contraction is a dynamic process involving a combination of these two strategies to sustain a given force demand over time. Given the complexity of the NMJ and the associated muscles, it follows that peripherally derived mechanisms could underlie fatigue in various ways. Depleted energy supplies during starvation or intense physical exercise, for example, cause muscles to stiffen since energy is required for muscles to relax. Energy-rich ATP allows the thick muscle filaments to disengage from the thin filaments against which it pulled to initiate muscle contraction. An energy-depleted state therefore disrupts muscle physiology, resulting in the experience of exhaustion. Peripherally derived fatigue occurs also in disease states such as myasthenia gravis\(^\text{16}\) in which a dysregulated immune system attacks the neurotransmitter receptors at the NMJ. Having severed the link between the neuron and its associated muscle(s), muscle fatigue and eventual paralysis invariably result.

Central fatigue is defined generally as the progressive exercise-induced failure in voluntary activation of the muscle, stemming from impairments in excitation and/or excitability of spinal motor neurons.\(^\text{17}\) Multiple etiologic factors involving complex and incompletely understood mechanisms could contribute to central fatigue, but the presence of central fatigue can be noninvasively demonstrated by a technique called twitch interpolation.\(^\text{18}\) The underlying assumption of this technique is that if additional force can be evoked through the superimposition of motor nerve stimulation during a maximal voluntary effort, force generation was impeded by central fatigue.

As it relates to fatigue, the motor neuron is influenced by factors both intrinsic and extrinsic to the cell. Like other neurons in the body, motor neurons are innately excitable, meaning they can initiate self-propagating and sustainable nervous impulses in response to external stimuli but also spontaneously. Decreased excitability, such as by the loss of a neuron’s insulating myelin sheath may be highly relevant to MS-specific fatigue, in which myelin loss features prominently. Not only a passive insulator, myelin also modifies and reorganizes the neuron’s membrane components that underlie its electrical properties so as to enhance the efficiency of electrical propagation. The loss of myelin as occurs in multiple sclerosis, therefore, would have profound effects on brain transmission that could present as fatigue.

III. THE EXPERIENCE OF FATIGUE IN ADULTS WITH MS

The role of fatigue in MS cannot be overstated. It affects upwards of 90% of MS patients and is considered the most disabling symptom by as many as 75%.\(^\text{7-11}\) Although it is often reported as one of the first symptoms, it has not been shown to be associated with brain atrophy or lesion load.\(^\text{19,20}\) Interwoven into all three dimensions of fatigue (physical, cognitive, psychological), MS fatigue is difficult to pinpoint, harder yet to define. This is true of fatigue generally; it has been described as a multifaceted syndrome with multiple sites of origin. Physical fatigue in MS is experienced not simply as an inability
to maintain a given effort, as is customary in exercise physiology. It is also an inability to
do simple tasks or even to get out of bed in the morning. Cognitive fatigue in MS often
includes issues of impaired focus, declarative memory, and behavioral control. MS affects
the psychosocial domain as well. Nearly 40% of people with MS feel that friends and
family perceive their fatigue negatively, for example. A significant challenge to treating
MS fatigue, therefore, is to disentangle contributions from impaired brain function versus
psychosocial factors such as disease coping, action control, and depression. Furthermore,
personality adds an additional layer of complexity into the mix. Considered the interme-
diary between physical disability and psychosocial outcomes, personality contributes to
the amorphous, unpredictable nature of MS fatigue. Although no personality traits have
shown to separate independently from depression when their role in the genesis of MS
fatigue is assessed,21 there is emerging evidence that fatigue may be a driver of disease
rather than simply a consequence of other factors.22

Despite major advances in pharmacological treatments targeting relapse and pro-
gression in MS, few treatments are available to combat the more insidious symptoms
that affect patients’ quality of life relating to mood, pain, and fatigue. Off-label phar-
macological interventions aimed at relieving fatigue such as modafinil, amantadine, or
acetyl-L-carnitine are sometimes prescribed; however, recent trials have shown that
evidence of their effects on fatigue in MS are conflicting and largely inconclusive.23–26
In addition to the limited evidence of their efficacy for treating fatigue, these drugs are
also accompanied by a host of possible side effects. Modafinil, FDA-approved to treat
narcolepsy, can cause insomnia, anxiety, headache dizziness, and heart palpitations; these
are similar to the possible side effects from the FDA-approved antiviral drug amanta-
dine.24,27 Acetyl-L-carnitine, which is required for fatty acid transport into mitochondria
to generate cellular energy, has a shorter list of potential secondary effects but can cause
nausea and gastrointestinal irritation.24 The possibility of creating additional symptoms by
using these medications despite limited evidence of fatigue reduction, combined with the
pervasiveness and reportedly overwhelming effects of fatigue in the lives of MS patients,
highlight the fact that fatigue management must be addressed on non-pharmacological
fronts as well. While exercise is not an instinctive prescription for fatigue, there is mounting
evidence that physical activity can lead to improvements in MS-related fatigue (as well as
other symptoms). Historically, MS patients were warned against exercise due to concerns
that investing energy in physical activity may worsen fatigue, and that increasing body
temperature may exacerbate other MS symptoms and even lead to relapse.28 However,
these apprehensions regarding safety have since been largely dispelled. Meta-analyses
of clinical trials studying the effects of exercise on relapse and other adverse events have
found no increased risk of relapse (and indeed, one reported a slightly reduced risk of
relapse in MS patients following an exercise regime), and that adverse events reported
were primarily related to musculoskeletal injuries which typically occur with exercise,
regardless of whether a subject has MS or is healthy.29,30

Since the general acknowledgement of fatigue as a primary source of disability and
the widening acceptance that exercise is safe in MS patients, a wealth of studies have been
undertaken to investigate the clinical effects of physical activity on fatigue in MS. The
breath of this literature is too vast to explore comprehensively here; however, multiple reviews and meta-analyses have examined the subject with the general consensus that exercise leads to improvements in MS fatigue.29,31–34 These studies, taken together with the evidence that physical activity does not pose harm for MS patients, suggest that exercise is a valuable intervention tool to help alleviate fatigue symptoms in MS patients. Obvious advantages of this type of intervention over pharmacologic ones include low cost and low likelihood of side effects.

IV. MEASURING FATIGUE IN MS

As previously mentioned, fatigue is reported in over 90% of MS patients.7–9 Most of these patients identify fatigue as their most disabling symptom,10,11 making it a critical target for therapeutic intervention. Despite this, few effective treatments have been developed for clinical use. This is due mainly to the roadblocks hindering development of fatigue treatments, including the difficulty of measuring fatigue, the expanding but still limited understanding of the mechanisms that drive it, and challenges in separating aspects of peripheral and central fatigue.

Peripheral fatigue is most easily evaluated, using objective tools that address muscle fatigue directly using endurance tests such as the Six-Minute Walk and dynamometer. These tests have been found to be reliable in tracking clinically significant changes specifically in MS patients.35–37 Conversely, no wholly objective tools exist to quantify central fatigue. Thus, more subjective measures such as patient questionnaires provide the bulk of central fatigue assessments. A large variety of questionnaires have been developed for fatigue assessment. For MS in particular, commonly used assessments include the Modified Fatigue Impact Scale (MFIS), Fatigue Severity Scale (FSS), and Checklist Individual Strength (CIS20R). Though there are confounding factors in the data obtained from such questionnaires due to their subjective nature, the MFIS, FSS, and CIS20R have demonstrated both validity and reliability specifically in MS patients.38–41

A. FSS

The FSS was developed in 1989 by Krupp et al.42 to assess fatigue in patients with MS and lupus. The questionnaire includes a series of 9 questions each graded on a 7-point Likert scale, addressing the impact of fatigue on a patient’s physical function in every day life and exercise, and in their family and social life. The questions are heavily weighted towards addressing the impacts of peripheral fatigue.

B. MFIS

The MFIS is a permutation of the 40-question fatigue impact scale (FIS) originally created by Fisk et al.43 and was modified by the National MS Society to eliminate redundancies in content. In its modified form, the MFIS comprises 21 questions scored on a 5-point Likert scale. An advantage of the MFIS over the FSS is its subdivision into how fatigue impacts physical, cognitive, and psychosocial function, allowing some interpretation of peripheral versus central fatigue in a patient. It has been proposed that MFIS is more
sensitive than the FSS to changes in fatigue over time, making it a preferred tool for following fatigue during rehabilitation.\(^{41}\)

**C. CIS20R**

Similar to the MFIS, the CIS20R also has a multidimensional assessment of fatigue, addressing both peripheral and central aspects. It contains 20 questions ranked on a 7-point Likert scale which allow scoring of a patient’s subjective feeling of fatigue, loss of concentration, loss of motivation, and loss of physical activity.\(^{44,45}\) Though it was not initially developed as a tool for MS, it has shown good correlation with both FSS and MFIS scores in MS patients.\(^{38}\)

**V. POTENTIAL MECHANISMS BEHIND A BENEFICIAL EFFECT OF EXERCISE ON MS-RELATED FATIGUE**

Although existing studies investigating the effects of exercise on MS-related fatigue have been heterogeneous in terms of their study population, sample size, control groups, and exercise plan, the overall interpretation is that exercise can have a positive effect on reducing fatigue. Based on current understanding, there are a few hypotheses that can explain the beneficial effects of exercise on MS-related fatigue.

