Astrocyte MiRNA profiling in Multiple Sclerosis and normal Human Brains

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory, neurodegenerative demyelinating disease of the central nervous system (CNS). The conventional role of astrocytes in MS is the formation of glial scar in the later stage of the pathology, but their interaction with other glial and immune cells as well as their involvement in the lesion development in the early pathology are also recognized nowadays. In our study, we measured the expression of a set of miRNAs (microRNAs) related to multiple astrocyte functions in astrocytes from brain sections of MS patients and compared them with those from normal controls. We found a differential expression of miRNAs between MS patients and normal controls. An upregulation of ischemic-related miRNAs in active lesions from white matter and a low expression of inflammatory-related miRNAs in MS lesions were observed. MiRNAs thought to be involved in neuroprotection and in regulation of the blood brain barrier were also altered. The overall miRNA expression also showed a tendency for several miRNAs to return to normal levels in inactive lesions. In addition, we demonstrated the long term effect of interleukin-1β (IL-1β) on miRNA expression in primary human fetal astrocytes. In summary, these findings may help us illustrate the roles of astrocytes in MS and how they respond to injury and inflammation.

Résumé

La sclérose en plaque (SP) est une maladie inflammatoire chronique, neurodégénérative et liée à une perte de gaines de myéline dans le système nerveux central (SNC). Les astrocytes jouent un rôle conventionnel dans la SP en étant impliqués dans la formation de la cicatrice gliale durant les dernières étapes de la pathologie. De plus, les astrocytes interagissent non seulement avec leur environnement glial mais aussi avec les cellules du système immunitaire et leur implication dans le développement des lésions lors des premiers stades de la maladie est reconnue par l'ensemble de la communauté scientifique. Dans notre étude, nous avons mesuré l'expression d'un ensemble de microARNs (miARNs) qui sont reliés aux fonctions essentielles des astrocytes dans des coupes de cerveaux de patients SP et avons comparé ces résultats aux cerveaux de patients contrôles. Nous avons pu mettre en évidence une différence dans l'expression de miARNs entre ces deux groupes. En effet, nous avons démontré une augmentation de l'expression de miARNs, associés à une ischémie, dans les lésions actives de la matière blanche et une faible expression de miARNs inflammatoires dans les lésions SP. Les miARNs apparentés à la neuroprotection et à la régulation de la barrière hémato-encéphalique ont aussi été altérés. Certaines expressions miARNs ont démontré une tendance au retour à un niveau normal dans les régions des lésions inactives. De plus, nous avons pu mettre en évidence l'effet à long terme de l'interleukine-1β (IL-1β) sur l'expression des miARNs dans les astrocytes primaire de fœtus humains. En conclusion, nos observations nous apportent une meilleure illustration du rôle des astrocytes dans la SP et de leur réponse en situations de dommages et inflammation.

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Chapter 1. Introduction and Literature review

Astrocyte function

In 1870s, Camillio Golgi described a type of neuroglial cells with staining and drawings of their star-like processes, hence the name astrocytes as we refer to them today (Parpura and Verkhratsky, 2012). Astrocytes show morphological differences between different regions. Based on their morphology, astrocytes can be categorized as in two main types: Protoplasmic and fibrous by Kölliker and Andriezen (Kettenmann and Verkhratsky, 2008). Protoplasmic astrocytes can be found in gray matter with many ramified branches, while fibrous astrocytes in the white matter have longer and thinner processes. Moreover, astrocytes also show distinct morphological differences across different species (Oberheim et al., 2009). In physiological conditions, astrocytes have a supportive role. They can store glycogen, provide energy to neurons, and clear excess glutamate in synaptic cleft to prevent excitotoxicity (Brown and Ransom, 2007; Coulter and Eid, 2012; Mangia et al., 2011). They also support the survival of other surrounding cells such as neurons and oligodendrocytes by expressing factors such as brain-derived neurotrophic factor (BDNF) (Dreyfus et al., 1999) and platelet derived growth factor (PDGF) (Gard et al., 1995). Furthermore, astrocytes contribute to the regulation and maintenance of blood brain barrier (BBB), which prevents larger molecules from the circulation traveling into the brain but allowing smaller molecules to freely pass in. Astrocytes express water channel proteins such as aquaporin 4 (AQP4) that plays a role in balancing water and iconicity (Verkman, 2002). Overall, these functions of astrocytes help maintain the homeostasis of the local environment and proper neural transmission in healthy condition, but astrocytes also play important roles in the context of injury and inflammation.

Astrocyte function in injury and inflammation

Under inflammatory conditions, astrocytes secrete various chemokines and cytokines. Astrocytes and the secreted molecules participate in regulation of immune responses and the

eventual glial scar formation, which may either facilitate the repair process or worsen the injury. Astrocytes respond to pro-inflammatory signals such as IL-1β, tumor necrosis factor-α (TNF-α), IL-17, and many other secreted molecules are involved in the responses to these injury and infections (Burns et al., 2012).

IL-1β is a pro-inflammatory cytokine, which not only signals astrocytes, but also produced by astrocytes. It can trigger hypoxia inducible factor-1α (HIF-1α) and other chemokines production such as C-C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine ligand 2 (CXCL2) to increase BBB permeability for infiltrating immune cells (Ambrosini et al., 2003; Argaw et al., 2006; Rothhammer and Quintana, 2015; Wang et al., 2014). TNF-α, in addition to its pro-inflammatory main role as a pro-inflammatory cytokine, also inhibits glutamate transporters on astrocytes (Bezzi et al., 2001; Olmos and Llado, 2014; Sitcheran et al., 2005). IL-17 binds to its receptor and recruits the adaptor CIKS (connection to IκB kinase and stress-activated protein kinases, also known as Act1), eventually leading to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway in astrocytes (Kang et al., 2012; Waisman et al., 2015). CIKS-deficient astrocytes show an impairment of IL-17 mediated gene induction (Kang et al., 2010). The same group has studied mice lacking CIKS in neuroectoderm-derived cells (of which astrocytes being the main type of cells responsive to IL-17) and observed a reduction in experimental autoimmune encephalomyelitis (EAE) severity. EAE is a demyelination animal model for MS. A similar result has been shown with mice deficient in either IL-17A, IL-17 receptor, or the adaptor CIKS (Kang et al., 2012).

Astrocytes also impact other cells by secreting factors (trophic or otherwise). For example, in response to inflammatory signals, astrocytes release vascular endothelial growth factor (VEGF), which increases BBB permeability (Argaw et al., 2012). Activated astrocytes undergo reactive astrogliosis; several types of conditions can lead to reactive astrogliosis, such as mechanical injury, ischemia, and inflammation (de Pablo et al., 2013; Wanner et al.,

2013). Depending on the environment, activated astrocytes can produce a spectrum of cytokines including IL-1, IL-6, TNF-α, and tumor growth factor-β (TGF-β) (da Cunha et al., 1993; Sterka et al., 2006; Van Wagoner et al., 1999).

Astrocytes may return to their normal state if the injury does not persist; however, if the injury state worsens, astrocytes will proliferate and form a physical barrier known as a glial scar in order to contend with the injury, preventing a massive infiltration of lymphocytes which may further damage the tissue (Faulkner et al., 2004). On the other hand, if this persists, the glial scar formed around the inflammation area will hinder axon regeneration; hence, it remains to be understood if glial scarring is beneficial or detrimental in a context-specific manner (Sofroniew, 2009). Reactive astrocytes undergo several morphological and functional changes including hypertrophy, which is an enlargement of cells characterized by the upregulation of glial fibrillary acidic protein (GFAP), a molecular marker of astrocytes (Wilhelmsson et al., 2006).

Astrocyte markers and heterogeneity

Among all the available astrocyte markers, the most commonly used marker is GFAP. GFAP is a type of intermediate filament that helps maintain the shape and structure of astrocytes (Gilbert, 2008). GFAP expression can be significantly upregulated in astrocytes upon activation in MS or Alzheimer's disease, while the disadvantage of using GFAP as the marker is that it is less effective for protoplasmic astrocytes (Eng, 1985). Despite that, it still can be readily detected in human protoplasmic astrocytes, and is considered the most widely used astrocyte marker to date (Oberheim et al., 2009).

Some of the other common astrocyte markers include nuclear factor 1 A-type (NF1A), glutamate aspartate transporter (GLAST), aldehyde dehydrogenase 1 family member L1 (ALDH1L1), connexin 43 (CX43), S100 calcium-binding protein B (S100β), CD44, aldolase C, and glutamate transporter 1 (GLT1), in order of their appearance during development (Roybon et al., 2013). Astrocytes derived from mouse embryonic stem cells (mESC) express

markers including GFAP, AQP4, S100β, vimentin, and NF1A, but the coexpression of these markers are not commonly observed, which suggests the heterogeneity of the astrocyte population (Roybon et al., 2013). Astrocyte differentiation can be induced with fibroblast growth factors (FGFs), and these mESC-derived astrocytes express maturation markers including glutamate transporters GLT1 and GLAST, aldolase C, CX43, and ALDH1L1 with a decreased expression of GFAP under FGF1 treatment.

In addition to the two most well known types: Protoplasmic and fibrous astrocytes, there are other subpopulations of astrocytes, such as interlaminar astrocytes and varicose projection astrocytes (Oberheim et al., 2009). Astrocytes from different regions have been shown to have distinct gene expression profiles (Yeh et al., 2009). Moreover, there is a difference of astrocyte markers expression across species and developing periods. Because of the heterogeneity of astrocytes, there is a need to identify a more comprehensive molecular marker of astrocytes and / or for a specific subpopulation. Although many other markers have been proposed since the discovery of GFAP, a marker which identifies all astrocyte subpopulations and not other cell types is yet to be discovered. Further investigation of the subpopulation of astrocytes and their molecular profiles can help us better understand the important functions of astrocytes in different regions, developmental periods, and context of diseases such as MS.

