miRNAs in Microglia: Important Players in Multiple Sclerosis Pathology

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Abstract
Microglia are the resident immune cells of the central nervous system and important regulators of brain homeostasis. Central to this role is a dynamic phenotypic plasticity that enables microglia to respond to environmental and pathological stimuli. Importantly, different microglial phenotypes can be both beneficial and detrimental to central nervous system health. Chronically activated inflammatory microglia are a hallmark of neurodegeneration, including the autoimmune disease multiple sclerosis (MS). By contrast, microglial phagocytosis of myelin debris is essential for resolving inflammation and promoting remyelination. As such, microglia are being explored as a potential therapeutic target for MS. MicroRNAs (miRNAs) are short non-coding ribonucleic acids that regulate gene expression and act as master regulators of cellular phenotype and function. Dysregulation of certain miRNAs can aberrantly activate and promote specific polarisation states in microglia to modulate their activity in inflammation and neurodegeneration. In addition, miRNA dysregulation is implicated in MS pathogenesis, with circulating biomarkers and lesion specific miRNAs identified as regulators of inflammation and myelination. However, the role of miRNAs in microglia that specifically contribute to MS progression are still largely unknown. miRNAs are being explored as therapeutic agents, providing an opportunity to modulate microglial function in neurodegenerative diseases such as MS. This review will focus firstly on elucidating the complex role of microglia in MS pathogenesis. Secondly, we explore the essential roles of miRNAs in microglial function. Finally, we focus on miRNAs that are implicated in microglial processes that contribute directly to MS pathology, prioritising targets that could inform novel therapeutic approaches to MS.

Keywords
epigenetics, gene regulation, microglia, miRNA, multiple sclerosis, neurodegeneration

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Role of Microglia in Central Nervous System Health and Inflammation
Microglia represent an unusual population of mononuclear phagocytic cells. First observed and characterised by Pio del Rio-Hortega in the early 20th century, microglia are central nervous system (CNS) resident immune cells and one of the four main cell types in the CNS (along with neurons, oligodendrocytes and astrocytes), making up 5–15% of total brain cells (Lawson et al., 1990; Aguzzi et al., 2013; Sierra et al., 2016). Unlike the other three cell types however, microglia do not arise from neurogenic progenitors but rather have a myeloid origin. Microglia originate from erythromyeloid progenitors (EMPs) in the embryonic yolk sac, before undergoing a wave of migration to the developing brain parenchyma, where they proliferate and distribute throughout the brain (Alliot et al., 1999; Monier et al., 2007; Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013). Following colonisation of the brain, microglia maintain a self-sustaining population with very little integration of peripheral macrophages and monocytes.
Microglia exhibit distinctive functions that critically mediate both development and adult homeostasis of the healthy CNS. One central function of these cells is effectorcytosis, the removal of apoptotic cells and debris to promote immune tolerance and tissue repair (Colonna and Butovsky, 2017; Li and Barres, 2018). During development, microglia regulate neurogenesis and refine synaptic architecture by eliminating immature neurons and excessive synapses (Paolicelli et al., 2011; Schafer et al., 2012). Additionally, microglia secrete neurotrophic factors including IGF-1, FGF and BDNF, essential molecules that promote neuronal survival and function (Araujo and Cotman, 1992; Takahashi et al., 2005; Ueno et al., 2013). Microglia are also implicated in the development and ongoing maintenance of myelin in the CNS. Myelin is produced by oligodendrocytes and is a fundamental component of correct neuronal function, insulating axons to support rapid neurotransmission (Saab et al., 2013). Adult microglia phagocytose inflammatory myelin debris, and specific subsets of microglia regulate the proliferation and growth of oligodendrocytes and their progenitor cells during white matter development and in remyelination following injury (Miron et al., 2013; Hagemeyer et al., 2017; Lloyd et al., 2017; Li et al., 2019a). Microglia thus exert a profound influence on many aspects of the formation and ongoing maintenance of the CNS.

By far the most well studied aspect of microglia is their role in immunity. As resident immune cells in the brain, microglia are first responders to inflammatory and pathological stimuli (Li and Barres, 2018). In the healthy CNS, microglia constantly survey their environment using a subset of around 100 cell surface proteins described collectively as the ‘microglial sensome’ (Davalos et al., 2005; Nimmerjahn et al., 2005; Boche et al., 2013; Hickman et al., 2013). In response to homeostatic disturbances, microglia have the capacity to ‘activate’, triggering proliferation and dramatic shifts in morphology and function to effect an appropriate response (Marissa et al., 2018). These responses include phagocytosis of damaged cells and debris, pathogen recognition, antigen presentation and secretion of chemokines and cytokines (Amici et al., 2017; Herz et al., 2017). Microglial activation is multifaceted and dynamic, and cells can adopt numerous phenotypes, often labeled as either ‘neuroprotective’ or ‘neurotoxic’, each of which significantly influences the outcomes of inflammation (Block et al., 2007; Glezer et al., 2007) (Figure 1). Neurotoxic microglia (often labelled ‘M1’ or “classically activated”) adopt a reactive ameboid morphology, release pro-inflammatory molecules including interleukins (IL-6, IL-23, IL-1β), TNF-α and reactive oxygen species (ROS) (Boche et al., 2013) (Figure 1). Conversely, neuroprotective microglia (often called “M2” or “alternatively activated”) promote homeostasis, tissue repair and suppress inflammatory responses, mainly via uptake of apoptotic debris and release of anti-inflammatory cytokines including TGF-β and IL-10 (Boche et al., 2013; Amici et al., 2017) (Figure 1). This paradigm of “good” and “bad” microglia is not black and white, microglial activation is multi-faceted whereby these cells can adopt many phenotypes with significant overlaps in gene expression (Ransohoff, 2016b). Microglia-mediated inflammation is similarly complex, microglia play key roles in both promoting and suppressing inflammatory processes, and therefore careful control of microglial phenotype is critical for appropriate immune responses.