First, exercise therapy may improve general physical and cardiorespiratory fitness, resulting in an increase of energy reserves. This is accompanied by a concomitant decrease of the negative effects of physical inactivity, which could in turn alleviate MS-related fatigue.\(^{52}\) Individuals with MS have reduced muscle performance, impaired muscle activation, and/or reduced muscle quality.\(^{56}\) Additionally, their muscles show signs of physical inactivity, weakness, and muscle fiber atrophy.\(^{47}\) Furthermore, there is an observed shift of muscle fiber composition from type I to type IIa fibers consistent with that seen in spinal cord injury, as well as decreased oxidative enzyme activity that results in lower energy supply.\(^{57}\) Although such muscular defects are consistently observed in patients with MS, studies have shown that MS-related fatigue has a central origin with impaired cortical motor activation and diffuse axonal dysfunction, underlying a failure to drive motor neurons sufficiently.\(^{48,49}\) This may point to a direct central nervous system (CNS) fatigue, rather than a consequence of intramuscular or peripheral changes.

Work by Colcombe et al. has shown that increased cardiorespiratory fitness is associated with decreased age-related decline in brain tissue density.\(^{50}\) To that extent, frontal brain regions that are normally associated with a wide range of higher-order cognitive functions, such as attention, memory processes, and motivation, were especially affected and showed significant volume increase with aerobic exercise training.\(^{51}\) Overall, research in the field suggests that aerobic exercise and cardiorespiratory fitness have beneficial effects on cognition and brain function. These include angiogenesis and growth of capillaries in the brain,\(^{52}\) increase in length and number of neuronal connections,\(^{53}\) and neurogenesis in the hippocampus.\(^{54,55}\) Cumulatively, these structural changes render the brain more plastic and adaptive.\(^{56}\) A recent article demonstrated an association between prefrontal cortex hyper-connectivity and cognitive fatigue in MS. Extensive networks of hyperconnectivity arise...
from inappropriate and persistent task-oriented disengagement processes. The maintenance of such high-energy-demanding networks rapidly depletes neuronal energy reserves. It can be hypothesized that an increase in cardiorespiratory fitness could subsequently increase the energy reserves available for high-order brain functions. This in turn could improve neuronal functioning and possibly alleviate MS-fatigue symptoms.

Second, regular physical activity may provide neuroprotection, partially by influencing neuroactive factors, and thereby reduces any long-term disability. Exercise intervention can attenuate cognitive deterioration by influencing neuronal growth factors to promote neuronal and axonal health. Several lines of evidence suggest that exercise may offset neurodegenerative processes and mitigate neurocognitive decline through insulin-like growth factor (IGF)-mediated mechanisms. IGF signaling has important roles in cell survival, CNS growth, and myelination during development, as well as neuroprotective functions in the adult brain. It has been shown that disease progression can be abrogated in the Experimental Autoimmune Encephalomyelitis (EAE) mouse model of MS via effective IGF-1 stimulation of regulatory T cells. Research suggests that many of the brain-related beneficial effects conferred by exercise, such as neurogenesis, plasticity, and energy regulation, are mediated by IGF-1. Further, IGF-1-mediated increase in brain and neuronal functioning could potentially mitigate symptoms such as MS-related fatigue. Brain-derived neurotrophic factor (BDNF), a potent neuroprotective growth factor associated with CNS neurogenesis, learning, memory, and cell survival, is significantly elevated by exercise. Lower levels of BDNF were shown to be associated with an exacerbation of prostate cancer-related fatigue, suggesting that persistent mental, emotional, and physical fatigue symptoms could be influenced by neurotrophic factor levels. Since BDNF expression is decreased in both chronic fatigue syndrome (CFS) and MS, we postulate that exercise-induced BDNF may lessen fatigue-symptom severity.

Third, physical activity may regulate oxidative stress-induced damage and, in addition to its immunomodulatory effect, could influence the complex interactions between cytokines, hormones, and growth factors. The exact causal factors of MS remain unknown; however, immune cell infiltration in the CNS, vitamin D deficiency, genetic risk alleles, viral infection, hypoxia, and oxidative stress have all been implicated. Among these, microglia-mediated inflammation and oxidative stress have been heavily linked to the pathophysiology of MS. Increased oxidative stress and/or mitochondrial dysfunction have also been implicated in the pathophysiology of several other fatigue-associated disorders such as CFS, fibromyalgia, and systemic lupus erythematosus (SLE). More specifically, among SLE patients, those who presented with debilitating levels of fatigue had higher markers of oxidative stress, suggesting a potential link between the two. It can be postulated that a similar oxidative stress-mediated mechanism could contribute to MS-related fatigue.

Microglia are resident macrophages of the CNS that mediate immune responses against pathogens and remove debris from sites of injury. Under normal physiological conditions, a bidirectional cross-talk between resident brain cells (neurons, astrocytes, and oligodendrocytes) and microglia tightly controls microglial number and function. In MS, there is an observed disruption of this cross-talk, which normally suppresses...
excessive microglial activation, and results in microglial “priming” even at distant sites of
damage. Following exposure to pro-inflammatory cytokines that are present in the brain of
MS patients, these “pre-activated” or “primed” microglia can conform to a cytotoxic
phenotype and result in further cellular damage, axonal degeneration, and exacerbated
inflammatory response. Evidence suggests that physical exercise can modulate cytokine production by promoting a more anti-inflammatory milieu, and this could potentially counteract the immune dysregulation associated with MS. A recent study involving prostate cancer patients undergoing radiotherapy treatment showed that physical exercise reduced cancer-related fatigue and decreased levels of pro-inflammatory cytokines. It can be postulated that similar exercise-mediated reduction of pro-inflammatory cytokines may help alleviate MS-related fatigue.

Alternatively, microglia/macrophage-derived reactive oxygen species (ROS) and reactive nitrogen species (RNS) are released into the extracellular space, thereby triggering axonal injury and damage to neighboring cells. Free radical-mediated damage to mitochondria and mitochondrial DNA (mtDNA) can result in harmful mtDNA mutations and mitochondrial dysfunction. This in turn can disrupt the activity of important mitochondrial respiratory chain enzymes that are crucial for oxidative phosphorylation and energy generation. Exercise training has been shown to increase brain mitochondrial biogenesis and maintain normal cellular bioenergetics. Further, exercise has been shown to reduce oxidative stress in hypertension and obesity-related liver diseases; both of which are associated with mitochondria-rich tissues such as the heart and liver. It could be assumed that exercise may have a similar effect by reducing oxidative stress-induced damage in the brain, another high-energy-requiring tissue. This once again demonstrates how physical activity can have a positive effect on MS symptoms and potentially alleviate MS-related fatigue.

Finally, physical activity may mitigate abnormalities in cytokine production and the neuroendocrine system, as well as normalize the hypothalamic-pituitary-adrenal (HPA) axis deregulation. The HPA axis is a stress response system that regulates many body processes such as the immune system, mood, emotions, and energy storage, to name a few. The reciprocal regulation between the HPA axis and immune systems is mediated partially via stress hormones that may influence disease progression in MS. Exercise has been shown to be an effective regulator of the HPA axis, leading to increased circulation of major stress hormones such as catecholamines and glucocorticoids. Such increased circulation can reduce neuroinflammation associated with the disease by promoting anti-inflammatory and inhibiting pro-inflammatory cytokine synthesis. It can be postulated that the elevation of stress hormone levels associated with regular physical activity may play a neuroprotective role by modulating immune dysregulation present during MS disease progression.

More recent studies are providing additional information on both the underlying physiological basis of MS-related fatigue and the potential role of exercise in mitigating this symptom. Mulero et al. conducted a 6-month, 3 times per week aerobic exercise study, and compared circulating mRNA using a whole genome microarray between healthy controls and fatigued MS patients. Comparing between the two groups, differential expression was

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found between 889 transcripts at baseline. The same comparison 6 months after exercise cessation astoundingly showed no significant differences between MS and healthy control, suggesting that all transcripts previously found to be differentially expressed in fatigued MS patients reverted to healthy control levels following exercise. A small subset of six inflammation-related genes were validated from the microarray data, all of which showed decreased expression following the exercise program, and this was illustrated particularly in MS patients who were fatigued at baseline but were scored as non-fatigued at the end of the program. This study again included a small, non-randomized group of patients, and the data require considerable additional validation and controls to confirm the microarray results at both the mRNA level and the functional (i.e., protein) level. That being said, it represents the only unbiased analysis that we know of that investigates changes in gene expression in the framework of MS, fatigue, and exercise, and further analysis of the data may reveal previously unreported mechanisms that warrant examination.