Astrocytes in MS

Astrocytes were considered to be passive responders in the immune responses, and their roles in MS pathology have gained attention with the discovery of their direct involvement in neuromyelitis optica (NMO). NMO, also known as Devic’s disease, was once considered as a variant of MS. One of the characteristics of NMO is the presence of antibodies against the water channel protein AQP4 in astrocytes (Lennon et al., 2004). In MS, astrocytes play a major role in the development of lesions by secreting molecules that regulate immune responses. In MS and EAE, astrocytes respond by secreting various cytokines that are

involved in the pathology, such as interferon-γ (IFN-γ), IL-1β, IL-6, and IL-10. IFNγ receptor KO mice show more severe EAE with increased axonal loss and demyelination through elevated production of pro-inflammatory mediators such as IL-1, IL-6 and TNF (Hindinger et al., 2012). The pro-inflammatory IL-1β induces NF-κB dependent CCL2, CCL20, and CXCL2 and activates HIF-1α, which increases BBB permeability (Argaw et al., 2006; Li et al., 2014). In addition, astrocytes also produce IL-1β themselves when stimulated, providing a feed-forward mechanism for sustained inflammatory state; thus, immunomodulatory drugs such as FTY720 and resveratrol are applied in EAE to inhibit IL-1β production (Wight et al., 2012; Wu et al., 2013). In addition, mice deficient in another pro-inflammatory cytokine, IL-6, are more resistant to EAE due to an impaired priming phase for T cell response that may lead to a detrimental effect in EAE, while the EAE susceptibility is restored in transgenic mice that are globally IL-6 deficient with overexpressed IL-6 under GFAP promoter (Campbell et al., 1993; Eugster et al., 1998). IL-10, an anti-inflammatory cytokine, downregulates NF-κB pathway and the induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in astrocytes from Theiler's Murine Encephalomyelitis Virus (TMEV) demyelination mice model (Molina-Holgado et al., 2002a; Molina-Holgado et al., 2002b). In addition to the previously mentioned cytokines, cell stress response such as upregulation of heat shock protein 60 (Hsp60) is also observed in human astrocytes treated with IL-10 (Bajramovic et al., 2000). Although not fully understood, it is thought that astrocytes may negatively contribute to the MS pathology.

Cytokine and channel protein expression of astrocytes in MS and EAE

Molecular readouts of astrocytic expression of channel proteins and cytokines in MS can give us insights into the differential gene expression and potentially help us unveil the MS pathogenesis and diagnostics. For example, AQP immunoreactivity may be used to distinguish NMO and MS. A study with a cohort of 20 active demyelinating lesions shows loss of AQP4 in 18 out of 20 biopsy MS samples and an increase in two samples (Popescu et

al., 2015). The two cases with increased AQP4 were later identified as MS, while for those with lost AQP immunoreactivity, all the cases with available serum were later shown positive for AQP4-IgG, suggesting a diagnostic method for NMO. In addition, immunohistochemistry studies have shown increased expression of various cytokines in astrocytes from MS brains, including IL-15 (Saikali et al., 2010), IL-27 (Lalive et al., 2017), IL-22 and IL-22 receptor 1 (Perriard et al., 2015), and complement components (Ingram et al., 2014), while some molecules with decreased expression include the potassium channel KIR4.1 (Schirmer et al., 2014). In an EAE mice model, astrocytes upregulate the sodium channel Nav1.5, which is related to gliosis (Pappalardo et al., 2014). Furthermore, transcriptomic studies in MS and EAE have also been performed by several groups. Studies have utilized laser capture micro-dissection (LCM) to determine the molecular profile of astrocytes. LCM-captured white matter astrocytes in spinal cords from EAE rats show that transcripts of key inflammatory regulators IL-1β and TNFα were detected either in only some samples or not upregulated (Guillot et al., 2015). Astrocytes in normal appearing white matter from MS patients showed upregulation of genes associated with iron homeostasis and oxidative stress, suggesting a neuroprotective role of these astrocytes (Waller et al., 2016). In addition to these immunohistochemistry and transcriptomic studies, miRNA profiling studies have also been performed in MS to better understand MS and in search for potential biomarkers of MS.

MiRNA functions and MiRNA in astrocytes

MiRNAs are involved in regulation of important astrocyte functions. They were first reported in *C. elegans* but only considered as a distinct class of gene regulators later (Lee et al., 1993; Reinhart et al., 2000). They are small non-coding RNAs that regulate gene expression post-transcriptionally, with the length of around 22 nucleotides (Ha and Kim, 2014). In RNA silencing, miRNAs usually bind to the 3’ untranslated region (UTR) of the target (Bartel, 2009). MiRNAs can be loaded to the Argonaute protein, forming a complex that pairs up with the target to repress the target expression or lead to degradation of the

target mRNA (Hutvagner and Zamore, 2002; Yekta et al., 2004).

In addition to its regular function of post-transcriptional regulation of gene expression, miRNAs can also bind to Toll-like receptors (TLRs), such as miR-21 and miR-29a, acting as a TLR ligand (Fabbri et al., 2012). MiRNAs can be transported through vesicles such as exosomes and microvesicles or travel in a vesicle-free manner associated with other proteins, and miRNAs regulating important functions in astrocytes may not necessarily be endogenous. For instance, neuron-to-astrocyte exosomal transfer of miR-124a regulates GLT1 expression (Morel et al., 2013). Astrocytes also secrete exosomes to regulate gene expression in other cells, as there is an astrocyte-to-neuron exosomal transfer of miR-26a, which is dysregulated in neurological disorders such as depression and Alzheimer's disease (Lafourcade et al., 2016). The crosstalk between astrocytes and neurons show how closely they are associated and that astrocytes are an essential component in proper neuronal functions.

Some miRNAs have shown differential expression across different cell types and developing periods in the brain. For example, miR-26 and miR-29 are more highly expressed in astrocytes compared to neurons from embryonic mouse brain (Smirnova et al., 2005). In addition, a comparative miRNAs expressions study in rat astrocytes, neurons, oligodendrocyte, and microglia, shows that miR-143 and miR-146a are specifically enriched in astrocytes (Jovicic et al., 2013). While little is known about the *in situ* miRNA profiles of different types of astrocyte, our group has previously performed comparative studies of miRNA expression from different brain regions in which astrocytes are involved still provide insightful remarks to the studies on astrocyte functions.

MiRNA profiling in MS

MiRNAs profiling studies have been done in MS and EAE, including those from circulating miRNAs and those with miRNAs from white matter lesion, either from a selection of mixed cell types or homogenized tissue, but very few studies have been done for astrocytic miRNAs alone (Junker et al., 2009; Lescher et al., 2012; Siegel et al., 2012). The majority of

the miRNA profiling studies in MS have utilized cerebral spinal fluid (CSF), serum, and blood. Although fewer miRNA profiling studies of MS and EAE have been performed with the brain lesions, in situ, studies such as Junker et al. on MS white matter lesions (Junker et al., 2009), Lescher et al. on both MS and EAE lesions (Lescher et al., 2012), and Noorbakhsh et al. on normal-appearing white matter (NAWM) of MS patients (Noorbakhsh et al., 2011) exist. Many miRNA profiling studies have been done in MS, either at tissue level or cell level, including blood (Yang et al., 2017), serum (Niwald et al., 2017), serum exosome (Selmaj et al., 2017), CSF (Haghikia et al., 2012), peripheral blood mononuclear cell (PBMC) (Mameli et al., 2016), T cells (Sheng et al., 2015) and B cells (Aung et al., 2015). Some miRNAs have been shown as biomarkers of MS. For example, miR-145 has been identified as a marker of MS in the serum from relapsing-remitting MS (RRMS) patients (Edwards et al., 2010). There are also studies investigating the relationship of differentially expressed miRNAs and mRNAs in MS (Sheng et al., 2015; Yang et al., 2017). Furthermore, some miRNAs are linked with disease progression and are expressed differently in serum between different stages of the disease. While only 15% of MS patients have a progressive disease course (primary progressive MS, PPMS), most patients begin with RRMS, and among these patients, more than half develop secondary progressive MS (SPMS) (Antel et al., 2012). Between different disease courses, several miRNAs have been shown to have differential expression, including miR-27a between progressive MS and RRMS (Regev et al., 2016) and miR-572 between SPMS and RRMS versus PPMS (Mancuso et al., 2015). Drugs and treatments have been reported to alter the expression of miRNAs in MS patients, including members of miR-29 family in PBMC after treatment with interferon-β (IFN-β) (Hecker et al., 2013), miR-20a and -145 in blood after IFN-β treatment (Ehtesham et al., 2017), and miR-26a and -155 in PBMC after natalizumab treatment (Mameli et al., 2016). Comparisons between NMO and MS miRNA profiles have also been done, though the difference was found insufficient to differentiate the two (Chen et al., 2017). In summary, many miRNA profiling studies have

been performed on blood or CSF from MS patients, while fewer *in situ* studies from either brain tissue or cells, especially from astrocytes, have been reported. Further investigations on MS miRNA profiling will help illustrate how miRNAs are involved in gene regulation in MS.

Expression of miRNAs in astrocytes in injury, inflammation and disease

MiRNAs have been shown to regulate many functions in astrocytes, especially in injury and inflammation responses. For example, some miRNAs such as miR-146a and miR-155 are known to have critical roles in inflammation (Iyer et al., 2012; Tarassishin et al., 2011). Similar to miR-146a, miR-147b also inhibits IL-6 and COX-2 release in human fetal astrocyte culture with IL-1β treatment (van Scheppingen et al., 2018). In addition, miR-21 is upregulated during spinal cord injury (SCI) and has a role in the hypertrophic response of the astrocytes (Bhalala et al., 2012). Downregulation of miR-21 in murine postnatal astrocytes has been shown to be associated with a decrease in GFAP expression (Sahni et al., 2010).