Consequently, dysregulation of microglial activation is a major contributor to neurodegeneration. Microglia-mediated phagocytosis of debris and apoptotic cells is essential for promoting repair and regeneration. Conversely, chronic microglial ‘neurotoxic’ activation is a hallmark of CNS diseases including Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), Huntington’s disease and the focus of this review, multiple sclerosis (MS; Perry and Teeling, 2013; Ransohoff, 2016a; Colonna and Butovsky, 2017). In these diseases, microglial activation is heavily skewed towards inflammation and neurotoxicity, whereby microglia fail to transition to a protective phenotype to promote repair. Secretion of inflammatory cytokines and reactive oxygen/nitrogen species promotes inflammation and exacerbates tissue damage which ultimately culminates in neurodegeneration (Cunningham, 2013).

Microglia Are Essential Mediators of MS

MS is an autoimmune disease of the CNS that affects over 2 million people worldwide (Hassan-Smith and Douglas, 2011). The disease is characterised by chronic inflammation, oligodendrocyte death and damage to the protective myelin sheath of axons (demyelination) that leads to axonal loss and progressive neurological disability (Ferguson et al., 1997; Popescu and Lucchinetti, 2012). The pathological hallmark of MS is the formation of demyelinating lesions, which are associated with neuronal damage and an influx of infiltrating immune cells. The aetiology of MS remains unclear and the cellular pathology of disease onset and progression is complex and often heterogeneous. Around 85% of patients are diagnosed with relapsing-remitting MS (RRMS) which presents with alternating episodes of acute inflammatory attack and subsequent periods of remission (Weinschenker et al., 1989; Dobson and Giovannoni, 2019). In many cases, RRMS can transition into a secondary progressive phase of disease (SPMS) with no remission stages and cumulative neurodegeneration. A smaller proportion (10-15%) of MS patients present with a progressive...
course from the onset of disease (primary progressive multiple sclerosis, PPMS) (Hassan-Smith and Douglas, 2011).

Historically, MS research has focused on the role of the adaptive immune system and particularly the roles of autoreactive T and B cells that infiltrate the CNS and drive lesion pathology. However, recent developments have recognised the importance of resident innate immune cells and especially microglia in MS aetiology and pathogenesis. (Barnett and Prineas, 2004; Gandhi et al., 2010; Kasper and Shoemaker, 2010). Studies in
both human MS lesions and the experimental autoimmune encephalomyelitis (EAE) mouse model observe microglial migration, proliferation and activation in the onset of inflammation and demyelination, even prior to T cell infiltration (Barnett and Prineas, 2004; Ponomarev et al., 2005). Activated microglia produce reactive oxygen species/nitrous oxides (ROS/NO) and secrete pro-inflammatory cytokines in these lesions, actively contributing to oligodendrocyte loss and subsequent neurodegeneration (Luo et al., 2017) (Figure 1). While ablation of microglia in EAE delays disease onset and progression, it also impacts the potential for remyelination and recovery (Lu et al., 2002; Heppner et al., 2005). This highlights the importance of ‘neuroprotective’ microglial activation and clearance of myelin debris as essential for alleviating inflammation and stimulating OPC proliferation and remyelination (Figure 1). In addition, priming of microglial activation with anti-inflammatory reagents prior to EAE onset alleviates symptoms and actively promotes tissue repair (Bhasin et al., 2007). Similar results have been observed in the cuprizone mouse model, which utilizes the toxic copper chelator cuprizone to generate focal demyelination in the CNS while minimizing a peripheral immune response. The onset and progression of demyelination in this model is accompanied by microglial proliferation and activation (Binder et al., 2008; Voss et al., 2012). Genetic tracing of microglia and peripheral macrophage populations in a lysolecithin-dylcholine induced demyelination model has identified that microglial activation actively limits peripheral macrophage infiltration and proliferation in the CNS, suggesting that microglia are the main drivers of the innate immune response to acute demyelination (Plemel et al., 2020). Therefore, clearance of myelin debris by microglia is critical for promoting remyelination, which is driven by a transition to a neuroprotective microglial phenotype (Neumann et al., 2009; Voss et al., 2012; Lampron et al., 2015). This is observed in a lysolecithin-induced model of demyelination, whereby oligodendrocyte proliferation is dependent on activated microglia clearing debris to reduce inflammation (Miron et al., 2013). Finally, studies in human MS pathology show that microglial activation is implicated in the onset and progression of lesion activity in acute, relapsing and progressive subtypes of MS (Zrzavy et al., 2017). However, these activation states are dynamic, and microglial phenotype shifts with lesion severity and disease progression (Zrzavy et al., 2017). While lesion onset is associated with pro-inflammatory activation of microglia, evidence shows that upon ingesting myelin debris, microglia adopt an “intermediate” phenotype and express numerous anti-inflammatory markers, suggesting that these cells have the potential to promote tissue repair (Boven et al., 2006; Vogel et al., 2013; Zrzavy et al., 2017).

Overall, data from human lesions and animal models highlight the complexity of microglia in MS pathogenesis (Figure 1). In response to environmental cues, generally cellular debris or pathogens, microglia adopt numerous phenotypes and functions that are heavily context dependent. Microglia make significant contributions to inflammation, demyelination, and axonal loss in MS, but are also essential for the transition to a regenerative CNS environment. Neurodegeneration is driven by neurotoxic microglia that fail to transition into a neuroprotective phenotype. How and why this imbalance in microglial phenotypes occurs remains poorly understood. One proposed mechanism for microglial dysregulation is reported by Lloyd et al., who recently observed that the transition to a regenerative state is preceded by necroptosis of pro-inflammatory microglia, followed by repopulation of neuroprotective microglia, highlighting a dysregulation of this necroptotic wave as a potential source for chronically inflammatory microglia in demyelination (Lloyd et al., 2019).

Clearly, further understanding of the mechanisms that regulate microglial phenotype and population dynamics in MS will be essential for the development of therapeutic approaches that modulate these cells to a protective phenotype during neuroinflammation.