The value of resistance training for fatigue in MS has also been investigated recently from a mechanistic perspective. A cohort of fatigued MS patients participated in a 12-week resistance training regime, and blood and cerebral spinal fluid (CSF) samples were collected at baseline and regime completion for analysis of selected cytokine concentrations. All patients in the study showed reduced fatigue following the exercise program. Significant reductions of cytokine levels in plasma and/or serum were observed subsequent to completion of resistance training, including TNF-α, IL-6, IL-17, and IL-1ra, among others. Interestingly, none of the assayed cytokines were changed in the CSF. While this study too contained a small, non-randomized cohort and requires additional controls, these data along with those shown by Mulero et al. indicate the potential for both aerobic and resistance exercises to both ameliorate fatigue in MS and provide valuable clues as to how this improvement may be driven by underlying physiology.

Specific forms of exercise used in the studies discussed above to investigate underlying mechanisms of fatigue improvement include 30 minutes of continuous walking, and a short cardiovascular warm-up followed by resistance training using typical gym equipment covering all major muscle groups over a 60-minute time-span. A follow-up study suggested that a combination of both cardiovascular and resistance training can also be beneficial in alleviating inflammation, though fatigue was not specifically addressed. The anti-inflammatory effects of exercise do appear to increase in proportion to exercise intensity. The first high-intensity resistance training study involving whole-body exercises was that conducted by Kierkegaard et al., which as described above demonstrated significant reductions in peripheral, pro-inflammatory cytokine levels, and these reductions were associated with improvements in fatigue and health-related quality of life. In parallel with these high-intensity studies, low-impact training programs are also being investigated. A meta-analysis examining yoga as a therapeutic intervention revealed no change in fatigue status in MS patients. Nevertheless, important ancillary factors such as group exercise and engaged, competent supervision contributed to program success. It is important to note that the majority of studies we have reviewed are preliminary findings based on a small sample size and inconsistent and/or infrequent outcome measurements. An important next step in the field is to validate the many preliminary findings in large,
randomized controlled trials. Only through such trials will we realize the true potential of physical exercise for relief of MS fatigue. By illuminating those factors that are not merely epiphenomena but are critical mediators of MS-fatigue relief, the promise of a new generation of an affordable and accessible treatment approach can be realized.

VI. CONCLUSION

Of the many crippling symptoms experienced by those living with MS, fatigue is often described as the most disabling. It pervades all three dimensions of fatigue, sparing none of its physical, cognitive, and psychosocial domains. Although wakefulness-promoting agents such as modafinil may provide symptom relief, an incomplete understanding of underlying disease activity raises questions about whether the benefits of such medications outweigh their potential side effects. The subjective nature of fatigue, no matter its cause, raises questions about how best to treat it. Fatigue evaluation, although generally self-reported and lacking objectivity, has nevertheless demonstrated clear benefit in tracking lethargy both over time and in response to therapeutic interventions. Such evaluations indicate a clear role for physical exercise in the management of MS fatigue. Exercise to treat fatigue may at first glance seem counterintuitive; indeed, it was previously discouraged for fear of increased relapse activity. However, a growing number of studies and a recent, large-scale meta-analysis argue strongly that such fears are unfounded and that exercise effectively benefits fatigue management. An aggregation of existing studies points to three general mechanisms underpinning the therapeutic benefit of exercise in treating MS fatigue: (1) increases in cardiorespiratory fitness (i.e., increased energy reserves), (2) neuroprotection/remyelination, and (3) anti-inflammatory activity. Thus, exercise not only alleviates the subjective experience of fatigue but may also be correcting underlying pathophysiology relating to, for example, unchecked nervous system inflammation. The affordability and availability of exercise make it a potent combatant in the fight against MS fatigue.

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Transgenic expression of neuronal dystonin isoform 2 partially rescues the disease phenotype of the dystonia musculorum mouse model of hereditary sensory autonomic neuropathy VI

Andrew Ferrier1,3,†, Tadasu Sato1,2,†, Yves De Repentigny1, Sabrina Gibeault1, Kunal Bhanot1, Ryan W. O’Meara1,3, Anisha Lynch-Godrei1,3, Samantha F. Kornfeld1,3, Kevin G. Young5 and Rashmi Kothary1,3,4,*

1Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada K1H 8L6 2Division of Oral and Craniofacial Anatomy, Graduate School of Dentistry, Tohoku University, Sendai 980-8575, Japan 3Department of Cellular and Molecular Medicine and 4Department of Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5 5National Research Council of Canada-Human Health Therapeutics, Ottawa, Ontario, Canada K1A 0R6

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A newly identified lethal form of hereditary sensory and autonomic neuropathy (HSAN), designated HSAN-VI, is caused by a homozygous mutation in the bullous pemphigoid antigen 1 (BPAG1)/dystonin gene (DST). The HSAN-VI mutation impacts all major neuronal BPAG1/dystonin protein isoforms: dystonin-a1, -a2 and -a3. Homozygous mutations in the murine Dst gene cause a severe sensory neuropathy termed dystonia musculorum (dt). Phenotypically, dt mice are similar to HSAN-VI patients, manifesting progressive limb contractures, dystonia, dysautonomia and early postnatal death. To obtain a better molecular understanding of disease pathogenesis in HSAN-VI patients and the dt disorder, we generated transgenic mice expressing a myc-tagged dystonin-a2 protein under the regulation of the neuronal prion protein promoter on the dtTg4/Tg4 background, which is devoid of endogenous dystonin-a1 and -a2, but does express dystonin-a3. Restoring dystonin-a2 expression in the nervous system, particularly within sensory neurons, prevented the disorganization of organelle membranes and microtubule networks, attenuated the degeneration of sensory neuron subtypes and ameliorated the phenotype and increased life span in these mice. Despite these improvements, complete rescue was not observed likely because of inadequate expression of the transgene. Taken together, this study provides needed insight into the molecular basis of the dt disorder and other peripheral neuropathies including HSAN-VI.

INTRODUCTION

Hereditary sensory and autonomic neuropathies encompass a diverse group of inherited disorders of the peripheral nervous system, characterized by progressive sensory neuron degeneration and varying degrees of autonomic dysfunction (1). The genetic spectrum of hereditary sensory and autonomic neuropathies (HSANs) covers both autosomal dominant and autosomal recessive forms. Autosomal dominant traits typically present in the second and third decade of life with marked sensory involvement and little autonomic and variable motor involvement, while autosomal recessive forms typically show an early onset pattern (i.e. congenital or during childhood) with overt sensory and autonomic dysfunction (2). Based on age of onset, mode of inheritance and predominant clinical features, a classification system was devised representing HSAN types I–V (3). Over the past 15 years, 12 disease-causing HSAN alleles have been identified, albeit the pathological mechanisms in over two-thirds of HSAN patients remain unresolved. As such, understanding the functional implications of known or novel genetic
defects is urgently needed to better understand the molecular basis of these disorders.

A recent study has identified deleterious homozygous mutations in the BPAG1/DST gene that impart a frame-shift mutation resulting in a newly described hereditary autonomic sensory neuropathy in four infants (4). The dystonin protein is exceptionally large (>600 kDa) and is capable of interacting with all cytoskeletal filaments. The frame-shift mutation, which reduces dystonin-a transcript expression, starts at Glu4995 and leads to a loss of the C-terminus, which harbors the microtubule-binding domain (MTBD), a domain common to all major dystonin-a isoforms (5). The patients’ clinical features are reminiscent of familial dysautonomia (also known as HSAN-III, or Riley-Day syndrome), although the course and outcome of disease are more severe and are ultimately fatal. This newly reported dystonin-related neuropathy was termed HSAN-VI and shares clinical features reminiscent of those seen in dystonia musculorum (dt) mice. Indeed, both human patients and dt mice suffer from limb contractures and dysautonomia.

The dt phenotype is characterized as a loss of limb coordination beginning between 11 and 14 days postnatal (6,7). As the disease progresses, which it does rapidly, limb movement becomes more uncoordinated, while writhing and twisting of the trunk become increasingly prominent features. Among the pathological features displayed by dt mice, degeneration is most apparent in large and medium-sized proprioceptive primary sensory afferents of the dorsal root ganglion (DRG) and cranial nerves (8–10).

In both humans and rodents, the DST gene is characterized as having tissue-specific promoters and an abundance of exons that are alternatively spliced yielding differentially expressed transcripts (11). Within the nervous system, three prominently large (>600 kDa) neuronal dystonin isoforms exist (schematically represented in Fig. 1A), dystonin-a1, -a2, and -a3, each of which have distinct cellular localizations and are capable of interacting with microtubules (MTs) and actin filaments (12,13). Each isoform is endowed with a functional N-terminal actin binding domain, a plakin domain, a spectrin repeat-containing rod domain in the middle of the molecule and a MTBD at the C-terminus (14). Isoform uniqueness is achieved through alternative splicing of the first 5′ exons. Dystonin-a1 encodes a short N-terminal domain that includes an actin-binding domain, while dystonin-a2 possesses a transmembrane domain capable of interacting with various organelle membranes, and dystonin-a3 harbors a myristoylation domain, aiding in anchoring to the plasma membrane (12,13).