In the context of infections and neuropathies other than MS, differential miRNA expression profiles have been shown by many groups. For example, there is a dysregulation of miRNA expression in astrocytes with Zika virus infection (Kozak et al., 2017). In a pentylenetetrazol-induced rat model of epilepsy, Saikosporin (SSa) treatment is found to be beneficial. SSa induces miR-155 expression through AP-1, and miR-155 targets GLAST to regulate glutamate uptake in hippocampal astrocytes (Gao et al., 2017). Upregulation of miR-155 in an Alzheimer's disease murine model and in astrocyte culture with amyloid beta (Aβ) stimulation shows that through repressing its target suppressor of cytokine signaling 1 (SOCS1), and miR-155 can contribute to the production of IL-6 and IFN-β (Guedes et al., 2014). Using NeAL218, which includes miR-218 and 3 transcription factors (NEUROD1, ASCL1, and LMX1A), human astrocytes *in vitro* and mouse astrocytes of a Parkinson's disease model *in vivo* can be converted to induced dopamine neurons for potential therapeutic applications (Rivetti di Val Cervo et al., 2017). Furthermore, miRNAs are also involved in glioma cell proliferation. In glioma cells, miR-219 is downregulated compared to normal

astrocytes and negatively regulates Sal-like protein 4 (SALL4) to act as a tumor suppressor (Jiang et al., 2017). MiR-181b also targets SALL4 in glioma cells and inhibits cell proliferation (Zhou et al., 2017). As of date, thousands of miRNAs have been discovered, and the presence of many has been confirmed in astrocytes, but their targets and biological significances remain to be elucidated.

Project objective and Rationale

MiRNAs are involved in regulation of many important functions of astrocytes, and we are interested in how astrocytes respond in the context of MS lesion developmental pathology, particularly during injury and inflammation. We have previously shown a differential expression of miRNAs between anatomical regions and developmental time points in astrocytes (Rao et al., 2016). In this study, we performed miRNA profiling from astrocytes in different MS lesions (*in situ*) and primary fetal astrocytes (*in vitro*) in an inflammatory state treated with IL-1β. We think that our study provides insights as to how astrocytes respond in MS pathology and how they react and participate in injury and inflammation.

Connecting statement 1 (Chapters 1-2)

The following chapter comprises the main component of the Master's thesis project. The manuscript draft is currently being prepared for journal submission.

Chapter 2. Manuscript:

Astrocyte MiRNA Profiling in Multiple Sclerosis and Normal Human Brains

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Abstract

Multiple sclerosis (MS) is a demyelinating neurodegenerative disease. Although for a long time, astrocytes were not considered central to the pathogenesis of MS, their roles in MS have gained attention in the recent years. We investigated the expression of a selected microRNA (miRNA) panel in astrocytes in situ from brain sections of MS patients using laser capture microdissection. Our findings showed an upregulation of ischemic-related miRNAs in active lesions from white matter. Other miRNAs considered to be mediators of neuroprotection and changes in the blood brain barrier, were also altered. The lower expression of the two prominent inflammatory-related miRNAs, miR-155 and miR-146a, in astrocytes from MS patients suggests a less prominent role for these miRNAs in astrocytes in the context of MS pathology. The overall miRNA expression profile showed a differential miRNA expression between normal controls and MS samples. In addition, we found expression differences between different lesion pathologies. In summary, these findings will

help us illustrate the roles of astrocytes in MS and how they respond to injury

Introduction

Multiple sclerosis (MS) is a complex inflammatory demyelinating disease with accompanied axonal damage. Although the exact etiology is uncertain, there is a striking immune component operating on a genetic basis, and involving numerous environmental factors (Belbasis et al., 2015; Hillert, 2010). MS usually presents as a relapsing-remitting disease which gradually becomes progressive, though the pathogenetic mechanisms of this switch remain unclear. The lesions demonstrate many elements, such as oligodendrocyte degeneration and demyelination, immune infiltration by lymphocytes and monocytes, alterations in the blood-brain barrier, and reactive astrogliosis or glial scarring. Attempts at repair with remyelination are frequently present (Chari, 2007; Plemel et al., 2017). Although in the past, and continuing today, most pathogenetic studies have centered on the immune system, in recent years the role of the macrophages has become a focus of investigation. For a long time the astrocyte was considered to be a cell reacting non-specifically to damage and causing the gliotic scar, it has become clear that these cells play a major role in the complex interaction of glial and immune cells that lead to damage and possible repair.

Astrocytes are the most abundant cell type of the central nervous system (CNS). They perform numerous physiological roles and react during pathological conditions. In a healthy brain, they play a supportive role by secreting trophic, pro- and anti-inflammatory molecules, hosting glycogen stores, supplying lactate to neurons, and protecting cells in the milieu from excitotoxic stress through glutamate uptake (Brown and Ransom, 2007; Coulter and Eid, 2012; Mangia et al., 2011; Sofroniew, 2009). Besides these homeostatic functions, these cells also form and maintain the blood-brain barrier (BBB) and support neurogenesis and angiogenesis (Jensen et al., 2013; Sofroniew, 2009). Following injury of any kind, including inflammatory conditions, astrocytes become reactive, secrete chemokines and / or cytokines and proliferate to promote astrogliosis (Dong and Benveniste, 2001; Jensen et al., 2013).

Astrogliosis may be beneficial or detrimental for tissue repair (Faulkner et al., 2004; Sofroniew, 2009).

In MS, astrocytes have long been known to have important roles during and after lesion development. They secrete molecules that regulate immune responses to protect cells and tissues from damage including trophic, pro- and anti-inflammatory molecules. In the context of MS and experimental autoimmune encephalomyelitis (EAE), astrocytes secrete cytokines such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-6, and IL-10 (Rothhammer and Quintana, 2015). They also form glial scars and assist in the overall tissue repair process (Brosnan and Raine, 2013; Ludwin et al., 2016). The importance of these cells in the generation of the MS lesions has also been emphasized with the seminal discovery of their direct involvement in neuromyelitis optica (NMO). NMO is associated with antibodies against the water channel protein aquaporin 4 (AQP4) in astrocytes (Lennon et al., 2004), and the role of AQPs can be deemed to be important in the context of MS pathology.

Astrocyte morphology does reflect to some degree the state of inflammation and injury during MS lesion development. MS lesions have been categorized in many ways. Commonly, they are referred to as acute or active, chronic active, and chronic inactive, based on microglial and macrophage activity, astrocyte morphology, the degree of ongoing demyelination, reactivity of lymphocyte and other immune cells (Ludwin et al., 2016). A more recent classification has changed the categorization into active, mixed active / inactive and inactive lesions by doing away the terms acute and chronic, which often refer to timelines and clinical progression, which cannot be measured pathologically (Kuhlmann et al., 2017). Astrocytes in active lesions can be described as hypertrophic, displaying swollen cytoplasm and processes. In contrast, cells in the inactive lesion have smaller cell body, with thickened processes aligned in a parallel orientation, which constitutes glial scar. In mixed active / inactive lesions, there is an inactive center while the rim is relatively active, with signs such as inflammatory cellular infiltration, by macrophages and microglia and lymphocytes,

phagocytosis, and active demyelination. Regional differences (white vs. gray matter) have been commonly observed in the quality and quantity of inflammatory responses of many cell types including those of astrocytes. “In the demyelinative sub-pial, intra-cortical and cortical component of leukocortical lesions, there is also reactive astrocytosis, although mirroring the inflammatory infiltrate, the reaction is far more muted than in the corresponding white matter” (Ludwin et al., 2016).

MicroRNAs (miRNAs) are small, non-coding RNAs that post-transcriptionally regulate gene expression. MiRNAs are first synthesized as long primary transcripts and then cleaved by the enzyme Dicer to form mature miRNAs (Hutvagner and Zamore, 2002). They promote mRNA degradation by their incorporation into the RNA-induced silencing complex (RISC), a RNA-protein complex which mediates mRNA degradation, or represses the translation of a target gene by binding to its corresponding mRNA (Hutvagner and Zamore, 2002). MiRNAs have been shown to regulate many functions in astrocytes, covering both constitutive and inflammatory functions. While there are many reports on astrocyte miRNA expression profiles from body fluids, brain tissue and cell culture analyses in the context of MS pathology, there are very few *in situ* studies, especially ones based on specifically identified single cells. Further, not many studies have investigated miRNAs in adult human brain derived astrocytes. We have previously shown a regional variation of miRNAs expression profiles between white and gray matter astrocytes (Rao et al., 2016). In the present study, we have used white and gray matter astrocytes, specifically identified by glial fibrillary acidic protein (GFAP)-immunostaining and individually laser-captured from different pathologically-classified MS lesions and normal control brains. We used the same miRNA panel as in the previous study; these miRNAs had been specifically chosen as we were interested in miRNAs that could be implicated in damage and repair, and weighted toward immune and inflammatory disease (Rao et al., 2016). It should be noted that although hundreds of miRNAs have been identified, the function of about half of these remains

unknown. The miRNAs were selected based on the following criteria:

1. MiRNAs that are upregulated in ischemia

2. MiRNAs that are known to be involved in immune and inflammation functions

3. MiRNAs that are highly expressed in astrocytes or enriched in astrocytes compared to other cell types in brain

4. MiRNAs that regulate AQPs

5. MiRNAs that have characterized functions in astrocytes, such as those involved in glutamate transport or related to apoptosis

Table 1. shows the diverse functions relevant to astrocytes that have been ascribed to the studied miRNAs.