**Microglial Transcriptomics: The Link Between Genotype and Phenotype**

Recent technological advances in microglial isolation and whole transcriptome/epigenome analysis have vastly improved understanding of microglial phenotypes and their unique gene signatures (Eggen et al., 2017; Grabert and McColl, 2018). Changes associated with microglial plasticity and activation are driven by dramatic changes to transcriptional landscape, meaning that transcriptomic studies are a powerful tool to dissect microglial phenotypes. Several studies have profiled transcriptomes that define microglia isolated during developmental, homeostatic and inflammatory states (Crottì and Ransohoff, 2016; Eggen et al., 2017).

Studies in isolated mouse and human microglia have identified enriched signature in mouse and human homeostatic microglia including $CX3CRI$, $TMEM119$, $TREM2$, $P2RY12$, $MERTK$, $PROS1$, $SALL1$ and $SIGLEC$ (Gautier et al., 2012; Beutner et al., 2013; Hickman et al., 2013; Butovsky et al., 2014; Gosselin et al., 2014; Zhang et al., 2014b; Bennett et al., 2016; Grabert et al., 2016; Matcovitch-Natan et al., 2016; Gosselin et al., 2017). A consistent finding is that the top genes enriched in microglia are broadly related to microglial immune function, cytokine production, cell motility and phagocytosis (Eggen et al., 2017). Interestingly, a common damage-associated microglia (DAM) signature has been identified in numerous neurodegenerative diseases, including AD, PD, ALS and MS (Keren-
Shaul et al., 2017; Krasmann et al., 2017). This is associated with a downregulation of the microglial homeostatic signature genes such as P2RY12, TMEM119, MERTK and ARG1 and upregulation of inflammatory genes associated with the ‘neurotoxic’ microglial phenotype, including ITGAX, CCL2, CLEC7A, CCL2, CD86, TNF and activation of the TREM2-APOE pathway (Krasmann et al., 2017; Zrzavy et al., 2017; Sousa et al., 2018) (Figure 1). Genetic studies in MS patient cohorts and mouse models of demyelination have identified dysregulation of microglial genes in both demyelinating and remyelinating contexts (Figure 1). Many of the 200 MS susceptibility genes identified by large scale genome wide association studies are associated with microglial function (Patsopoulos, 2016; International Multiple Sclerosis Genetics Consortium, 2019). Direct sequencing of microglia from white matter lesions of MS patients has shown upregulation of inflammatory pathways including NF-KB and APOE related genes and varying downregulation of homeostatic markers including P2RY12 and TMEM119 (Zrzavy et al., 2017; van der Poel et al., 2019). This is supportive of the DAM signature and promotion of inflammation that may contribute to disease (Zrzavy et al., 2017; Jordão et al., 2019; van der Poel et al., 2019). However, sequencing studies have also identified genetic signatures that support a neuroprotective role for microglia, again highlighting the complexity of microglial phenotype, particularly in the context of demyelination. This ‘neuroprotective’ signature is characterised by a downregulation of NF-kB inflammatory signaling and upregulation of key anti-inflammatory genes including ARG1, IFNB1, IL4, IL10, SOCS1/3 and TGFB1 that inhibit inflammation and are conducive to tissue repair (Olah et al., 2012; Butovsky et al., 2014; Butovsky and Weiner, 2018) (Figure 1).

Environmental and epigenetic modifiers have a profound influence on microglial gene expression and cell function. Many factors strongly influence microglial transcriptomics including brain region, sex and ageing with significant influences on microglial function and disease outcomes (Perry and Teeling, 2013; Gosselin et al., 2014; Grabert et al., 2016; Askew et al., 2017; Galatro et al., 2017; Gosselin et al., 2017; Hanamsagar et al., 2017; Spittau, 2017; Olah et al., 2018; Villa et al., 2018). Defining epigenetic mechanisms that finely regulate immune function may provide novel therapeutic approaches to targeting microglia in MS. An example of an abundant epigenetic mechanism that regulates microglial identity and immune activation are the non-coding ribonucleic acid (RNA) species known as microRNAs (miRNAs).

**miRNAs: Biogenesis, Function and Therapeutic Potential**

miRNAs are small (21–25 nucleotides), non-coding RNAs that negatively regulate gene expression via binding to and suppressing translation of target messenger RNA (mRNA), or by promoting mRNA degradation (Gebert and MacRae, 2019). Since their discovery in nematodes over 20 years ago, thousands of miRNAs have been identified, many of which are highly conserved throughout evolution (Lee et al., 1993; Gebert and MacRae, 2019). Approximately 2700 and 2000 miRNAs have been described and characterised in the human and murine genomes respectively (miRBase) and more than 60% of the mammalian coding genome is predicted to be directly regulated by at least one miRNA (Friedman et al., 2009; Kozomara and Griffiths-Jones, 2014).

miRNAs are generated by a series of steps that convert a stem-loop containing primary miRNA (pri-miRNA) transcript into a functionally active mature miRNA (Figure 2). miRNAs are transcribed by RNA polymerase II and while many reside in individual loci or in polycistronic transcriptional units, the majority of miRNAs are encoded within introns of the protein coding genes (Rodriguez et al., 2004; Kim et al., 2009). The canonical miRNA maturation pathway involves a two-step enzymatic process coupled to nuclear export into the cytoplasm via exportin-5 (Gebert and MacRae, 2019). Pri-miRNA is sequentially processed by two RNAseIII endonucleases, Drosha and Dicer which produces a ~21 base imperfectly paired miRNA duplex (Ketting et al., 2001; Lee et al., 2003; Yi et al., 2003). One strand of the duplex is then incorporated into a RNA induced silencing complex (RISC) to form a mature active miRISC complex, made up of many proteins including the RNA splicing protein Argonaute (Liu et al., 2004; Fabian and Sonenberg, 2012; Sheu-Gruttdauria and MacRae, 2017).