While the dystonin-a isoforms mediating disease in HSAN-VI remain enigmatic, loss-of-function of dystonin-a1 and -a2, but not dystonin-a3, in the murine nervous system is causal in the dtNle734Tg84 disorder (15,16). As such, numerous studies and speculations have been involved in parsing apart the biological functions of individual dystonin-a isoforms. Initial inspection of dystonin-a transcripts found dystonin-a2 to be the more unique isoform and least likely to be compensated for by any related protein (e.g. microtubule actin crosslinking factor and plectin) (16). Moreover, dystonin-a2 expression predominated in sensory ganglia—the most severely affected tissue of dt mice—and poorly expressed in non-neuronal tissues (17). These initial descriptions of the dystonin-a2 variant suggested that it might be the principle mediator of dt pathogenesis (16,18). Support for this notion was later corroborated through isoform-specific loss-of-function analyses in cell culture model systems. Indeed, depletion of dystonin-a2 perturbs membrane organization [endoplasmic reticulum (ER) and Golgi apparatus] and transport flux through the secretory pathway (19,20).

In a separate study exploring the individual roles of dystonin-a1 and -a2 isoforms in mediating dt sensory neuron degeneration, dystonin-a2 was determined to be the principle initiator of neuronal degeneration (20). Silencing dystonin-a2 in neurons in culture elicited distinct neurodegenerative pathologies, including Ca2+ dyshomeostasis, unfolded protein response induction, caspase activation and apoptosis. In addition to these aberrations, ER structural integrity was also compromised, presumably through uncoupling of cytoskeletal filaments, dystonin-a2 and ER membranes. Taken together, these studies suggest that dystonin-a2 is the chief contributor in the demise of dt sensory neurons. Nevertheless, dystonin-a1 is also involved in fundamental neuronal processes, including anterograde and retrograde trafficking (21,22). As disturbance of these processes are well-known to underlie many neurological diseases (23), including HSANs (1), dystonin-a1 likely also remains an important contributor in dt pathogenesis.

To determine the dystonin-a isoform mediating dt pathogenesis, and to gain insight into viable pathogenetic mechanisms underlying HSAN-VI, we generated and characterized transgenic mice expressing dystonin-a2 under the nervous system-specific prion protein promoter (PrP-dystonin-a2). These transgenic mice were subsequently crossed onto the dtNle734Tg84 background, producing homozygous (PrP-dystonin-a2/PrP-dystonin-a2; dtNle734Tg84) transgenic mice. We find restoring dystonin-a2 expression within the nervous system greatly diminishes the severity of the dt disorder—due, in part, to prolonged survival of specific sensory neuron subtypes—and significantly extends life span. Despite these improvements, complete rescue was not observed likely because of inadequate expression of the transgene.

RESULTS

Generation and characterization of the PrP-dystonin-a2/PrP-dystonin-a2 transgenic mouse model

Transgenic mice harboring full-length dystonin-a2 cDNA were generated and used in rescue experiments using dtNle734Tg84 mice. Full-length dystonin-a2 cDNA was placed under the control of a strong neuronal promoter, the 3.5 kb mouse PrP promoter (Fig. 1B). In addition, a myc/his epitope tag was included in frame at the 3′ end to facilitate detection of the transgene product. The mouse PrP promoter was previously demonstrated to yield high expression in neuronal tissues of various transgenes (24). Although the PrP promoter can exhibit low expression in other cell types (e.g. astrocytes and skeletal muscle), we are mainly concerned with expression within neuronal tissues.

To assess whether the PrP-dystonin-a2 transgene is functional and the myc-tag can be detected via immunochemical labeling, immortalized F11 sensory neurons were transfected with the construct. The transgene product displays a characteristic perinuclear/cytoplasmic staining pattern aligning with cytoskeletal filaments, particularly MTs (Fig. 1C). This pattern is very similar to what we had previously shown (13,18).

Purified
PrP-dystonin-a2 cDNA transgene fragment was microinjected into the pronuclei of one-cell mouse embryos and transgenic founder lines were established thereafter. To identify mice harboring the transgene, a genotyping assay was developed. Amplification of endogenous DNA from non-transgenic mice produces a 318 bp DNA fragment from within the Dst gene, while amplification of the transgene DNA produces a 220 bp DNA fragment (Fig. 1D and E). From five transgenic founder mice generated, two lines (founder lines F542 and F559) were bred to establish independent strains. These transgenic lines appeared normal and were fertile.

**PrP-dystonin-a2 mice express the transgene in a neuronal specific manner**

Using primers specific to the myc-his tag, transgene expression was assessed in multiple neuronal tissues including DRG, spinal cord and brain for both PrP-dystonin-a2 transgenic lines (Fig. 2A). At P7 stage, all neuronal tissues from heterozygous mice exhibited PrP-dystonin-a2 transgene expression, while no expression was evident in tibialis anterior muscle (Fig. 2B and C). PrP-dystonin-a2 transgene expression was also assessed in P7 heterozygous tissues by immunohistochemistry. Using an
anti-myc monoclonal antibody to assess protein expression (this tag is placed in frame with the dystonin-a2 isoform), a positive signal was detected in the cerebral cortex and cerebellum of both transgenic lines; however, transgene expression in DRG tissue sections was faint and difficult to discern in mice from line 542 (data not shown). For this reason, transgenic line 559 was used and bred to homozygosity to increase relative transgene expression levels. Encouragingly, brain (P10) and DRG (P10 and P58) tissue sections of F559 homozygous (PrP-dystonin-a2/PrP-dystonin-a2) mice display the transgene product (Fig. 3A). Indeed, robust PrP-dystonin-a2 transgene expression was seen throughout the sensory neurons of the DRG. In comparison, as expected, there was no signal in age-matched brain and DRG tissues taken from wild-type mice (Fig. 3A).

To further assess the pattern of transgene product expression, we have examined both DRG sections and primary cultures of DRG neurons by confocal microscopy. The transgene product is largely localized to the perinuclear region of the cell bodies of sensory neurons in DRG sections (Fig. 4A and B). As well, we observe a similar pattern in the cell bodies of DRG neurons in primary culture (Fig. 4C). Again, this is a very similar pattern to what we had previously shown (13,18).

The DRG contains distinct cell populations including proprioceptive large and medium-sized sensory afferents (muscle sensory) and small-sized (skin and visceral sensory) sensory neurons, each of which has specific physiological properties. Large- and medium-sized sensory neurons innervate muscle tissues and transmit proprioceptive and tactile information, whereas small-sized sensory neurons innervate and relay information from the skin and viscera. Dystonin-a is expressed throughout the DRG and not restricted to single subgroup (17). To ensure PrP-dystonin-a2 transgene was expressed in all sensory neuron subtypes, sensory neurons were cultured from P5 PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice for 5 days in vitro and immunolabeled with anti-c-myc. Different sized sensory neurons (small 100–400 μm, medium 400–700 μm and large 700–1200 μm) were categorized as previously described (10), using the Axiovision 4.6 software (Carl Zeiss) circumference-measuring tool. Anti-c-myc staining was replete throughout the PrP-dystonin-a2/PrP-dystonin-a2 sensory neuron subtypes, whereas no anti-myc labeling was observed in dtTg4/Tg4 sensory neurons (Fig. 3B). To address how the levels of expression of dystonin-a2 from the transgene compared with endogenous dystonin-a2, we...
extracted RNA from the DRGs of P15 wild-type, $dt^{Tg4/Tg4}$ and $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice. We have performed real-time quantitative reverse transcription-polymerase chain reaction (RT–qPCR) and our analysis shows that dystonin-a2 transcripts are not detectable in $dt^{Tg4/Tg4}$ samples compared with the wild-type. Further, the level of dystonin-a2 transcripts in the $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ sample, although higher than the mutant alone, does not reach the same level as in the wild-type (Fig. 5).

These initial observations suggested that $PrP-dystonin-a2/PrP-dystonin-a2$ transgenic mice express the dystonin-a2 transgene throughout the nervous system, most importantly in all sensory neuron subtypes. $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice are viable, fertile and phenotypically similar to wild-type mice.

Postnatal phenotypic characteristics of $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice

To characterize whether the PrP-dystonin-a2 transgene confers protection in $dt^{Tg4/Tg4}$ sensory neurons and ameliorates the $dt$ phenotype, $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice were crossed onto the $dt^{Tg4/Tg4}$ background. $dt^{Tg4/Tg4}$ mice heterozygous for the PrP-dystonin-a2 transgene mice died at approximately the same age as $dt^{Tg4/Tg4}$ mice (P21) and displayed poor locomotor coordination, unsteady gait and postural instability (data not shown). In comparison, homozygous $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice exhibited significant improvements in all these properties, as discussed below. This suggests that differences in dystonin-a2 protein levels in heterozygous versus homozygous transgenic mice likely influence the extent of rescue in $dt$ mice.