There are two main goals of the study. The first is to compare the changes in miRNA expression in different areas and stages during the course of MS. We believe that this will help us understand how astrocytes respond to injury and how they interact with other cells in MS pathogenesis. Secondly, we also want to understand how the astrocyte, a cell with multiple roles, reacts to injury conditions. Are most of the functions upregulated or only a few essential ones? Are some downregulated, perhaps in order to maintain the essential functions during stress condition?

Materials and Methods

Immunohistochemistry and laser capture micro-dissection (LCM)

Human brain tissue sections from patient autopsies and biopsies were obtained from the archives of the Montreal Neurological Institute, Queen's University, St.Michael's Hospital (University of Toronto), and the Ottawa Hospital, and following ethical approval from the relevant hospitals and the Chief Coroner of Ontario. The overall study was approved by the Ethics Committee of McGill University. For each category in white matter, there were 8, 3, 7, and 6 cases for active lesions, mixed lesions, inactive lesions, and normal controls. For each

category in gray matter, there were 5, 5, and 4 cases for active lesions, inactive lesions, and normal controls. Mixed lesions in the gray matter were less commonly found in our patient cohort and were not included in the study. For each patient, multiple blocks of brain sections were used in the study where appropriate.

Formalin-fixed paraffin-embedded (FFPE) tissue sections from both MS patients and normal controls were studied as described previously (Rao et al., 2016). Sections were GFAP-immunostained with an auto-stainer (Ventana cat # 760-4345, clone #EPG724, Cell Marque, M). Secondary antibody conjugated to 3,3'-diaminobenzidine (DAB) were used for cell visualization. Each sample was composed of a capture of about 30-40 $GFAP^+$ cells with PALM-LCM system (Carl Zeiss). Captures across samples were carefully normalized for both the laser power and capture area. Collected samples were immediately lysed in Trizol® and stored at −80°C until further processing. For lesion categorization, additional guide slides with sections stained with Haematoxylin-eosin with luxol fast blue and anti-myelin basic protein (MBP) to identify demyelination, and anti-CD68 (for myeloid cells) were also used. Lesions were categorized by the Kuhlmann method (Kuhlmann et al., 2017), based on the criteria described in Table 2. These features are illustrated in Fig. 1. We have included three lesion categories for the white matter (active, mixed active / inactive and inactive) and two categories for the gray matter (active and inactive). In the gray matter, there were insufficient areas of inactive demyelination with an active rim to allow for meaningful comparison for mixed lesions.

To show the presence of downstream effects of ischemia-related miRNAs, the images for astrocytic expression of HIF (Hypoxia Inducing Factor) and VEGF (Vascular Endothelial Growth Factor) shown here, were taken from previous studies on active MS lesions performed by one of the authors (SKL): HIF (unpublished data), VEGF (Ludwin, 2006).

RNA Isolation, Preamplification Polymerase Chain Reaction (PCR), Quantitative PCR (qPCR), and Statistical Analyses

Total RNA was extracted from astrocytes using standard Trizol® protocols, followed by DNase treatment (Qiagen, Valencia, CA). Multiplexed reverse transcription (RT) reactions were carried with a mix of miRNA-specific RT primers and a TaqMan® MicroRNA RT kit. The resulted cDNAs were subjected to a preamplification reaction using a pool of TaqMan® miRNA-specific probes to increase the number of target copies, as previously described by our group and in other studies (Li et al., 2008; Moore et al., 2013; Rao et al., 2016). Expression assays for each miRNA were performed using specific miRNA TaqMan probes. The expressions of each miRNA were compared to the expression U47, a stable human small nuclear RNA (snRNA), to calculate the relative expressions. Analysis of miRNA expressions was performed using Δ cycle time (CT) values. Analysis of variance (ANOVA) is a commonly used statistical analysis in the field of bioscience. However, it relies on several assumptions that may not be applicable to our dataset. When experiments are performed, multiple data collections may be performed across samples. ANOVA assumes that each data point are independent and do not correlate with each other, which does not fit in the case described previously. Therefore, we have chosen the GLMM, which takes into account the repeated measurement of technical and biological replicates that are not covered in the rather simple ANOVA.

Data was analyzed using a general linear mixed model (GLMM) with fixed effects for different test groups and random effect for technical replicates and biological replicates when applicable. The GLMM method takes advantage of data from multiple technical replicates while correctly allowing for correlation among repeated measures. Overall fit of the model was evaluated using a Chi-squared test, and individual effects with t-tests using Satterthwaite's approximation for the denominator degrees of freedom. The graphs are plotted with GraphPad Prism software (version 5.0), with each point representing the average of two technical replicates for one sample in the scale of -ΔΔCT (DDCT) relative to the controls. Data are presented as mean ± standard error of the mean. Test results with a p

value < 0.05 were considered to have statistical significance.

For a few selected pairs of comparisons with respect to 3 miRNAs: MiR-124a, -125b, and -365, which did not meet the statistical significance in GLMM analyses, we performed simple Student's T-test. This was done as we observed some differential trends under selected comparisons.

Results

MS pathology

A low magnification view of the active plaque showed the interface between the preserved blue myelin and the very cellular demyelinated plaque, with only a hint of myelin or myelin debris (Fig. 1A-1). Within the plaque, there was extensive myelin breakdown debris lying within macrophages with vacuolated cytoplasm (Fig. 1A-2). Large pink hypertrophic reactive astrocytes are seen lying between smaller macrophages with vacuolated cytoplasm often containing blue myelin debris (Fig. 1B-1). GFAP stains demonstrate the hypertrophic astrocytes with large cell bodies and extensive processes (Fig. 1B-2). In Fig. 1C, the mixed plaque was stained with CD68. The inactive center (right) showed only a few stained microglial cells with small curvilinear bodies, and the active rim (left) contained numerous macrophages with large amoeboid cell bodies. In Fig. 1D, there was a hypocellular well-demarcated plaque in the center surrounded by myelinated (blue) tissue of varying intensity. The pink area on the left represented the overlying occipital cortex. Cells with small bodies and scant pink cytoplasm were lying within a fibrillary matrix of cell processes (Fig. 1E-1). These features were well-demonstrated on GFAP staining (Fig. 1E-2).

Astrocyte morphology

Astrocytes immunostained for GFAP in normal appearing white matter and gray matter showed the typical fibrous (Fig. 2A) and protoplasmic (Fig. 2E, F) morphology respectively.

Both white and gray matter astrocytes showed more prominent processes and enlarged cell bodies in active lesions, displaying a reactive and hypertrophic morphology (Fig. 1B, Fig. 2B, F). Astrocytes in mixed white matter lesions depicted relatively finer processes, a premonitory sign serving to indicate the consequential formation of glial scar (Fig. 2C). Astrocytes with less conspicuous cell bodies that have very fine processes made up both inactive white and gray matter lesions (Fig. 1E, Fig. 2D, H). As shown in Fig. 2E and 2F, we captured only the cell body and not the processes using LCM.

Overall summary of miRNA expression in astrocytes captured from white matter and gray matter

The summary of miRNA expression in astrocytes from gray and white matter in MS is illustrated in Tables 3 and 4. MiRNAs: MiR-29a, -99a, 100, -143, -145, -146a, -155, were found to be have lower expression in astrocytes from the three pathology groups of active, mixed and inactive cases compared to controls, while miR-181a had a lower expression only in mixed and inactive lesions compared to control. MiR-365 expression was lower in astrocytes from mixed lesions compared to controls. MiRNAs that were higher in astrocytes from active lesions compared to controls were miR-34a, -124a, -146b, -210, -214, -320, and -449. While comparing the two pathology groups of mixed and inactive lesions to the active ones we found that miR-34a, -124a, -146b, and -449 were lower in the mixed and inactive lesions. MiR-100, -181a, -320, and -365 had higher expression in active lesions compared to mixed lesions, and miR-365 had a higher expression in inactive lesions compared to mixed lesions.

In the gray matter we found that some miRNAs were expressed lower in astrocytes from active lesions compared to both control and inactive lesions. These include miR-29a, -99a, -100, -143, -145, -146a, -146b, -155. In addition, miR-181a was found to have a lower expression in astrocytes from active lesions compared to controls. Only miR-100 was found

to have a lower expression in inactive lesions compared to controls. No significance in the expressions of miR-21 and -129 were found between control and different MS lesions in both gray and white matter (data not shown).

With regards to miRNAs-125b, 124a, and -365, we did not find significant expression differences between any of the conditions when we compared all the conditions together (did not pass the Chi-squared test ($0.05<p<0.1$)). For these three miRNAs, we chose to perform T-tests for selected pathological and control conditions (Fig. 3 and Table 4). MiR-125b was found to be more highly expressed in astrocytes from the active lesions compared to those from the mixed lesions in the white matter (Fig. 3A). MiR-124a was more highly expressed in the inactive lesions compared to the controls in the gray matter (Fig. 3B). Expression of miR-365 was lower in the in the active lesions compared to the controls in the gray matter (Fig. 3C).

Overall, when we compared astrocyte miRNAs expression profiles between controls and inactive MS lesions, we found many miRNAs to be down regulated in the white matter; the same was not found in those from the gray matter. However, between the white matter astrocytes from mixed and inactive lesions, we did not find many miRNAs to be differentially expressed. For the comparison between controls and active lesions, only white matter astrocytes showed upregulation of many miRNAs in our panel while the gray matter astrocytes did not increase the expression of any miRNA in our panel. A summary of miRNAs in our panel with their functions are listed in table 1.

Ischemia-related miRNA expression

All three miRNAs, miR-34a, -210, and -214 were expressed more highly in astrocytes from white matter active lesions compared to controls; their expression in mixed and inactive lesions returned to levels closer to those from controls (Fig. 4). MiR-34a expression in astrocytes from active lesions was also higher compared to mixed and inactive lesions in

white matter.