Gene silencing by miRNAs involves miRISC complexes, guided by their constituent miRNA, binding to a target mRNA strand and inhibiting translation. Target recognition is not driven by complementarity of all ~21 bases of the miRNA sequence, but rather a seven base pair ‘seed’ site, generally bases 2–8 (Wang, 2014). The majority of miRISC complexes bind to the 3’ untranslated region (UTR) of their targets, but many can bind the 5’ UTR and/or coding regions. Once bound, the miRISC directs suppression of mRNA translation either by AGO-2 mediated cleavage of the target strand or recruitment of deadenylation complexes by the protein GW182 that lead to mRNA decay and a net decrease in protein expression (Baek et al., 2008; Karginov et al., 2010; Braun et al., 2011; Chekulaeva et al., 2011; Eichhorn et al., 2014). miRNAs can profoundly impact gene expression in a target cell. This is because a single miRNA can potentially target hundreds of mRNA transcripts and in turn a single mRNA transcript can be bound by many miRNAs (Selbach et al., 2008; Friedman et al., 2009). Consequently, miRNAs can
target multiple molecular pathways simultaneously and regulate major aspects of cell physiology including differentiation, maturation, activation and immune function. miRNA targets also include transcription factors (TFs), which are often master regulators of broad transcriptional programs and cellular events (Enright et al., 2003). In turn, TFs can specify expression patterns of miRNAs by binding upstream of miRNA transcriptional units. Therefore, these two classes of regulators can form complex and synergetic regulatory relationships that promote specific cellular states (Arora et al., 2013).

Much of the biological functionality of miRNAs is driven by specific temporal and spatial expression domains. High specificity of miRNA expression enables fine control of complex mechanisms and pathways that regulate cell specific functions (O’Brien et al., 2018). However, miRNAs have been predominately characterised in tissues and bulk samples. Tissues are composed of multiple, unique cell types and as a result the cellular source of specific miRNA signals can be lost. Studies of individual cell types help to resolve these issues (McCall et al., 2017). The number of ubiquitous miRNAs is fewer than previously thought, and as more single cell data becomes available the number of catalogued cell type specific miRNAs across species is predicted to increase significantly (Londin et al., 2015; McCall et al., 2017).

**Figure 2.** Biogenesis Pathways of miRNAs. miRNAs can be generated from a dedicated miRNA locus or spliced from the introns of premRNA transcripts (miRtrons). A stem-loop containing pre-miRNA is produced either by Drosha or the spliceosome complex dependent on origin. The pre-miRNA is exported from the nucleus to the cytoplasm via exportin-5 where the stem loop is cleaved by the RNase III protein Dicer to generate an imperfect double stranded miRNA. One strand of this duplex is then incorporated into an RNA induced silencing complex (RISC) of which the main catalytic component is Argonaute (AGO). The other strand is usually degraded. The functionally active miRISC complex guided by the seed site of its constituent miRNA strand will seek out and bind to target miRNA transcripts in the cytoplasm and signal them for cleavage and degradation. Alternatively, double stranded miRNA complexes can be packaged into extracellular vesicles (EVs) and released into the extracellular space to be taken up by neighboring cells and influence target gene expression in a different cellular environment.
It is now of critical importance to interrogate the function of miRNAs within specific cell types to fully understand their biology in health and disease.

Considering the broad influence miRNAs have on cell physiology, it is not surprising that aberrant miRNA regulation is implicated in a range of pathologies including cancers, immune disorders and neurological diseases of the CNS (Sébastien and Bart De, 2007; O’Connell et al., 2010). In recent years, hundreds of miRNAs have been highlighted as key modulators for a range of CNS functions and related physiological and pathological conditions (Cao et al., 2016). In addition, disease-associated changes in circulating miRNA levels are being harnessed as diagnostic biomarkers (Condrat et al., 2020). Therefore, selecting miRNAs that target either single genes or multiple genes/pathways of interest provides a unique opportunity for the development of therapeutics to specifically modulate cell activity. miRNA mimics and inhibitors can be synthesised and are amenable to delivery via vehicle in vivo to modulate target miRNA activity (Ming Ming, 2016). Targeted delivery of miRNA technology to diseased cells is a promising approach for correcting gene expression and cell function to prevent or reverse disease progression.

**miRNA Dsyregulation Is Implicated in MS Pathogenesis**

miRNAs represent potential diagnostic markers for, and pathological agents of, MS pathogenesis. Many dysregulated miRNAs are reported in MS patient samples derived from serum, whole blood, peripheral blood mononuclear cells (PBMCs), cerebrospinal fluid (CSF) and brain lesions (Aslani et al., 2017; Dolati et al., 2018a). The observation of aberrant miRNA expression in both central and peripheral tissues suggests a global role for miRNAs in MS pathology.

Otaegui et al. profiled peripheral miRNA expression in MS, identifying dysregulated miRNAs in PBMCs associated with disease relapse and remission (Otaegui et al., 2009). Interestingly, upregulation of the miRNA machinery components Drosha, Dicer and DGCR8 is observed in PBMCs of MS patients, further suggesting a dysregulation of miRNA biogenesis and function in MS pathology (Jafari et al., 2015). Numerous studies in MS patients have expanded this work to identify specific dysregulated miRNA profiles from subsets of T cells, B cells and monocytes within the PBMC population [reviewed extensively in (Aslani et al., 2017; Dolati et al., 2018a)].

There are fewer studies of miRNAs in MS lesions but these have nevertheless identified miRNAs regulating the resident CNS cells central to MS pathology (Junker et al., 2009; Noorbakhsh et al., 2011; Tripathi et al., 2019; Fritsche et al., 2020). Despite considerable lesion and neurological heterogeneity observed in MS, collated findings suggest that there are conserved miRNA profiles that influence gliosis, inflammation, demyelination and remyelination (Teuber-Hanselmann et al., 2020). Junker et al. identified 20 upregulated and 8 downregulated miRNAs in active MS lesions compared to normal appearing white matter (NAWM) (Junker et al., 2009). These included miR-155, miR-326 and miR-34a, all of which simultaneously target the CD47 transcript and promote macrophage phagocytosis (Junker et al., 2009). Other dysregulated miRNAs included miR-146a, miR-219 and miR-388 which influence numerous cellular processes including T cell differentiation and remyelination (Junker et al., 2009; Wang et al., 2017). These studies have identified key miRNAs that target important molecular pathways as potential therapeutic targets for MS. However, studies in human CNS tissue are not without limitation. MS lesion samples are obtained postmortem with considerable delay to analysis, which can confound miRNA expression and detection (Moreau et al., 2011). Nevertheless, they are an essential resource for identifying dysregulated miRNAs that may be central to human CNS pathology.