The life span of $dt^{Tg4/Tg4}$ mice is $\approx 2–3$ weeks (25). Here, we find the life span of $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice was significantly increased (mean age 55 days) compared with $dt^{Tg4/Tg4}$ mice (mean age 22 days, $P < 0.0001$) (Fig. 6A). Indeed, 60% of $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice assessed lived past P50, while 20% survived past 123 days ($n = 10$). Despite this increase in survival, $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ life span was compromised compared with control $PrP-dystonin-a2/PrP-dystonin-a2$ mice. In addition, $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice surviving past 120 days manifested additional phenotypes related to dysautonomia. Two mice (P123 and P126) showed blepharoptosis (drooping of the upper eyelid) and conjunctivitis (an early symptom of dry eye). While these dysautonomic features were not observable among the younger $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ mice, these features are occasionally seen in P20 $dt^{Tg4/Tg4}$ mice. This suggests that it takes a significantly longer period of time

Figure 3. Immunohistochemical staining demonstrating robust PrP-dystonin-a2 transgene expression in neuronal tissues. (A) Representative tissue sections from brain (P10) and DRGs (P10 and P58) of homozygous PrP-dystonin-a2/PrP-dystonin-a2 (PrP/PrP) transgenic mice. Immunohistochemical staining with anti-c-myc antibody produced a perinuclear/cytoplasmic staining pattern in P10 cortical brain tissue, while a strong perinuclear staining pattern was observed in P10 and P58 DRGs. As expected, DRGs from wild-type (WT) non-transgenic mice stained with anti-c-myc revealed no specific staining (scale bars = 20 μm). (B) Analysis of transgene expression in cultured DRG neurons. Again, as expected, there was no anti-c-myc staining in P10 $dt^{Tg4/Tg4}$ sensory neurons (left panel), whereas staining was present in large-, medium- and small-sized sensory neurons of P10 PrP-dystonin-a2 mice (right panel). Scale bar = 50 μm. Sections or cells were counterstained with DAPI to label the nuclei.
for PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice to manifest dysautonomic features compared with dtTg4/Tg4 mice. From birth to P10, dtTg4/Tg4 mice are indistinguishable in size and phenotype from their wild-type littermates. At approximately 2 weeks of age, however, arrest in weight-gain and ambulating abnormalities is evident. We therefore assessed whether PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice show improved growth and ambulation. A progressive gain in weight in PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice was observed between postnatal days P16 and P22, albeit this progressive weight gain was not equivalent to PrP-dystonin-a2/PrP-dystonin-a2 control mice (Fig. 6B). In comparison with dtTg4/Tg4 mice, PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice showed a normal growth rate between P16 and P22, reminiscent of PrP-dystonin-a2/PrP-dystonin-a2 control mice (Fig. 6C). As dtTg4/Tg4 mice succumb to death at ~P20, analyses between PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 and dtTg4/Tg4 mice could not be performed past this time point.

To assess whether the transgenic rescue mice have improved coordination, the mice were subjected to the pen test—a test used to measure grip strength and coordination—and the duration of time the animal was able to stay on the pen was recorded. PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice had only minor improvements in this test at early time points compared with dtTg4/Tg4 mice and were unable to stay on the pen following the P24 time point (Fig. 6D and E). PrP-dystonin-a2/PrP-dystonin-a2 mice, on the other hand, performed normally. Despite poor performance on the pen test, PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice showed a delay in onset of hind limb clasping, a hallmark phenotypic feature of dtTg4/Tg4 mice (Fig. 6F). Indeed, dtTg4/Tg4 mice typically present this pathological reflex at P15, or earlier, whereas PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice only begin to show this phenotype at P21. This pathological reflex was non-existent in PrP-dystonin-a2/PrP-dystonin-a2 mice. Gait was assessed by using an in-house gait box as previously described (26). While P20 dtTg4/Tg4 mice exhibited an aberrant gait, the stride of P20 PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice was indistinguishable from that of P20 PrP-dystonin-a2/PrP-dystonin-a2 control mice (Fig. 6G). Taken together, restoring dystonin-a2 expression on the dtTg4/Tg4 background does improve features of the dt phenotype, albeit attenuation of the phenotype is transient.

### The PrP-dystonin-a2 transgene delays sensory neuron degeneration in dt mice and improves neuromuscular junction maturation

The degeneration of dt sensory neurons is the primary contributor to the overt ataxia and dystonia observed in dt mice (17,27). We previously found the demise of dtTg4/Tg4 sensory neurons commences at P15, concomitant with the onset of dt pathogenesis and the dt phenotype (20). The PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mouse does not manifest impaired locomotion or dystonia at P15, suggesting delayed sensory neuron degeneration. To address this notion, quantitative TUNEL labeling on lumbar DRG tissue sections was performed.

As expected, P15 PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 DRG tissue sections displayed a dearth of TUNEL-labeled neurons, while P15 dtTg4/Tg4 DRGs tissue sections exhibited a significant increase in TUNEL activity (Fig. 7B, C; F; P < 0.001). In comparison with dtTg4/Tg4 DRGs, P15 PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 DRGs showed a significant reduction in the number of TUNEL-positive sensory neurons (Fig. 7D and F; P < 0.001), suggesting that the PrP-dystonin-a2 transgene confers protection and delays the onset of neurodegeneration. As PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice eventually present ataxia, sensory neuron viability was assessed at a later time point. At P21, a significant increase in TUNEL-positive sensory neurons (P < 0.05) was observed compared with the P15 time point (compare Fig. 7D with 7E). Despite this increase, the percentage of dying cells was significantly less than that observed in P15 dtTg4/Tg4 sensory neurons (Fig. 7F; P < 0.001). These observations indicate that the aforementioned phenotypic improvements in PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice are due, in part, to increased viability in dtTg4/Tg4 sensory neurons. Furthermore, while the PrP-dystonin-a2 transgene confers protection in PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 sensory neurons, they still undergo degeneration, albeit at a slower rate.

To assess the impact of the transgene on the pathology in the axons, we have performed histopathology on dorsal root sections from P15 wild-type, dtTg4/Tg4 and PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice. Toluidine blue staining on thin sections was used to assess axon caliber. We have measured the axon caliber in at least 500 individual myelinated axons from cross-sections (n = 3 for each genotype). Note that there was no statistically significant difference in the axonal caliber between wild-type and dt. As well, there was no change in axon caliber after transgenic rescue of dt. Thus, this aspect of the morphology of axons is not affected in the dt mouse. As well, we have...
assessed the number of axonal swellings per dorsal root section (n = 3 for each genotype). Our quantification revealed that there were no axonal swellings observed in wild-type sections, 2 ± 1 axonal swellings in dt sections and 1.66 ± 1.53 axonal swellings in the rescued mice. Thus, this analysis revealed that axonal swellings were still present in the dorsal roots of rescued mice (Fig. 8). Interestingly, however, we did observe a relative increase in the density of axons in these mice, suggesting that axonal loss has been attenuated. Similarly, we have performed histopathology on muscles from P15 mice to assess neuromuscular junction (NMJ) and endplate maturity. In general, there was an increase in the number of immature endplates and NMJs in muscles from dtTg4/Tg4 mice compared with wild-type (Fig. 9, top and middle rows). By comparison, there was an improvement, albeit modest, in these defects in the PrP-dystonin-a2/dtTg4/Tg4 mice compared with wild-type sections, 2 ± 1 axonal swellings in dt sections and 1.66 ± 1.53 axonal swellings in the rescued mice (Fig. 9, bottom rows). In particular, the incidence of neurofilament accumulation at the presynaptic site in PrP-dystonin-a2/dtTg4/Tg4 cultures was reduced compared with dtTg4/Tg4 mice.

Finally, we have performed an analysis of muscle spindle morphology in 30 cross-sections of tibialis anterior muscles (n = 3 for each genotype) (Fig. 10). This analysis revealed that there was a significant degeneration of muscle spindles, regardless of their size, in dtTg4/Tg4 samples (Fig. 10D). Interestingly, this degeneration was substantially rescued in the PrP-dystonin-a2/dtTg4/Tg4 mice.

Dystonin-a2 protects proprioceptive sensory afferents and improves MT integrity

Various HSAN disorders are characterized by the demise of a specific sensory neuron subtype (1,28). Interestingly, while dystonin-a is expressed throughout different sensory neuron populations, large and medium proprioceptive sensory neurons are most affected by the dt disorder (8,10). Consequently, movement is severely impaired in dt mice. To address which population of sensory afferents the PrP-dystonin-a2 transgene protects, sensory neurons from PrP-dystonin-a2/dtTg4/Tg4 and dtTg4/Tg4 mice were co-labeled with β-III tubulin (a neuronal marker) and caspase-3 (an apoptosis marker) (n = 3) (Fig. 11A–C). Furthermore, to differentiate between different-sized sensory neurons, soma circumference was measured and grouped by size as discussed above. Primary sensory neurons derived from P5 mice and cultured for 5 days in vitro (5 DIV) revealed a dearth of caspase-3 labeling for each genetic background (data not shown). As such, sensory neuron cultures were challenged with starvation media (serum-, glucose- and amino acid-free media) for 24 h following 5 DIV and cell viability was assessed thereafter. For cells to maintain viability following starvation, they activate cell-survival mechanisms (e.g. autophagy and robust intracellular transport).

In initial analysis, there was no difference in the average number of small and medium sensory neurons between PrP-dystonin-a2/dtTg4/Tg4 and PrP-dystonin-a2/dtTg4/Tg4 cultures following 24 h serum-free treatment (Fig. 11D). However, a significant increase in caspase-3 labeling was noted in cultures following 24 h serum-free treatment (Fig. 11D). Furthermore, no significant difference in caspase-3 labeling was observed in small and medium sensory neurons between PrP-dystonin-a2/dtTg4/Tg4 and PrP-dystonin-a2/dtTg4/Tg4 cultures (Fig. 10E). These observations suggest expression of the PrP-dystonin-a2 transgene on the background imparts protection in small and medium sensory neurons.