To demonstrate downstream function secondary to these ischemia-reactive miRNA changes, immunostaining for HIF-1 and VEGF in white matter MS lesions (Fig. 5) revealed positively stained astrocytes. $VEGF^{+}$ microglia were also observed.

Inflammation-related miRNA expression

In white matter, miR-146a and miR-155 expressions were lower in MS lesions compared to controls (Fig. 6). MiR-365 had a lower expression in astrocytes from mixed lesions compared to those from other groups. MiR-146b was more highly expressed in active lesions compared to other groups. In the gray matter, miR-146a, -146b, and miR-155 expressions were lower in astrocytes from active lesions compared to those from other groups.

MiRNAs enriched in astrocytes

MiR-99a and miR-143 shared a similar expression profile pattern across both groups and regions. These two miRNAs had lower expression in astrocytes from all MS lesion groups compared to those from controls in the white matter (Fig. 7). In the gray matter, the two miRNAs had lower expression in astrocytes from active lesions compared to those from other groups. MiR-449 was more highly expressed in astrocytes from active lesions compared to those from other groups in the white matter while it was not found to be differentially expressed in gray matter astrocytes from MS lesions compared to controls.

MiRNAs regulating AQPs

In the white matter, both miR-100 and -145 expression were lower in astrocytes from MS lesions compared to controls, while expression of miR-100 was also lower in mixed lesions compared to active lesions (Fig. 8). MiR-320 had a higher expression in astrocytes

from active lesions compared to controls and to those from mixed lesions. In the gray matter, miR-100 had a lower expression in astrocytes from both active and inactive lesions compared to those from controls. MiR-145 had a lower expression in astrocytes from active lesions compared to controls. Both miR-100 and miR-145 showed lower levels in gray matter astrocytes from active lesions compared to those from inactive lesions.

Glutamate transport and apoptosis-related miRNAs

MiR-29a had a lower expression in astrocytes from MS lesions compared to controls in the white matter, while miR-181a had a lower expression in astrocytes from mixed and inactive lesions compared to controls in the white matter (Fig. 9). MiR-181a also had a lower expression in mixed lesions compared to active lesions. MiR-124a was more highly expressed in astrocytes from active lesions compared to those from other groups in the white matter. MiR-29a had a lower expression in active lesions compared to other groups in gray matter. MiR-181a had a lower expression in gray matter astrocytes from active lesions compared to controls.

Discussion

MiRNA profiling in MS

In this study, we profiled miRNAs in specifically-identified astrocytes laser-captured singly from brain sections of autopsied and biopsied MS cases. The miRNAs in our panel include those that are inflammation mediators, ischemia responders, regulators of AQPs, glutamate transporters, astrogliosis, and those that are enriched in astrocytes. We do acknowledge the exclusion of many other important miRNAs that are relevant for their contribution in MS pathology such as miR-132, miR-491, and miR-572 (Mameli et al., 2016; Mancuso et al., 2015; Noorbakhsh et al., 2011). We employed multiplexed RT and pre-amplification reactions to quantify miRNAs expression in qPCR. The multiplexing steps disallowed us

from including many other important and pertinent miRNAs in our panel. The quality of GFAP immunostaining achieved in our study we believe was optimal to visualize astrocytes in various stages of lesion pathology as well in normal appearing tissue specimens. We believe this is the first miRNA study on in situ MS lesions where astrocytes have been specifically identified and individually laser-captured, giving us a marked advantage in assessing the functional status of these cells.

MiRNAs expression profile comparison of astrocytes between normal controls, active, mixed, and inactive MS lesions

MiRNAs upregulated in astrocytes from active white matter lesions did not also show a difference in expression between inactive lesions and controls. In the gray matter, these same miRNAs (with the exception of miR-146b) were not differentially expressed in astrocytes from active lesions compared to controls as well as between those from inactive lesions and controls (Table 3). This finding of comparable expression levels for a subset of miRNAs, between astrocytes from inactive and normal controls may possibly be indicating an overall sign of lesion tissue returning to ‘normalcy’.

Expression profiling of astrocytes laser-microdissected from MS lesions

There are not many reports of miRNA expression profiles of laser-captured astrocytes from MS lesions. The Junker study seems to be the only report which has clearly provided evidence for the detection of miR-155 in astrocytes captured from MS lesions (Junker et al., 2009). However, that study does not quantify the expression of this miRNA relative to astrocytes captured from normal-appearing white matter (NAWM). Even though we cannot directly compare our results with this finding, it validates the expression of miR-155 in astrocytes derived from the human adult brains, as previously shown by our group (Rao et al., 2016). It is also important to note that this observation is contrasted with our previous finding

of marked miR-155 increase in microglia from active MS (Moore et al., 2013).

While comparing the miRNAs expression between control and active lesions as reported by Junker et al., the higher expressions of miRNAs: miR-34a, miR-146b, miR-214 and miR-320a are in agreement with our results obtained in astrocytes from active MS lesions. Our study also included astrocytes from mixed active / inactive and inactive lesions in which these miRNAs were not found to be upregulated compared to controls. In contrast to the Junker study which reported higher levels of miRNAs: miR-155 and miR-146a in active white matter lesions, we found these miRNAs to be down regulated in astrocytes captured from active and mixed lesions. This contrast in findings can be explained by the simple difference between our study which used laser-captured astrocytes for profiling while the Junker study used whole tissue specimens for expression analyses, and could have included microglia and macrophages. We did not find miR-21 to be differentially expressed in astrocytes from MS lesions unlike the higher expression detected in MS lesion tissues in the Junker study. We did not detect miRNAs: MiR-214 and miR-365 to be differentially expressed in astrocytes from inactive lesions compared to controls, which contrasts the higher expression of the two miRNAs in inactive lesions in the Junker study.

In our study, no significant differences of expression of miR-21 and miR-129 were found across all groups. MiR-21 is a miRNA that increases after spinal cord injury in mice, and overexpression of miR-21 attenuated the hypertrophic response of astrocytes to spinal cord injury (Bhalala et al., 2012). Overexpression of miR-129 in glioblastoma cells showed increased expression of pro-apoptotic Bax and decreased expression of the anti-apoptotic Bcl-2 (Zeng et al., 2018). These miRNAs without significant changes may suggest that the cells returned to normal or still performed certain functions in spite of the pathological changes in MS.

MiR-338 was differentially expressed (see Table 3). The miR-338 differential expression will not be discussed further, since it is considered as an oligodendrocyte-specific miRNA,

and its function has not been characterized in astrocytes to our knowledge (Zhao et al., 2010).

Ischemia-related miRNAs

MiRNAs miR-34a, -210, and -214, upregulated in an ischemia model of middle cerebral artery occlusion in rats (Jeyaseelan et al., 2008), were also upregulated in astrocytes from active white matter lesions in our study. This is suggestive of the presence of a hypoxic or ischemic pathology in our active white matter lesion samples. Ischemic or hypoxic pathology in MS lesions is a well-established phenomenon, and we believe our findings reflect this (Lassmann, 2003). Studies have shown that upregulation of miR-34a in astrocytes or astrocytic cell lines may result in increased expression of GFAP and augmented nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, activating astrocytes (Hu et al., 2017). Upregulation of miR-34a in astrocytes from active white matter lesions in our study indicates that these cells are reactive. In agreement with our finding, miR-210 was found to be upregulated in primary rat astrocytes under oxygen-glucose deprivation, a model of ischemia associated metabolic stress (Ziu et al., 2011). Also, miR-210 expression is upregulated in plasma from relapsing-remitting MS (RRMS) patients compared to controls (Gandhi et al., 2013). The function of miR-214 in astrocytes remains unknown. This miRNA was downregulated in CD4+ T cells from RRMS patients (Ahmadian-Elmi et al., 2016). Our finding on this miRNA in astrocytes is in concurrence with the earlier mentioned Junker study that has reported upregulation of miR-214 in active white matter MS lesions (Junker et al., 2009). Further, α-synuclein is negatively regulated by miR-214 as shown in a neuroblastoma cell line in the context of Parkinson's disease (Wang et al., 2015b). To date, there exist no studies that establish miR-214's targeting of α-synuclein in astrocytes. Assuming that miR-214's function of the negative regulation of α-synuclein is conserved in astrocytes, its upregulation in active white matter lesions in our study may be implying a neuroprotective role; increased expression of α-synuclein in astrocytes causes injury to BBB

(Gu et al., 2010). Unlike in the white matter, miRNAs: MiR-34a, miR-210, and miR-214 were not differentially expressed in both active and inactive gray matter-derived astrocytes compared to controls. This differential expression profile perhaps points to regional differences in ischemic or metabolic injury contribution between gray and white matter lesions. However, in a scenario wherein both gray and white matter lesions of our study were to be experiencing ischemic stress, our results could be suggesting a differential capacity in astrocytes located in the different regions (gray and white matter) to respond to such an injury.

With immunohistochemistry, HIF-1and VEGF have been observed in astrocytes in white matter MS lesions. HIF-1 is a transcriptional factor which is upregulated in astrocytes to offer protection against ischemic conditions (Badawi et al., 2012; Hirayama and Koizumi, 2017). VEGF is a proangiogenic factor which is upregulated during ischemia and also regulated by HIF-1 (Liu et al., 1995; Pichiule et al., 1999; Weidemann et al., 2010). In addition, miR-210 overexpression has been shown to increase VEGF expression in astrocytes (Zeng et al., 2014). The observation of HIF-1^{+} and $VEGF^{+}$ astrocytes corresponds with our observation of ischemic-related miRNAs in the white matter active lesions, which suggests a similar response to ischemia of astrocytes from white matter active MS lesions.