In addition to miRNA profiles of disease related cell types and tissue, hundreds of dysregulated miRNAs identified in the serum, plasma and CSF of MS patients represent potential diagnostic markers for disease onset and progression (Gandhi et al., 2013; Ridolfi et al., 2013; Sondergaard et al., 2013; Kacperska et al., 2015; Quintana et al., 2017; Regev et al., 2018; Munoz-San Martin et al., 2019). For example, the pro-inflammatory miR-155 is consistently upregulated in the serum of MS patients and miR-145 is the strongest single marker for MS, with 90% sensitivity when differentiating MS patients from healthy controls (Keller et al., 2009; Piket et al., 2019). Meta analyses and bioinformatic studies of collated serum/plasma/whole blood data have identified enriched immune pathways targeted by dysregulated miRNAs (Luo et al., 2020). Importantly, one meta-analysis identified considerable heterogeneity and low reproducibility of reporter biomarkers across several studies (Piket et al., 2019). Only 9% of miRNAs were reported to be dysregulated in the same direction (in a minimum of 3 studies), highlighting challenges in identifying robust markers for MS (Piket et al., 2019). Such low reproducibility is not entirely unexpected, considering that miRNA expression is influenced by various factors including age, sex, treatment and disease course (Piket et al., 2019; Mycko and Baranzini, 2020). A major source of variability comes from the techniques used to isolate and sequence miRNA expression from patient samples. As of now, there is no standardized method for profiling circulating miRNAs, which will...
need to be addressed in order to resolve issues of low reproducibility (Piket et al., 2019).

With over 650 reported dysregulated miRNAs in MS, a major challenge within the field is identifying miRNAs that universally and robustly modulate MS onset and progression. This is especially important when collating miRNAs identified across independent studies and in different tissues (ie central versus peripheral). To begin to address these challenges, a pair of studies correlated dysregulated miRNAs in serum to disease activity (measured by T1/T2 weighted magnetic resonance imaging of lesion activity) and then validated the expression of strongly correlating miRNAs in MS white matter lesions, identifying 23 miRNA targets with strong, consistent directional changes and association with disease severity (Regev et al., 2017; Tripathi et al., 2019). Strategies such as this set an important precedent for prioritising therapeutically viable targets that directly contribute to MS pathology. However, many of these approaches still do not address cell specific miRNA expression.

Defining the role of miRNAs in specific cell types and their influence on molecular pathways remains a major obstacle to fully understanding the miRNA biology in the context of health and disease. Studies in serum/plasma and especially in the CNS are mostly performed with bulk RNA sequencing and make determining miRNA expression in each cell type difficult. While some studies of non-CNS tissues have been able to elucidate individual roles of miRNAs in distinct subsets of lymphocytes, this is considerably more difficult to achieve in the resident CNS cells implicated in MS lesion activity (Aslani et al., 2017; Dolati et al., 2018a). It will be crucial to identify miRNAs that are specifically dysregulated in microglia, neurons, oligodendrocytes and astrocytes in MS pathology, particularly when it comes to identifying the most effective targets for therapeutic intervention.

**Dysregulation of Microglial Enriched miRNAs Is Implicated in Aberrant Microglial Function and MS Pathology**

The mechanisms of miRNA regulation are especially suited to the rapidly induced regulatory cascades that define activation phenotypes of microglia during inflammation. Emerging evidence suggests that miRNAs regulate key molecular pathways of microglia and can profoundly influence the outcomes of inflammation. Among the first studies to identify a crucial role for miRNAs in microglia, Varol et al. (2017) characterised the miRNAome of adult mouse microglia. Of the 160 unique miRNAs expressed by microglia, 84 were specifically enriched relative to other tissue macrophage populations, supporting the notion that microglia have a unique miRNA signature (Varol et al., 2017). While microglia and monocyte/macrophage populations share similar functions and phenotypes, microglia have a unique ontogeny and are restricted entirely to CNS, exhibiting significant differences in gene expression (Gosselin et al., 2014; Melief et al., 2016; Gosselin et al., 2017; Healy et al., 2018). This study, amongst others, suggests that this difference extends to miRNAs, whereby a unique microglial miRNA signature may be essential for regulating microglial specific functions in immunity (Butovsky et al., 2014; Varol et al., 2017). In addition, Varol et al. have studied microglial function following post-natal conditional knockout of the Dicer gene, removing the miRNA biogenesis pathway from homeostatic microglia. The impact on homeostatic function was negligible, however the cells were hyper-responsive to inflammatory challenge by lipopolysaccharide (LPS) (Varol et al., 2017). Similarly, conditional microglial knockout of Dicer in the P301S tauopathy model exacerbated disease progression and promoted specific enrichment of inflammatory and neurodegeneration-associated genes in microglia (Kodama et al., 2020). Mounting evidence suggests that perturbation of miRNA activity in microglia is functionally significant during microglial activation following immune challenge. This sets a precedent for investigating the miRNA profiles of microglia in specific neurodegenerative contexts including MS.

Dysregulation of microglia-enriched miRNAs is explicitly implicated in MS pathology. The majority of studies in this field come from bulk tissue studies in EAE and cuprizone models, of which only a few have profiled microglia specifically. Most studies of MS patient tissues and animal models of MS profile bulk CNS tissue and do not profile individual cell types. As such, many miRNAs are implicated in microglial contribution to MS progression by previous association with microglial function as opposed to direct measurement of MS patient samples. Nevertheless, correlating miRNAs implicated in microglial activation and inflammation with those dysregulated in lesions/serum/blood of MS patients is a means of identifying miRNAs that may modulate microglial function in MS pathology. A comprehensive list of microglia-enriched miRNAs that are dysregulated in MS animal models, peripheral biomarker studies and MS lesions is provided in Table 1. The following section will detail the specific biology of some of the important MS-related microglial miRNAs presented in Table 1 in more detail.