Unlike small and medium sensory neurons, the average number of large sensory neurons following serum-free treatment was significantly different between genotypes. On average, there were 14 large sensory neurons per PrP-dystonin-a2/dtTg4/Tg4 culture and significantly fewer in PrP-dystonin-a2/dtTg4/Tg4 cultures (five large sensory neurons per culture, P < 0.05) and in the PrP-dystonin-a2/dtTg4/Tg4 culture (one large sensory neuron per culture, P < 0.01) (Fig. 11D). Coupled with this, 15% of large PrP-dystonin-a2/dtTg4/Tg4 sensory neurons were capase-3 positive, while ~52 and 100% of large PrP-dystonin-a2/dtTg4/Tg4 sensory neurons were capase-3 positive, respectively (Fig. 11D). These data indicate that although the PrP-dystonin-a2 transgene imparts slight protection to large sensory neurons, they remain vulnerable to dt pathogenesis.

In both HSAN-VI patients and dt mice, disorganized MT networks are thought to be a critical driver of disease pathogenesis (4,29,30). As such, we addressed whether the dystonin-a2...
transgene improves MT network integrity. $dt^{Tg4/Tg4}$ cultures exhibited notable accumulations of β-III tubulin throughout axons (arrowheads, Fig. 11C), whereas accumulations of β-III tubulin were absent from the axons of $PrP-dystonin-a2/PrP-dystonin-a2$ and $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ sensory neurons (Fig. 11A and B). Similar accumulations seen herein have been observed in $dt$ axons, and likely influence the bi-directional transport impairment observed in $dt$ sensory neurons (31). We therefore conclude that the increased viability among small- and medium-sized $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ sensory afferents is mediated, in part, by improved MT organization.

Figure 6. Postnatal characteristics of $PrP-dystonin-a2/PrP-dystonin-a2;dt^{Tg4/Tg4}$ transgenic rescue mice. The different groups analyzed were: control ($PrP/PrP, n = 10$), mutant ($dt^{Tg4/Tg4}, n = 7$) and transgenic rescue ($PrP/PrP;dt^{Tg4/Tg4}, n = 10$). (A) Kaplan–Meier survival curve analysis indicates a significant increase in lifespan of $PrP/PrP;dt^{Tg4/Tg4}$ mice (median lifespan, 55 days) when compared with $dt^{Tg4/Tg4}$ mice (median lifespan, 21 days) (**** $P < 0.0001$). A significant decrease in lifespan was also observed between $PrP/PrP;dt^{Tg4/Tg4}$ mice and $PrP/PrP$ mice (**** $P < 0.0001$), indicating that although there was some rescue, it was not complete. (B) Postnatal growth curve analysis suggests that $PrP/PrP;dt^{Tg4/Tg4}$ mice have a progressive gain in weight between postnatal days P16 and P52, and plateau thereafter. $PrP/PrP;dt^{Tg4/Tg4}$ mice do not show comparable increases in weight to that of control $PrP/PrP$ mice between P30 and P123 (**** $P < 0.0001$). This increase in weight at early postnatal days is comparable to $PrP/PrP;dt^{Tg4/Tg4}$ mice as these mice die at ~ P20. Two-way ANOVA, Bonferroni posttest, data are represented as mean ± SEM. (C) Performance analysis of $PrP/PrP$ control, $dt^{Tg4/Tg4}$ and $PrP/PrP;dt^{Tg4/Tg4}$ mice in the pen test at various post-natal days between P23 and P123. $PrP/PrP;dt^{Tg4/Tg4}$ mice show improvements in weight and grip at early postnatal days (P18 and P20) compared with $PrP/PrP$ control mice. Further comparative weight analysis was not feasible for $dt^{Tg4/Tg4}$ mice as these mice die at ~ P20. Two-way ANOVA, Bonferroni posttest, data are represented as mean ± SEM. (D) Representative images of hind limb clasping during tail suspension, a hallmark $dt$ phenotype. P15 $dt^{Tg4/Tg4}$ mice consistently displayed limb clasping (arrow), while P15 $PrP/PrP;dt^{Tg4/Tg4}$ mice exhibited splayed hind limbs, reminiscent of wild-type or $PrP/PrP$ control mice. Note that clasping was detectable in P21 $PrP/PrP;dt^{Tg4/Tg4}$ mice. (E) Representative examples of $PrP/PrP$ (P20); $dt^{Tg4/Tg4}$ (P20) and $PrP/PrP;dt^{Tg4/Tg4}$ (P20) mouse paw prints used for gait analysis (red = front paws, red arrow; blue = back paws, blue arrows). Note the similar gait between P20 $PrP/PrP$ and P20 $PrP/PrP;dt^{Tg4/Tg4}$ mice and the abnormal gait exhibited by P20 $dt^{Tg4/Tg4}$ mice.
Dystonin-a2 transgene preserves organelle integrity

The cytoskeleton and cytoskeletal stabilizing proteins are instrumental in organelle organization, movement and function (32–35). We previously demonstrated that dystonin-a2 loss of function leads to the structural disorganization of the ER and Golgi membranes through uncoupling of the cytoskeletal network (19,20,36). To address whether the PrP-dystonin-a2 transgene protects organelle integrity in PrP-dystonin-a2/PrP-dystonin-a2;dt<sup>Tg4/Tg4</sup> mice, we sought to evaluate the ultrastructure of control, dt<sup>Tg4/Tg4</sup> and PrP-dystonin-a2/PrP-dystonin-a2;dt<sup>Tg4/Tg4</sup> P5 sensory neurons (Fig. 12A–E). Representative EM micrographs of P5 WT sensory neurons displayed organized rough ER sheets (A) and Golgi stacks (A′) compared with the dilated rough ER sheets (B) and Golgi membranes (B′) observed in P5 dt<sup>Tg4/Tg4</sup> sensory neurons. Similar to control sensory neurons, we found P5 PrP-dystonin-a2/PrP-dystonin-a2;dt<sup>Tg4/Tg4</sup> sensory neurons were devoid of dilated ER (C) and Golgi (C′) membranes. Quantification of ER and Golgi membrane diameter indicated P5 control and PrP-dystonin-a2/PrP-dystonin-a2;dt<sup>Tg4/Tg4</sup> were comparable in size, and were significantly smaller in size compared with ER and Golgi membranes of dt<sup>Tg4/Tg4</sup> sensory neurons (Fig. 12D and E). These findings corroborate previous inferences that dystonin-a2 is critical in maintaining organelle structure. Moreover, as the PrP-dystonin-a2 transgene attenuates cellular demise, we conclude that stabilization of vital organelles is an important contributor to this cellular rescue.

DISCUSSION

Deleterious homozygous mutations in DST were recently reported to cause a dystonin-related neuropathy termed HSAN-VI. All three dystonin isoforms are impacted in HSAN-VI. The human and mouse DST gene are highly similar (16) and DST mutations in both humans and mice produce similar phenotypic features.

To gain a better understanding of the dystonin-a isoforms underlying both HSAN-VI and dt pathogenesis, we addressed whether dystonin-a2 expression in dt<sup>Tg4/Tg4</sup> mice is able to partially or completely rescue the dt phenotype and the underlying pathophysiology. We successfully generated transgenic mice expressing the PrP-dystonin-a2 transgene on the dt<sup>Tg4/Tg4</sup> background. Transgene expression was evident throughout neural tissues and was, most importantly, expressed throughout all sensory neuron subtypes. Of note, the transgene product was largely localized to the perinuclear region of the cell bodies of DRG sensory neurons. Increasing dystonin-a2 expression in neural tissues of dt<sup>Tg4/Tg4</sup> mice decreased disease severity and increased life span. We find these aforementioned improvements are due in part to improved MT and organelle integrity within proprioceptive sensory neurons, and as such, delay apoptosis of sensory neurons. Collectively, this study proposes dystonin-a2 loss of function is an important contributor to dt pathogenesis. Despite these improvements, complete rescue was not observed likely because of inadequate expression of the transgene.

Dystonin-a2 decreases disease severity and increases life span

By reintroducing dystonin-a2 expression within the nervous system of dt<sup>Tg4/Tg4</sup> mice, we extended life span to a mean age of 55 days coupled with a progressive gain in weight between P16 and P52 (Fig. 6). Why some rescue mice show prolonged survival compared with other rescue mice (e.g. 123 versus 46 days) is unclear. We suspect this variability in survival is due in part to disease modifiers and/or intrinsic genetic background differences. The PrP-dystonin-a2/PrP-dystonin-a2;dt<sup>Tg4/Tg4</sup> mice also showed a delay in the onset of hind limb clumping and exhibited a normal gait at P20 reminiscent of PrP-dystonin-a2/PrP-dystonin-a2 control mice. As hind limb clumping and aberrant gait in dt mice are provoked through the degeneration of proprioceptive sensory afferents, improvement
in these areas suggests this subgroup of sensory neurons are transiently protected by dystonin-a2 transgenic expression. Qualitatively, PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice also display a milder form of dt phenotype (less limb incoordination, fewer dystonic postures and little to no writhing).