Inflammation-related miRNAs

MiR-146a and miR-155 are both involved in NF-κB signaling and regulate it in an opposite manner. MiR-146a negatively regulates IL-1 receptor-associated kinase 1 (IRAK1), IRAK2, and *TNF receptor-associated factor* 6 (TRAF6), and the downstream impact of which weakens NF-κB signaling (Iyer et al., 2012). In contrast, miR-155 acts as a positive regulator of NF-κB signaling through its targeting of suppressor of cytokine signaling 1 (SOCS1) (Tarassishin et al., 2011). We have not found studies in astrocytes wherein the integrated effect of miR-146a and miR-155 on NF-κB signaling has been investigated.

However, in a transgenic mouse model, miR-155 has been shown to be a major regulator of NF-κB-related inflammatory processes relative to that of miR-146a (Mann et al., 2017). Immunohistochemical studies of NF-κB in MS lesions have reported its detection in hypertrophic astrocytes but only occasionally in the nuclei (Bonetti et al., 1999). One has to however note that it has been reported that myeloid cells significantly augment their NF-κB signaling, while astrocytes did not appear to be a major cell type with increased NF-κB signaling in the context of lesion development (Gveric et al., 1998). This would be in accordance with our previous observations on miR-155 in microglia (Moore et al., 2013). Lower detection of miR-155 corresponds to the diminished NF-κB signaling in astrocytes. Similar to the classical M1 / M2 categorization of microglia, reactive astrocytes can be termed A1 and A2 as described by Liddelow et al. (Liddelow et al., 2017). The former can be induced by reactive microglia and found in neuroinflammatory conditions including acute RRMS, while the latter can be found in ischemic conditions. On the other hand, metal-sulfate stress induced NF-κB signaling has been shown to induce miR-146a expression in a human astroglial cell line (Pogue et al., 2011). Limited detection of NF-κB in MS lesion astrocytes as mentioned earlier correlates with the decreased expression of miR-146a in astrocytes from MS lesions. This suggests that our findings of the lower expression of these two miRNAs in MS lesions correspond to the minor NF-κB signaling activity observed in MS astrocytes.

For miR-146b, the expression was higher in astrocytes from the active white matter lesions compared to those from controls, mixed, and inactive lesions. In the gray matter, the expression was lower in astrocytes from active lesions compared to those from inactive lesions and controls. The expression of miR-365 was lower in astrocytes from mixed white matter lesions compared to those from active lesions, inactive lesions, and controls. These two miRNAs have been shown to attenuate IL-6 secretion in fetal astrocyte cultures under IL-1β treatment (Moore et al., 2014). In the active white matter lesions, miR-146b was increased while the expression of miR-365 did not change compared to controls. In the mixed

white matter lesions, miR-146b did not change while miR-365 was found decreased compared to controls. One can only speculate about the possibility of temporal regulation being mediated by these miRNAs during lesion development. The same is true for regional differences in their expression (white matter vs. gray matter) observed in our study. Since it is unclear as to what factors influence the expression of these two miRNAs in astrocytes in an inflammatory milieu, their downstream effects need further investigation. Notwithstanding the temporal differences in the expression between these two miRNAs, one cannot neglect the importance of our finding of higher expression of miR-365 in astrocytes from inactive white matter lesions compared to those from mixed ones. This is the only miRNA in our panel found to be up regulated in astrocytes from such lesions. This miRNA may have an important role in long term tissue repair processes in the white matter.

Albeit with an insignificant Chi-squared test, miR-125b was downregulated in astrocytes from mixed white matter lesions compared to those from active white matter lesions. In the context of glial scarring, increased level of this miRNA following spinal cord injury in rats was shown to suppress scarring-related genes and enhance functional recovery (Diaz Quiroz et al., 2014).

Highly expressed miRNAs

MiR-99a and miR-143 were expressed lower in astrocytes from all of the white matter lesions and only in those from active gray matter lesions compared to controls. MiR-449a was more highly expressed only in active white matter lesions. The regional differences (white matter vs. gray matter) observed in the expression of these miRNAs clearly point to the differences in astrocytic response in the two regions during lesion development. These three miRNAs have been shown to be more highly expressed in astrocytes compared to other cell types in the brain (Butovsky et al., 2014; Jovicic et al., 2013). The functions of these miRNAs in astrocytes are unknown. As discussed earlier, expression profiles of ischemia

related miRNAs are indicating an ischemia-induced metabolic injury in the MS tissue samples analyzed in our study. MiR-143 is a known neuronal pro-apoptotic mediator during metabolic stress (Zeng et al., 2017). Downregulation of this miRNA in astrocytes from white matter and active gray matter lesions suggests a putative neuroprotective role. MiRNAs: MiR-99a (Chakrabarti et al., 2013), miR-143 (Wang et al., 2014), and miR-449a (Yao et al., 2015) are known as tumor suppressors in glioma cells. The tumor-suppressive effect of these miRNAs is relevant as potential modulators of astrocyte proliferation during astrogliosis. The decreased levels of miR-99a and miR-143 in astrocytes from white matter lesions and active gray matter lesions are consistent with their potential negative regulatory function of proliferation confirming astrogliosis in and around the lesions analyzed in our study. But, another negative regulator of proliferation, miR-449a was found increased in astrocytes from active white matter lesions, the potential impact of which may attenuate astrogliosis. Hence, it is difficult to assess the overall effect of the expression profiles of these three miRNAs as observed in our samples. We speculate the involvement of multiple miRNAs that have opposite regulatory roles fine tuning the extent of astrogliosis during various stages of lesion development.

AQP related miRNAs

MiRNAs: MiR-100, miR-145, and miR-320a are all negative regulators of AQP4 (Jeyaseelan et al., 2008; Sepramaniam et al., 2010; Zheng et al., 2017), while miR-320a also negatively regulates AQP1 (Sepramaniam et al., 2010). MiR-100 and miR-145 had lower expressions in astrocytes from white matter lesions and active gray matter lesions. However, miR-320a had a higher expression only in active white matter lesions compared to controls. In the gray matter, this miRNA was not found to be differentially expressed in astrocytes from both active and inactive lesions compared to controls. On the regional differences, it can be speculated that these may have arisen out of the anatomical differences in the distribution

of blood vessels (Ballabh et al., 2004). Regulation of AQPs expression during lesion formation and the following pathological course that determines the fate of the tissue is complex and riddled with contentions. Regarding the differential expression profiles, AQPs are known to be modulated by different factors as encountered under physiological, inflammatory and / or stress conditions. In normal primate brain, AQP1 was found to be preferentially expressed in white matter astrocytes, suggesting a clear difference in its regional distribution (Arcienega et al., 2010). Under inflammatory condition, IFN-γ has been shown to induce AQP4 but not AQP1 in human fetal astrocytes (Satoh et al., 2007). Under mitotoxic stress conditions as simulated by the treatment of 3-nitropropionic acid, the expression of AQP1 in rat astrocytes was found to be upregulated while the expression of AQP4 was downregulated (Hoshi et al., 2016). The miRNAs expression differences observed across regions and lesion pathologies in our study are perhaps reflecting the diversity of factors and location differences that contribute to the regulation of AQPs expression. It is also important to note that in the course of cerebral inflammation, both influx and efflux into the brain may be occurring to allow fluid, electrolytes and even cells in or to exclude them. Thus the timing is critical and may explain these apparent differences.

Glutamate transport and Apoptosis-related miRNAs

MiR-124a was upregulated in astrocytes from active white matter lesions compared to those from controls, mixed, and inactive lesions, while the expression was higher in astrocytes from inactive gray matter lesions compared to those from active gray matter lesions. Transfer of exosomes with miR-124a from neurons to astrocytes has been shown to upregulate glutamate transporter 1 (GLT1) (rodent analog of excitatory amino acid transporter 2 (EAAT2)) in mice (Morel et al., 2013). Elevated glutamate level has been reported in active white matter MS lesions (Srinivasan et al., 2005). The increased expression of miR-124a in active white matter cases may result in upregulation of GLT1 expression in

reactive astrocytes conferring potential neuroprotective effect. In normal-appearing gray matter sections from MS cases, the expression of EAAT2 has been shown to be lower compared to controls (Zeis et al., 2015), while it was higher in optic nerve from MS patients (Vallejo-Illarramendi et al., 2006). These findings point to the differential regional expression of glutamate transporter that may explain the regional difference in the expression of this miRNA observed in our samples.

MiR-181a had a lower expression in astrocytes from mixed and inactive white matter lesions. MiR-181a targets Bcl-2 family members and reduces metabolic stress-induced apoptosis in astrocytes (Ouyang et al., 2012). Antagomir of miR-181a increases Bcl-2, prevents the decrease of GLT1 level, and reduces neuronal loss in forebrain ischemia (Moon et al., 2013). Inhibition of miR-181a expression (under propofol treatment) in astrocytes upregulated Bcl-2 expression, offering protection against glucose deprivation (Lu et al., 2014).

Unlike miR-181a, miR-29a has been shown to be a negative regulator of apoptosis. MiR-29a had a lower expression in astrocytes from white matter lesions compared to controls, and had a lower expression in astrocytes from active gray matter lesions compared to those from inactive lesions and controls. MiR-29a targets a protein from the Bcl-2 family, the pro-apoptotic p53 upregulator of apoptosis (PUMA), and has a neuroprotective effect in forebrain ischemia in rats (Ouyang et al., 2013). MiR-181a and miR-29a, both are known regulators of apoptosis having opposite effects on survival. One can only speculate on our findings for miR-181a and miR-29a that potentially reflect opposing effects on apoptosis in astrocytes from both white matter and gray matter lesions. As mentioned earlier, miR-181a has been shown to regulate multiple targets while miR-29a has only been shown to target PUMA in astrocytes. The overall impact of expression changes in these two miRNAs in astrocytes remains open for further investigation.