**Microglia-Enriched miRNAs That Promote Neurotoxicity**

Numerous miRNAs have been identified which promote microglial inflammation in MS. The most well defined is
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target</th>
<th>Role in microglia</th>
<th>MS mouse model dysregulation</th>
<th>MS peripheral tissue</th>
<th>MS lesion dysregulation</th>
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<tbody>
<tr>
<td>miR-155</td>
<td>C-Maf, SMAD2, C/EBPβ, SOCS-1 (Louafi et al., 2010; Bala et al., 2011; Cardoso et al., 2012)</td>
<td>Promotes microglial inflammation (Guedes et al., 2013; Guo et al., 2019)</td>
<td>Upregulated in EAE (Lescher et al., 2012; Zhang et al., 2014a; Shakerian et al., 2018; Venkatesh et al., 2018)</td>
<td>Upregulated in PBMCs (Paraboschi et al., 2011; Waschbisch et al., 2011; Moore et al., 2013; Dolati et al., 2018b)</td>
<td>Upregulated in all MS lesion types (Junker et al., 2009; Moore et al., 2013; Fritsche et al., 2020)</td>
</tr>
<tr>
<td>miR-145</td>
<td>NURR1, ARF6 (Xie et al., 2017; Li et al., 2018)</td>
<td>Promotes microglial inflammation (Xie et al., 2017)</td>
<td>Downregulated in cuprizone (Han et al., 2020)</td>
<td>Upregulated in PBMCs (Ridolfi et al., 2013; Sondergaard et al., 2013) Upregulated in serum (Sharaf-Eldin et al., 2017) Upregulated in plasma (Sondergaard et al., 2013) Upregulated in CSF (Bergman et al., 2016)</td>
<td>Upregulated in active MS lesions (Tripathi et al., 2019)</td>
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<tr>
<td>miR-125b</td>
<td>IRF4, A20, STAT3 (Chaudhuri et al., 2011; Parisi et al., 2013)</td>
<td>Promotes microglial inflammation (Chaudhuri et al., 2011)</td>
<td>Upregulated in EAE (Bergman et al., 2013) Upregulated in cuprizone (Han et al., 2020)</td>
<td>N/A</td>
<td>Dysregulated in NAWM (Noorbakhsh et al., 2011; Guerau-de-Arellano et al., 2015)</td>
</tr>
<tr>
<td>miR-222</td>
<td>ITGB8 (Bai and Niu, 2020)</td>
<td>Promotes microglial inflammation (Bai and Niu, 2020)</td>
<td>N/A</td>
<td>Upregulated in serum (Regev et al., 2017)</td>
<td>Upregulated in active MS lesions (Tripathi et al., 2019)</td>
</tr>
<tr>
<td>miR-32</td>
<td>DUSP5 (Yan et al., 2018)</td>
<td>Promotes microglial inflammation (Yan et al., 2018)</td>
<td>Upregulated in Cuprizone (Han et al., 2020)</td>
<td>N/A</td>
<td>Downregulated in active MS lesions (Fritsche et al., 2020)</td>
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<tr>
<td>miR-142</td>
<td>SLCLA3 (Mandolesi et al., 2017), SIRT1 (Chaudhuri et al., 2013), SOCS1 (Talebi et al., 2017)</td>
<td>Promotes microglial inflammation (Mandolesi et al., 2017)</td>
<td>Upregulated in EAE (Lescher et al., 2012; Mandolesi et al., 2017; Talebi et al., 2017) Upregulated in Cuprizone (Han et al., 2020)</td>
<td>Upregulated in PBMC (Keller et al., 2009; Mandolesi et al., 2017) Upregulated in serum (Regev et al., 2018) upregulated in CSF (Mandolesi et al., 2017)</td>
<td>Upregulated in active MS lesions (Junker et al., 2009; Noorbakhsh et al., 2011)</td>
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<tr>
<td>let-7 (family)</td>
<td></td>
<td></td>
<td>Upregulated in EAE (Bergman et al., 2013; Bergman et al., 2013)</td>
<td>Downregulated in whole blood (7d, 7f, 7g, 7i)</td>
<td>Upregulated in active MS lesions (Junker et al., 2009; Noorbakhsh et al., 2011)</td>
</tr>
<tr>
<td>miRNA</td>
<td>Target</td>
<td>Role in microglia</td>
<td>MS mouse model dysregulation</td>
<td>MS peripheral tissue</td>
<td>MS lesion dysregulation</td>
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<tr>
<td>miR-223</td>
<td>NFAT5, C/EBPα, NFIA</td>
<td>Regulates macrophage/microglial proliferation (Ponomarev et al., 2011)</td>
<td>Upregulated in EAE (Bergman et al., 2013; Galloway et al., 2019)</td>
<td>(Cox et al., 2010) Upregulated in plasma (7c,7d,7f) (Gandhi et al., 2013)</td>
<td>(2009; Dutta et al., 2013; Fritsche et al., 2020) Downregulated in NAWM (Noorbakhsh et al., 2011; Guerau-de-Arellano et al., 2015)</td>
</tr>
</tbody>
</table>

(continued)
Table 1. Continued.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target</th>
<th>Role in microglia</th>
<th>MS mouse model dysregulation</th>
<th>MS peripheral tissue</th>
<th>MS lesion dysregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-204</td>
<td>SIRT1 (Li et al., 2015)</td>
<td>Inhibits microglial inflammation (Li et al., 2015)</td>
<td>N/A</td>
<td>Downregulated in PBMCs (Sievers et al., 2012)</td>
<td>Upregulated in active MS lesions (Junker et al., 2009; Fritsche et al., 2020)</td>
</tr>
<tr>
<td>miR-17</td>
<td>NOX2, NOX4 (Jadhav et al., 2014)</td>
<td>Inhibits microglial inflammation (Jadhav et al., 2014)</td>
<td>Upregulated in cuprizone (Han et al., 2020)</td>
<td>Downregulated in whole blood (Eftekharian et al., 2019)</td>
<td>N/A</td>
</tr>
<tr>
<td>miR-199β</td>
<td>IKKβ (Zhou et al., 2016a)</td>
<td>Inhibits microglial inflammation (Zhou et al., 2016a)</td>
<td>Upregulated in EAE (Bergman et al., 2013)</td>
<td>Downregulated in serum (Regev et al., 2016; 2018)</td>
<td>Upregulated in active MS lesions (Tripathi et al., 2019)</td>
</tr>
<tr>
<td>miR-7</td>
<td>Nlrp3 (Zhou et al., 2016b)</td>
<td>Inhibits microglial inflammation (Zhou et al., 2016b)</td>
<td>Upregulated in Cuprizone (Han et al., 2020)</td>
<td>Downregulated in serum (Keller et al., 2014)</td>
<td>Downregulated in active MS lesions (Tripathi et al., 2019)</td>
</tr>
</tbody>
</table>