Dystonin-a2 is protective in subpopulations of sensory neurons

Although dystonin-a expression is widely distributed throughout DRG sensory afferents, the postnatal consequence of dystonin-a absence varies among sensory neuron subpopulations (8–10). The dt mutation provokes a very selective degeneration, mainly affecting large- and medium-sized sensory neurons which encompass group Ia and Ib (large), and group II (medium-sized) sensory afferents (8). These proprioceptive sensory afferents make up 20% of DRG neurons and innervate specialized sense organs within the musculature, including muscle spindles and Golgi tendon organs (37). These sensory organs are critical for posture and stability and goal-directed movements. Multiple studies found that the degeneration of muscle spindles is concomitant with phenotypic onset and that aberrations in spindle function are most likely the cause of both the ataxic and dystonic movements seen in dt mice (6,8,27). Delineating which dystonin-a isoform(s) is/are responsible for the demise of these populations is paramount, as it will not only narrow down the principle dystonin-a isoform, but also propose potential pathological mechanisms underlying the dt disorder and similar human diseases, like HSAN-VI.

Here, we show that dystonin-a2 is necessary in the maintenance of postnatal sensory neurons as its expression significantly delays the progressive degeneration seen in dtTg4/Tg4 mice (Fig. 7). Moreover, cultured primary sensory neurons from PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice revealed that the dystonin-a2 transgene confers protection among medium-sized sensory neurons (group II sensory afferents), but not large sensory neurons when challenged with serum-free media (Fig. 11). Why large sensory neurons are so vulnerable in the dt disorder is unclear. Previous studies have found large sensory neurons are more vulnerable to diseased conditions than small-sized neurons (38). Moreover, large- and medium-sized sensory neurons possess high-energy demands, and require significant trafficking capacity, and hence may be particularly vulnerable to vesicular and axonal transport defects. As transgene expression was replete throughout all sensory neuron subtypes (Fig. 3), we conclude that dystonin-a2 expression is critical for the survival of medium-sized sensory neurons. Although no rescue was seen in large sensory afferents, this does not imply dystonin-a2 expression is dispensable in this population of sensory neurons. It is likely that the level of transgene expression was not high enough for the survival of large sensory afferents. PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice show partial, but not complete, improvements in both the pen test and gait analyses. This would imply that specific sensory nerves—likely large sensory afferents—are already dying or are not functioning optimally. Furthermore, the milder dt phenotype indicates that the progressive nature of dtTg4/Tg4 pathogenesis within proprioceptive sensory afferents is delayed.

There are several possibilities as to why PrP-dystonin-a2 transiently rescues medium-sized sensory afferents. The most likely reason is that expression of the dystonin-a2 transgene is not
equivalent to that of endogenous dystonin-a2. However, expression of dystonin-a1, or a yet-to-be-determined dystonin-a isoform, may also be needed for a complete rescue of medium-sized sensory afferents.

To address how dystonin-a2 was mediating a partial rescue, we investigated the status of MT networks within our primary sensory neuron culture system. Dystonin-a has long been known to be involved in the maintenance of the neuronal cytoskeleton, particularly MTs (29,30). More recently, through interactions with the microtubule-associated protein-1B, dystonin-a2 was found to be necessary in maintaining the acetylation status of MTs, and as such, preserved optimal protein trafficking (19). Here, we find that the dystonin-a2 transgene is capable of maintaining the integrity of MT networks within \( dTg4/Tg4 \) sensory axons. Indeed, we did not observe axonal accumulations of βIII-tubulin as seen in \( dTg4/Tg4 \) sensory axons (Fig. 11C). In light of the aforementioned studies, improved MT stability is a conceivable explanation as to the delayed degeneration observed herein.

Coupled with improvements in MT stability was the ultrastructural preservation of ER and Golgi membranes in \( PrP-dystonin-a2/PrP-dystonin-a2;dTg4/Tg4 \) sensory neurons (Fig. 12A–E). As organelle function is contingent upon structure, we conclude that preserved organelle structure enables organelles to fulfill their function and hence extend cellular viability. Previous studies demonstrated that dystonin-a2 mediates Golgi organization through the maintenance of MT acetylation (19). It is therefore likely that restoration of dystonin-a2 expression stabilizes MTs, which in turn contributes to the maintenance of Golgi membranes. Whether ER membranes are also organized via acetylated MT networks remains to be determined.

Taken altogether, both dystonin-a1 and dystonin-a2 are involved in fundamental biological functions within neurons, including vesicular trafficking and maintenance of MTs and organelle membranes. As similar biological processes are perturbed in both inherited and acquired peripheral neuropathies, our results should be of clinical interest. Moreover, results herein will provide needed biological insights into the newly identified dystonin-related HSAN-VI.

MATERIALS AND METHODS

Reagents

All chemicals were purchased through Sigma-Aldrich (St Louis, MO, USA) and all cell culture reagents were obtained from Invitrogen (Burlington, ON, Canada) except where indicated.
Ethics statement

All experimental protocols on mice were approved by the Animal Care Committee of the University of Ottawa. Care and use of experimental mice followed the guidelines of the Canadian Council on Animal Care.

Animals and cell culture

The \(dt^{Tg4/Tg4}\), PrP-dystonin-a2/PrP-dystonin-a2;\(dt^{Tg4/Tg4}\) and PrP-dystonin-a2/PrP-dystonin-a2 transgenic mice all shared the same mixed genetic background of CD1/C57BL6 and were used at pre-phenotype (P7) and phenotype stages (P15 and P58). The generation of the \(dt^{Tg4/Tg4}\) line and characterization of the mutation have been described previously (15,25,39). The onset of phenotype was generally assessed by the appearance of clasping of hind limbs after picking the mice up by their tails. \(dt^{Tg4/Tg4}\) mice were genotyped by PCR amplification of genomic tail DNA.

The dystonin-a2 cDNA was previously developed in the laboratory. In brief, dystonin-a2 cDNA was amplified from mouse brain RNA and cloned into the pEF1-myc/his vector.
(Invitrogen) encoding a C-terminal myc/his tag (13,18). The dystonin-a2-myc/his cDNA was thereafter cloned into the 3.5 kb PrP promoter and the expression vector pEF1myc/hisB (Invitrogen). The resulting transgene construct was microinjected into one-cell mouse embryos. Tail biopsies were obtained from potential founder mice, DNA was extracted and transgenic mice were identified by PCR amplification using sense oligo 5′ TTG TGC ACC AAT GCC TTC GC 3′ and antisense oligo 5′ GCC GGA CCT GAT AGA CAT GA 3′. These primers amplify a 318 bp fragment on the wild-type Dst gene and a 220 bp fragment from the PrP-dystonin-a2 transgene. Positive founder mice were bred with wild-type mice to establish two independent transgenic lines (founder lines 542 and 559). Heterozygous PrP-dystonin-a2/+ and homozygous PrP-dystonin-a2/PrP-dystonin-a2 mice were subsequently crossed to heterozygous dtTg4/+ mice, and the dtTg4 allele was thereafter bred to

Figure 11. PrP-dystonin-a2 transgene imparts neuro-protection in small and medium caliber sensory neurons but not in large caliber sensory neurons. (A–C) Primary DRG sensory neuron cultures were established from P5 mice of various genetic backgrounds [PrP/PrP (A), PrP/PrP, dtTg4/Tg4 (B), dtTg4/Tg4 (C)], and cultured for 5 days in vitro. Cells were challenged with serum-free media for 24 h and antigenic labeling of β-III tubulin (neuronal marker) and the apoptotic marker caspase-3 was conducted thereafter. Note the accumulation of β-III tubulin in axons of dtTg4/Tg4 sensory neurons (arrowheads in C). (D) The average number of small (soma area, 100–400 μm²) and medium caliber sensory neurons (400–700 μm²) was not significantly different between genotypes. (E) A significant increase in caspase-3 staining was observed in small- and medium-sized dtTg4/Tg4 sensory neurons compared with PrP/PrP (** P < 0.01) and PrP/PrP, dtTg4/Tg4 (** P < 0.01), indicating the transgene confers protection in these cell types. (F) The average number of large caliber sensory neurons (700–1300 μm²) is significantly different between genotypes PrP/PrP and PrP/PrP, dtTg4/Tg4 (P < 0.05); PrP/PrP and PrP/PrP, dtTg4/Tg4 (P < 0.01). However, there was no difference between PrP/PrP, dtTg4/Tg4 and dtTg4/Tg4. (G) A significant increase in caspase-3 staining was observed in dtTg4/Tg4 large caliber sensory neurons compared with PrP/PrP large caliber sensory neurons (* P < 0.05). No significant difference in caspase-3 staining was observed between PrP/PrP and PrP/PrP, dtTg4/Tg4 large caliber sensory neurons. Statistics: ANOVA, post hoc Tukey, n = 3/genotype. Scale bar = 20 μm.
homozygosity producing both PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice and PrP-dystonin-a2/PrP-dystonin-a2;dtTg4/Tg4 mice. The genotype of offspring mice was confirmed by PCR.