Astrogliosis-related miRNAs

In our panel, two of the previously discussed miRNAs, miR-126b and -145, are also related to astrogliosis. Astrogliosis is an important aspect of inflammatory responses during MS lesion development, and miR-125b has been shown to be a positive regulator of astrogliosis in a human astrocyte cell line under IL-6 treatment (Pogue et al., 2010). An increased trend of this miRNA in astrocytes from our active white matter lesions may reflect some similar increase in astrogliosis. MiR-145 is found to be a negative regulator of astrogliosis after spinal cord injury in murine astrocytes, with potential targets including GFAP and C-myc (Wang et al., 2015a). In white matter, we observed an increasing trend of miR-125 in active lesions while there is a decrease of miR-145 expression in MS lesions compared to the control. This suggests an increased profile of astrocyte reactivity that corresponds to our findings of astrocyte morphology under LCM, especially in white matter active lesions.

Conclusion

Although in the past astrocytes were not considered to be central players in the pathogenesis of MS, researchers now know that their interaction with many immune and glial cells may contribute to the pathology of MS. In our study, we found astrocyte miRNAs expression to be different not only between control samples and MS lesions but also between different pathological categories within the same region. We also found regional variation in the miRNAs expression pattern (white vs. gray). Our most striking results indicate a prominent response to ischemic pathology mainly in the white matter lesions during the active phase. Of significance as well, are the influence on miRNAs involved in neuroprotection, and miRNAs involved in regulation of the blood brain barrier during the inflammatory event. In our study, we found higher expressions of ischemia-related miRNAs in astrocytes from the white matter active lesions. Whether these are A1 or A2 astrocytes, or

perhaps both may exist in MS depending on anatomical regions and timing, remains to be investigated. The expression profiles obtained for miR-155 and miR-146a in our study possibly suggests a diminished pro and anti-inflammatory role of astrocytes compared to microglia, especially during the active phase of lesion development in both white and gray matter regions. However their presence in the normal brain may suggest a housekeeping role. The tendency of many miRNA to return to normal levels of expression in inactive lesions also suggests that astrocytes maintain the capacity for normal function after being stimulated in the early active stage. In addition, it is also clear that these remarkable cells are able to maintain much normal function, while reacting to multiple pathogenic insults simultaneously, and are also able to react differentially in different locations.

In summary, our study highlights the usefulness of miRNAs as molecular readouts of pathological processes that underlie disease progression. We believe our study brings to the fore the importance of astrocytes in MS pathology and sheds light on several processes and mediators that may be of interest in the context of development of novel therapeutics for MS.

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Tables

miRNA	Target / Function	PMID / Ref.
	Ischemia-related miRNA	
34a	Upregulated in middle cerebral artery occlusion (MCAO) model of ischemia	18258830
34a	Involved in cell cycle, differentiation, and apoptosis	22162084
34a	LPS increases the release of shedding vesicles (SV) and the expression of miR-34a in SV from astrocytes	26091620
	MiR-34a negatively regulates Bcl2 (SV transfer from glioblastoma (GBM) cell to neuroblastoma cell)	
210	Upregulated in MCAO model of ischemia	18258830
210	Highly expressed in astrocytes; overexpression of this miRNA induced neurogenesis in	24152581
	subventricular zone of adult mouse brain	
	Overexpression of miR-210 via lentiviral vector upregulates VEGF in the brain	
210	Expression of miR-210 is increased in astrocytes after oxygen-glucose deprivation (OGD).	21373187
214	Upregulated in MCAO model of ischemia	18258830
214	Invovled in proliferation, migration and angiogenesis in glioma;	26349993
	however potentially neuroprotective in non-neoplastic conditions	
	MiR-214 mimics negatively regulates α-synuclein (invovled in Parkinson's Disease (PD)).	
	Inflammation-related miRNA	
125b	Positive regulator of astrogliosis (GFAP/vimentin)	20347935
	Targets CDKN2A(cyclin-dependent kinase inhibitor 2A)	
146a	Negatively regulates the expression of Interleukin-1 receptor-associated kinase 1 (IRAK-1), IRAK-2	23028621
	and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF-6) in vitro (inflammation resolving function)	
	Expression is stimulated by IL-1	
146b	Acts as a pro-inflammatory molecule in microglia in amyotorophic lateral sclerosis,	24336079
	operating through P2X7 receptor activation. Acts in synergy with other pro-inflammatory miRNAs	
	such as miR-155 and and miR-125b.	
146b	MiR-146b / miR-365 mimics can both attenuate IL-1β induced IL-6 release in fetal astrocytes	Moore et al.
	MiR-146b mimics decreased IP-10 expression but not miR-365 mimics	(See ref.)
155	Negatively regulates the expression of SOCS1 gene expression in vitro (proinflammatory function)	22170100
	MiR-155 expression is upregulated by IL-1b/IFNr treated astrocytes compared to control	
365	In microglia, acts as a pro-inflammatory miRNA that interferes with the interleukin-6 and	24336079
	STAT3 pathway determining increased tumor necrosis factor (TNF) transcription	
365	MiR-146b / miR-365 mimics can both attenuate IL-1β induced IL-6 release in fetal astrocytes.	Moore et al.
	MiRNAs enriched in astrocytes	
99a	More highly expressed in astrocytes, microglia, and neurons compared to oligodendrocytes	24316888
99a	MiR-99a transfection in GBM enhances anti-tumor effect of photofrin(photodynamic/radiation threapy)	23409016
143	More highly expressed in astrocytes compared to neurons and oligodendrocytes	23516279
143	Negatively regulates N-RAS(neuroblastoma RAS viral oncogene homolog), which inhibits PI3K/AKT, MAPK/ERK signaling,	24980823
	acting as a tumor supressor(cell migration / invasion)	
449a	More highly expressed in astrocytes compared to neurons and oligodendrocytes	23516279
449a	Negatively regulates MAZ(myc-associated zinc-finger protein)	25487955
	blocking PI3K/AKT signaling and acting as tumor suppressor	
	MiRNAs regulating aquaporin	
100	Negatively regulates the expression of aquaporin 4 (AQP4). Down-regulation of AQP4 in astrocytes	18258830
	is neuroprotective in cerebral ischemia/reperfusion injury (MCAO model)	
	Expression of miR-100 is higher 48hr after reperfusion	
145	Negatively regulates AQP4 in oxygen-glucose deprived astrocytes.	29057271
	MiR-145 expression is decreased in OGD astrocytes.	
	Both miR-145 overexpression and AQP4 siRNA protect astrocytes from OGD injury	
320a	Negatively regulates the expression of AQP1 and AQP4 in vitro and in vivo, supporting a neuroprotective role	20628061
320a	Negatively regulates AQP1 in rat astrocytes from spinal cord	26850728
	Mice with ischemic reperfusion have less motor deficits when treated with miR-320a mimics.	
	Glutamate transport / apoptosis-related miRNAs.	
29a	Negatively regulates proapoptotic protein, p53 upregulated modulator of apoptosis (PUMA, a Bcl2 family protein) in vitro.	24038396
	MiR-29a mimic protects / miR-29a inhibitor aggravates cell injury and mitochondrial function after ischemia-like stresses	
124a	Positively regulates the expression of glutamate transporter, GLT1 protein in vitro potentially enhancing protection	23364798
	via exosome transfer from neurons to astrocytes	
129	Negatively regulates the expression of expression of Caspase-6 in vitro inhibiting apoptosis and may	25187728
	be neuroprotective (Inhibits cell proliferation in GI cancer)	
181a	Decreased miR-181a levels reduced glucose deprivation induced apoptosis;	21958558
181a	Pretreatment of miR-181a antagomir prevents the drop of GLT-1 level in CA1 region after ischemic injury	24002437
	Astrogliosis-related miRNAs	
125b	Positive regulator of astrogliosis (GFAP/vimentin)	20347935
	Targets CDKN2A(cyclin-dependent kinase inhibitor 2A)	
145	Negative regulator of astrogliosis after SCI (increased levels of this miRNA leads to decreased reaction,	25139829
	proliferation and migration and process formation)	
	Potential targets include GFAP / C-myc	
	Other miRNAs	
21	Overexpression of miR-21 in astrocytes attenuates the hypertrophic response to spinal cord injury (SCI),	23238710
	and may reduce chronic scarring.	
338	Upregulated in MS / EAE (not specifically in astrocytes)	21908875

Table 1. Functions of miRNAs in the panel.

	Demyelination (LFB or MBP staining)	Reactivity		Lymphocyte Infiltration
		Astrocyte	Myeloid cells	
Active	++++(ongoing myelin debris in macrophages)	+++ (hypertrophic)	+++	+++
Mixed Active/Inactive	+++ Inactive centre / active rim	+++	++	+
Inactive	+++ (absence of macrophages activity)	+++(fibrillary gliosis)	+/-	+/-

Table 2. Categorization of MS lesions. Active, mixed, and inactive lesions are categorized by the degree of demyelination, cell reactivity, and lymphocyte infiltration. The degree of each category is designated by + sign, whereas +/- signs represent rare or dubious detection.