Note. C-Maf = Musculoaponeurotic fibrosarcoma, SMAD2 = Mothers against decapentaplegic homolog 2, C/EBPα = CCAAT enhancer binding protein beta, SOCS-1 = Suppressor of cytokines signalling 1, NURR1 = Nuclear receptor related-1 protein, ARF6 = ADP-ribosylation factor 6, IRF4 = Interferon regulatory factor 4, STAT3 = Signal transducer and activator of transcription 3, ITGB8 = Integrin beta-8, DUSP5 = Dual-Specificity Phosphatase 5, SLCA3 = Solute carrier family 1 member 3, SIRT1 = Sirtuin 1, ASK-1 = Apoptosis signal-regulating kinase 1, IL-10 = Interleukin-10, MYC = myc-like oncogene, IL-6 = Interleukin 6, NFAT5 = Nuclear factor of activated T cells 5, C/EBPα = CCAAT binding protein alpha, NFI-A = Nuclear factor 1 A-type, STAT1 = Signal transducer and activator of transcription 1, CREB1 = CAMP Responsive Element Binding Protein 1, CCL8 = Chemokine ligand 8, TRAF6 = Tumor necrosis factor receptor associated factor 6, IRAK1 = Interleukin-1 receptor-associated kinase 1, CYBA = cytochrome b-245 alpha chain, NOS3 = Nitric oxide synthase 3, NOX2 = NAPDH oxidase 2, NOX4 = NAPDH oxidase 4, IKKβ = = Inhibitor of nuclear factor kappa-B kinase subunit beta, Nlrp3 = NODD-, LRR- and pyrin domain-containing protein 3, EAE = experimental autoimmune encephalomyelitis (EAE), PBMCs = peripheral blood mononuclear cells, CSF = cerebrospinal fluid, MS = multiple sclerosis, NAWM = Normal appearing white matter.
miR-155, a master regulator of neurotoxic microglia. miR-155 directly represses anti-inflammatory genes including suppressor of cytokine signalling 1 (SOCS1), SMAD2 and C/EBPβ which leads directly to the production of pro-inflammatory cytokines and reactive oxidative species including TNF-α and inducible nitrogen synthase (iNOS) (Louafi et al., 2010; Cardoso et al., 2012; Moore et al., 2013). miR-155 targeting of SOCS1 inhibits negative feedback of pro-inflammatory JAK/STAT signaling (Figure 3). Dysregulation of JAK/STAT signaling pathways is a significant contributor to EAE pathology (Li et al., 2014). Ectopic overexpression of miR-155 in microglia induces reactive gliosis and perturbs neurogenesis, while miR-155 knockdown suppresses production of reactive NO and its radicals and rescues the microglial BV2 cell line from LPS-induced damage (Cardoso et al., 2012; Woodbury et al., 2015; Yin et al., 2017). Inhibition of miR-155 also reduces EAE progression, suppressing both T helper cell differentiation and production of TNF-α, which are pro-inflammatory processes normally mediated by activated microglia (Zhang et al., 2014a). In addition, miR-155 is significantly upregulated following cuprizone mediated demyelination (Han et al., 2020). In MS, miR-155 expression consistently upregulated in both peripheral circulation and CNS lesions (Junker et al., 2009; Paraboschi et al., 2011; Waschbisch et al., 2011; Moore et al., 2013; Zhang et al., 2014a; Piket et al., 2019; Fritsche et al., 2020). Moore et al. (2013) showed that miR-155 directly activates primary human microglia in vitro and identified upregulation of miR-155 expression in microglia isolated from active demyelinating MS lesions (Moore et al., 2013). The prominent role of miR-155 in neurotoxic microglial activation and MS pathogenesis makes it a promising target for therapeutic mitigation of inflammation and neurodegeneration.

miR-124, which appears twofold, involving upregulation of internal Nf-κB signaling and promotion of neurotoxicity via intercellular communication with neurons.

Microglia-Enriched miRNAs That Promote Neuroprotection

Conversely, there are also numerous miRNAs that have a distinct anti-inflammatory effect on microglia. For example, miR-124 plays a pivotal role in maintaining microglial homeostasis and suppressing microglial activation via targeting through multiple signaling pathways. miR-124 represses C/EBPα, CREB1 and PU.1, decreasing TNF-α expression while increasing ARG-1 and IL-10 expression to attenuate the inflammatory response of microglia both in vitro and in vivo (Ponomarev et al., 2011; Yu et al., 2017) (Figure 3). miR-124 is significantly downregulated during EAE, however intravenous administration to restore miR-124 concentrations delays EAE progression and inhibits chronic activation of microglia (Ponomarev et al., 2011). In addition to its crucial role in microglial biology, miR-124 is also strongly enriched in neurons where it promotes neuronal survival and differentiation (Sun et al., 2015). miR-124 is actively shuttled between microglia and neurons via EVs, active transfer of neuronal miR-124 to microglia is proposed to help to maintain quiescence and to inhibit inflammatory activation (Ponomarev et al., 2013). In neurons, miR-124 suppresses AMPA glutamate receptor signaling pathways (Dutta et al., 2013). Interestingly, in a model of hippocampal demyelination in mice, neuronal miR-124 expression correlated positively with myelin loss and neuronal death, suggesting a detrimental role for miR-124 during inflammation (Dutta et al., 2013). Despite a strong anti-inflammatory function in microglia, a reported detrimental function of miR-124 in neurons during demyelination, coupled with active shuttling of miR-124 between the two cell types, complicate the case for therapeutic modulation of this miRNA. Further work will be required to ascertain the exact mechanisms of miR-124 in both microglia and neurons.