F11 cells (a fusion of embryonic rat DRG cells with a mouse neuroblastoma cell line, kindly supplied by Dr Paul Albert, University of Ottawa) were maintained in DMEM + 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/antimycotic. Cells were passaged at \( \approx 70\% \) confluency in 10 cm plastic Petri dishes and plated onto glass coverslips for use in immunofluorescence assays. Cell transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s directions.

**Gait measurements**

Gait assessment was performed using an in-house gait box as previously described (26). Both front and back paws were marked with red and blue ink, respectively. Mice were situated at the larger end of the gait apparatus with a light shone through to encourage the mice to walk toward the opposite
end, leaving their imprints on a piece of white paper. Imprints were thereafter inspected and compared among different genotypes.

**Pen test**

Balance and strength were assessed using the pen test as described (40). Mice were placed on a suspended pen at different time points. The latency to fall from the pen was measured with an apparatus with a plateau of 30 s. At each time point, individual mice were placed three consecutive times.

**RNA isolation and reverse transcription-polymerase chain reaction analysis**

All RNA used for experiments was extracted using the RNeasy® Mini Kit (Qiagen), as per the manufacturer’s protocol. For RT–qPCR analysis, RNA was isolated from transgenic P7, P15 and P21 tissues from the two PrP-dystonin-a2 founder lines (F542 and F559). To synthesize cDNA, equal amounts of RNA from brain, spinal cord, muscle and DRGs were reverse-transcribed in a standard reaction with MuLV reverse transcriptase (Invitrogen). PCR amplification using the sense oligo 5′ AAC AAA AAC TCA TCT CAG AAG AG 3′, and the antisense oligo 5′ ATG GTG ATG ATG ATG AG C 3′ (specific to the Myc/His cDNA), yielded a 62 bp fragment. Primers were chosen to flank the intronic region of the construct to selectively amplify the RNA transcript and prevent amplifying any contaminating genomic DNA. The reaction began with a 3 min incubation time at 94 °C followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 68 °C, with a final extension time of 10 min at 68 °C. Amplification of actin cDNA served as a control. The PCR products were electrophoresed on a 5% agarose gel containing ethidium bromide, and amplified fragments were visualized under UV transillumination. cDNAs encoding actin and TUNEL labeling

Ten micrometer lumbar DRG tissue sections were dissected, washed in phosphate buffered saline (PBS) and permeabilized in ice cold 0.1% sodium citrate/0.1% Triton X-100 for 5 min followed by 2 min in 2:1 ethanol/acetic acid on ice. Samples were rinsed for 2 min in PBS and incubated for 1 h at 37 °C with FITC-labeled dUTP in terminal deoxynucleotidyl transferase (TdT) buffer (30 nm Tris–HCl, pH 7.2, 140 mm sodium cacodylate and 1 mm cobalt chloride) and TdT according to the protocol provided by the manufacturer (Roche). Cells were washed in PBS, mounted in fluorescent mounting media (Dako, Burlington, ON, Canada) and analyzed with a Zeiss Axiovert 200 m epifluorescent microscope equipped with an AxioCam HRM digital camera and Axiovision 4.6 software (Zeiss, Toronto, ON, Canada).

**Primary culture of DRG neurons**

Spinal columns were removed from P5 mice and transferred to a dissection microscope. Total DRGs were isolated per mouse, and subsequently digested for 15 min each with collagenase A (Roche) and papain (Worthington, Lakewood, NJ, USA) solutions. DRG neurons were dissociated with flame polished glass Pasteur pipettes and seeded onto 18 mm laminin-2 (Millipore, Billerica, MA, USA) coated cover slips at a density of 50 000 per 12-well cell culture vessel. Cells were cultured in DMEM with 10% FBS and 1% Pen/Strep and placed in a 37 °C tissue culture incubator, under 8.5% CO₂. The following day, media were changed to neuronal maintenance media (DMEM base, 0.5% FBS, 1% Glutamax, 200 μg/ml triiodothyronine, 6.2 ng/ml progesterone, 5 ng/ml sodium selenite, 100 μg/ml bovine albumin serum, 5 μg/ml bovine insulin, 50 μg/ml holo-transferrin) supplemented with 200 ng/ml nerve growth factor and 1 μM 5-fluoro-2′-deoxyuridine. A three-quarter media change was carried out every other day. Cultures were fixed in 4% paraformaldehyde (PFA) before antigenic labeling. Primary antibodies used were anti-c-myc (1:800; Santa Cruz Biotechnology, Inc.), mouse polyclonal anti-βIII-tubulin (1:1000; Millipore) and rabbit polyclonal anti-cleaved caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA, USA). Antibodies were diluted in antibody buffer (PBS, 0.3% Triton X-100, and 3% BSA). Where DAPI staining is indicated, samples were incubated in DAPI stain (0.2 μg/ml in PBS) for 10 min and washed three times in PBS for 5 min. Samples were mounted in fluorescent mounting media (Dako) and analyzed with a confocal microscope (LSM 510 meta) equipped with an EC Plan-Neofluar 40×/1.30 NA oil DIC M27 objective using Zen 8.0 software.

Subpopulations of sensory neurons were visualized with an epifluorescent microscope (Axiovert 200M; Carl Zeiss) under a ×20 objective (Achromplan 0.25) equipped with a digital camera (AxioCam HRm; Carl Zeiss). Using Axiovision 4.6 software (Carl Zeiss), the circumference-measuring tool was employed in determining sensory neuron size.
**Immunohistochemistry**

Tissues (cortical, cerebellum, DRG and muscle) were collected from at least three $dt^{Tg4/Tg4}$, PrP-dystonin-a2/PrP-dystonin-a2, $dt^{Tg4/Tg4}$ and PrP-dystonin-a2/PrP-dystonin-a2 mice at multiple time points (P10, P15, P58) as previously described (18). Mice were anesthetized with tribromethanol (Avertin) and perfused transcardially with 3 ml of PBS followed by 10 ml of 4% PFA in PBS. Samples were embedded in OCT compound (Sakura), and frozen in liquid nitrogen. Cryostat sections of 10 μm thickness were stored at −20°C before use. Primary antibodies used were rabbit monoclonal anti-Myc (1:100, Abcam). Secondary antibodies used were anti rabbit Alexa-488 (1:2000, Molecular Probes). Antibodies were diluted as mentioned above. Samples were mounted in fluorescent mounting media (Dako) and analyzed with a Zeiss Axiovert 200 m epifluorescent microscope equipped with an Axiocam HRM digital camera and Axiovision 4.6 software (Zeiss).

Axonal assessment in the dorsal roots and morphological analysis of endplates was performed essentially as described previously (41). The number of muscle spindles was counted in serial paraffin cross-sections of P15 tibialis anterior muscle of three mice per genotype stained with hematoxylin and eosin. Big and small spindles were examined for degeneration from 30 sections per muscle using light microscopy.

**Cell preparation for transmission EM and morphometric analysis**

P5 primary sensory neurons were cultured as described above, washed with PBS and trypsinized (0.25% trypsin/0.5% EDTA) for 5 min at 37°C. To obtain a suitable-sized pellet of sensory neurons, an n = 2 was collected and combined for each genotype (PrP-dystonin-a2/PrP-dystonin-a2 control, $dt^{Tg4/Tg4}$ and PrP-dystonin-a2/PrP-dystonin-a2, $dt^{Tg4/Tg4}$). Cells were centrifuged at 1000g for 5 min and supernatant removed. Neurons were fixed for 1 h at room temperature in Karnovsky’s fixative (4% PFA, 2% glutaraldehyde and 0.1 M cacodylate buffer) and subsequently washed twice in 0.1 M cacodylate buffer. Cells were post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature, followed by 3 × 5 min washes in water. Cells were dehydrated twice for 20 min for each step in a graded series of ethanol from water through 30, 50, 70, 85 and 95% ethanol, and twice for 30 min in 100% ethanol (molecular sieves were used to dehydrate ethanol), followed by two washes for 15 min in 50% ethanol/50% acetone and twice for 15 min in 100% acetone. Neurons were infiltrated in 30% spurr/acetone for 15 h (overnight), then in 50% spurr/acetone for 6 h, and in fresh 100% spurr resin for overnight. Spurr was changed twice a day for 3 days at room temperature. All infiltration steps were performed on a rotator. Neurons were embedded in fresh liquid spurr epoxy resin and polymerized overnight at 70°C. Ultrathin sections (80 nm) of cells were collected onto a 200-mesh copper grid and let dry overnight. Grids were stained with 2% aqueous uranyl acetate and with Reynold’s lead citrate. Sections were observed under a transmission electron microscope (Hitachi 7100). Approximately 10 EM micrographs at × 20 000 magnification were examined per genotype.

**Statistical analysis**

Data were analyzed using Student’s t-test or factorial ANOVA. Following detection of a statistically significant difference in a given series of treatments by ANOVA, *post hoc* Dunnett’s t-tests or Tukey tests were performed where appropriate. P-values under 0.05 were considered statistically significant (shown as *); P-values under 0.01, 0.001 or 0.0001 were considered highly statistically significant (shown as **, ***,***, respectively).

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**Conflict of Interest statement.** None declared.

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