Another major regulator of the innate immunity is miR-146a, which is consistently upregulated in animal models and MS patient lesions, serum and CSF of MS patients (Junker et al., 2009; Fenoglio et al., 2011; Lescher et al., 2012; Bergman et al., 2013; Yang et al., 2014; Zhang et al., 2014a; 2017; Munoz-San Martin et al., 2019; Han et al., 2020). miR-146a expression is induced by NF-kβ mediated inflammation and initiates negative feedback of inflammatory microglial activation by targeting key genes (Including TRAF6 and IRAK1) in the NF-kβ and JAK/STAT pathways (Taganov et al., 2006) (Figure 3.). Mice treated with miR-146a mimetics exhibit reduced EAE score progression while miR-146a
deficiency manifests significantly worse disease (Li et al., 2017; Zhang et al., 2019). In addition, Zhou et al. identified a polymorphism within the miR-146a locus associated with increased likelihood of MS conversion and relapse, further supporting a critical role for this miRNA in microglial function and MS pathogenesis (Zhou et al., 2018). Lastly in terms of neuroprotective miRNAs is miR-199b, another promising biomarker of MS progression associated with microglial repair and inhibition of chronic activation. Upregulated in animal models and in MS patient lesions, miR-199b expression in serum is negatively correlated with clinical disability (EDSS) (Bergman et al., 2013; Tripathi et al., 2019; Han et al., 2020). The action of miR-199b is considered...
Microglia-Enriched miRNAs With Ambiguous/Context Dependent Function

While the miRNAs discussed above have discrete pro/anti-inflammatory roles in microglia, there are some that can promote both microglial repair and inflammation, often in a context dependent manner. A good example of this is miR-223, which has strong links to neurodegeneration and is upregulated in human monocytes, serum and brain lesions in MS (Junker et al., 2009; Martinelli-Boneschi et al., 2012; Sondergaard et al., 2013; Baulina et al., 2018; Galloway et al., 2019). miR-223 targets IKK kinase in the NF-κB pathway and master regulators of myeloid development including C/EBPα and NFI-A, therefore regulating aspects of inflammation and microglial proliferation/differentiation (Fazi et al., 2005; Li et al., 2010) (Figure 3.). However, its specific role in MS progression is unclear, with conflicting data reported in the EAE model in mice. Galloway et al. (2019) reported reduced microglial-mediated myelin debris clearance and increased remyelination in miR-223 knockout mice, defining this miRNA as protective. Conversely, other studies have implicated miR-223 as pathogenic in EAE, where its knockdown disrupts macrophage/microglial proliferation and impairs pathogenic T cell activation (Ifergan et al., 2016; Cantoni et al., 2017; Li et al., 2019b).

Conflicting evidence for the function of miR-223 in microglia could be due to biological and technical variability of animal models, peripheral biomarkers and tissue samples. Alternatively, it is possible that this molecule serves multiple functions that are cell and/or environment dependent. Incomplete data on miRNA expression across cell types hampers our current understanding of miRNA function. While correlation of study results provides a means to identify putative targets that are most likely important in MS progression, much remains to be determined regarding the specific biology of beneficial and detrimental miRNAs regulating microglia in disease pathology. What is becoming increasingly clear is that miRNAs regulate critical microglial inflammatory signaling pathways and transcriptional networks (Figure 3.). Furthermore, key miRNAs can regulate multiple targets within these cascades, and in some cases such as miR-155 target proteins in several pathways simultaneously, highlighting the significant impact that miRNAs can have on major cell functions (Figure 3.). Further interrogation of the roles of miRNAs enriched in microglia and the specific inflammatory pathways they regulate will be crucial for defining miRNA biology and prioritising targets for therapeutic intervention.

Emerging evidence from animal models, biomarker studies and MS patient lesions provides a strong framework for identifying relevant miRNAs in microglia. Although doubtlessly informative, it will be important to sequence miRNAs from purified microglia to confirm expression and understand the function of dysregulated miRNAs in CNS disease. Very few studies have directly assessed microRNA expression in microglia isolated from human CNS tissue. This will be particularly important in MS which is a uniquely human disease with specific autoimmune pathology. Determining the miRNAome of microglia isolated from active MS pathology will be an invaluable tool in the development of miRNA therapeutics for the modulation of microglia and thus of neurodegeneration.

Concluding Remarks

miRNAs have gained considerable importance in both the pathogenesis of MS and the immune function of microglia. Appropriate microglial responses to inflammation are dependent upon rapid shifts in transcription to express critical genes that inhibit pathogenic processes. In diseases such as MS this process often fails; microglia remain chronically activated and exacerbate neuroinflammation. In addition, mounting evidence shows that key microglia-enriched miRNAs are also implicated in MS onset and pathogenesis. Elucidating specific miRNA regulatory networks in microglia could inform new strategies to influence inflammation and repair in MS. Identifying regulatory feedback loops comprising of enriched miRNAs and master transcription factors will enhance our understanding of the critical transcriptional events that polarise microglia to either pro- or anti-inflammatory function. Expanding beyond endogenous miRNA expression, the role of circulating miRNAs (often packaged in EVs) can exert a major influence on inflammatory microenvironments and microglial function. Bidirectional shuttling of select miRNAs occurs between microglia and other cell types, including neurons and oligodendrocytes to directly impact cell function. Our knowledge of the extent and complexity of miRNA regulation on microglia and its impact on the CNS environment in both health and disease is incomplete. Focused studies in this area will be invaluable for informing novel therapeutics in microglial biology and neurodegenerative conditions such as MS.

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Author Contributions
A. D. W and M. B. conceived and planned manuscript. L. T. N. contributed to the conception and illustration of Figures. A. D. W. wrote the manuscript in consultation with M. B. and L. T. N.

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