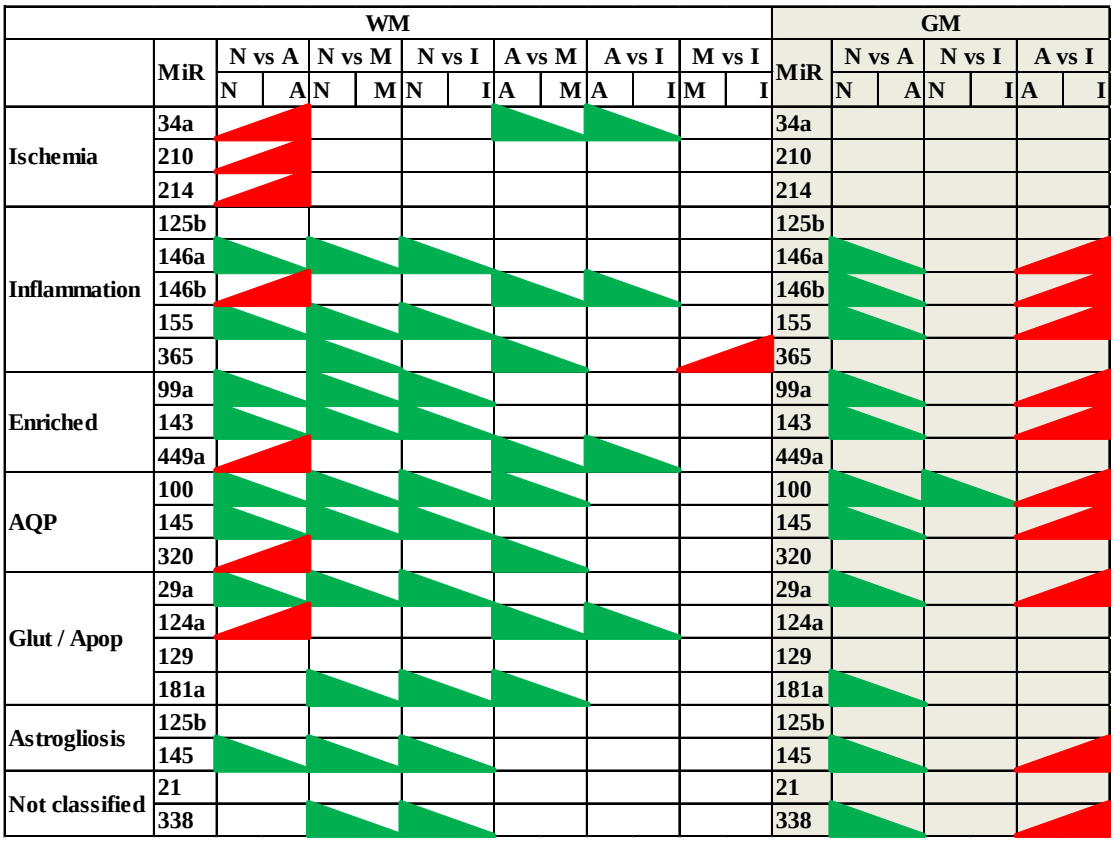


Table 3. MiRNA expression in astrocytes captured from white matter or gray matter and studied using GLMM. The expression of miRNAs is shown in groups of their functions, as in Ischemia (ischemia-related miRNAs), Inflammation (inflammation-related miRNAs), Enriched (miRNAs enriched in astrocytes), AQP (miRNAs regulating aquaporin), Glut / Apop (miRNAs regulating glutamate transport or apoptosis) Astrogliosis (astrogliosis-related miRNAs), and Not classified. WM = white matter; GM = gray matter; N = normal control; A = active lesion; M = mixed lesion; I = inactive lesion.

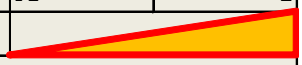
WM		
MiR	A vs M	
	A	M
125b		
GM		
MiR	A vs I	
	A	I
124a		
GM		
MiR	N vs A	
	N	A
365		

Table 4. MiRNA expression in astrocytes captured from white matter or gray matter as studied statistically with the T test (Chi-squared test $p > 0.5$). WM = white matter; GM = gray matter; N = normal control; A = active lesion; M = mixed lesion; I = inactive lesion.

Figures

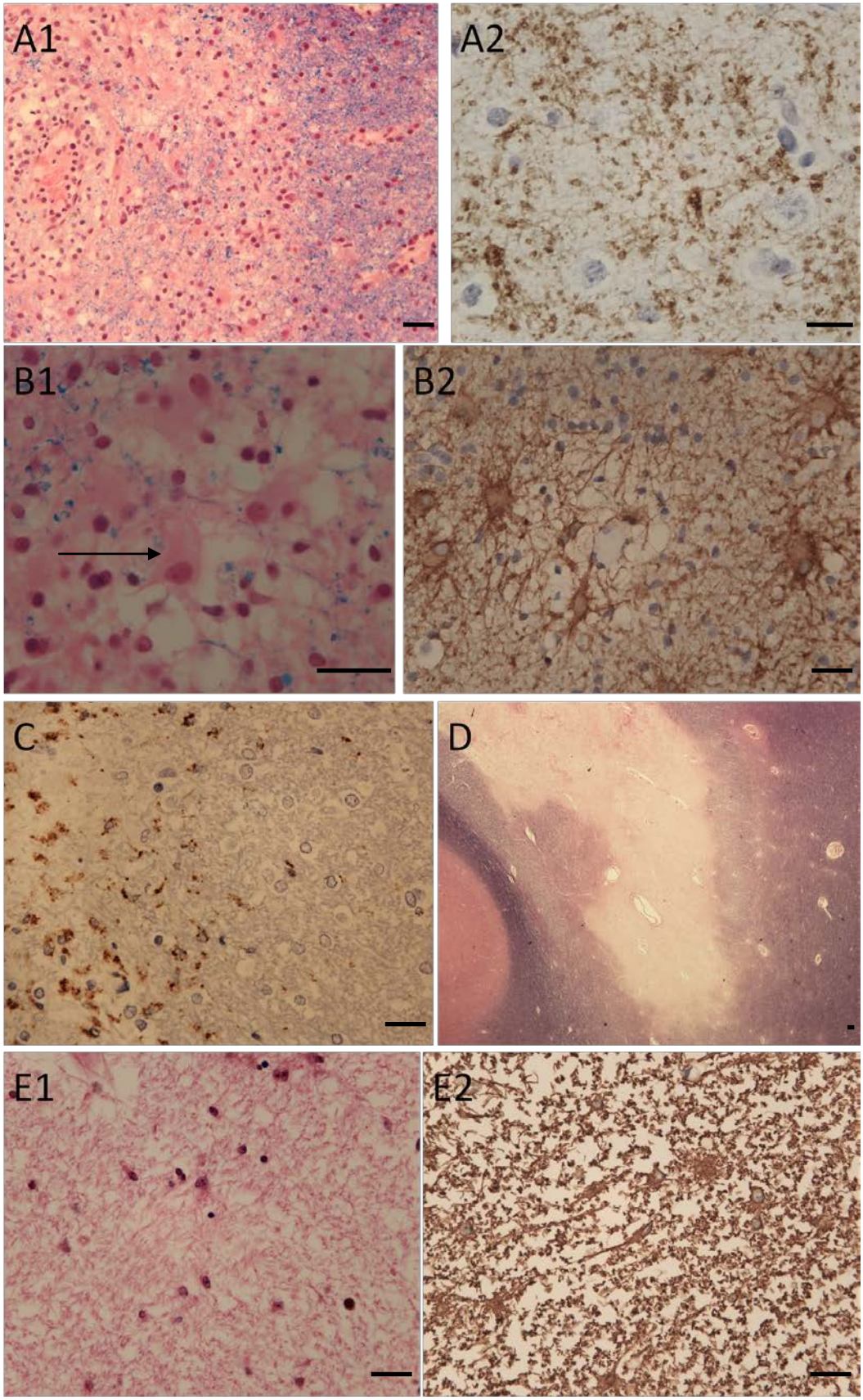


Figure 1. Pathological features of MS lesions. Active plaque stained with LFB and H&E (Fig. 1A-1) and myelin basic protein (Fig. 1A-2) (right). Astrocytes in an active plaque stained with LFB and H&E (Fig. 1B-1, indicated by the arrow) and GFAP (Fig. 1B-2). Mixed plaque stained with CD68 for macrophages and microglia (Fig. 1C). Inactive plaque stained with LFB and H&E (Fig. 1D). Astrocytes in an inactive plaque stained with H&E (Fig. 1E-1) and GFAP (Fig. 1E-2). Approximate scale bar = 50μm

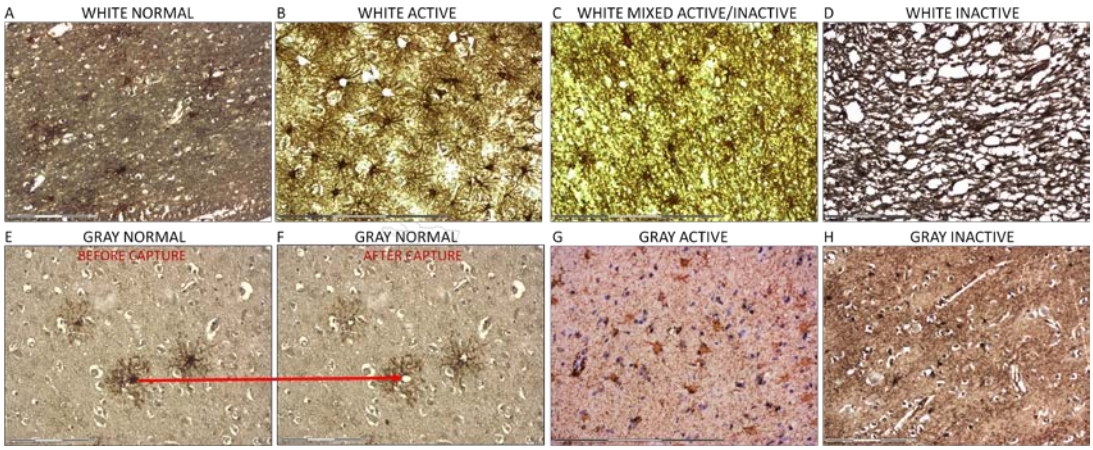


Figure 2. Morphology of astrocytes. Astrocytes from normal appearing white matter (A), active (B), mixed (C), and inactive (D) lesions. Astrocytes from normal appearing matter before (E) and after (F) capture. Astrocytes from active (G) and inactive (H) gray matter.

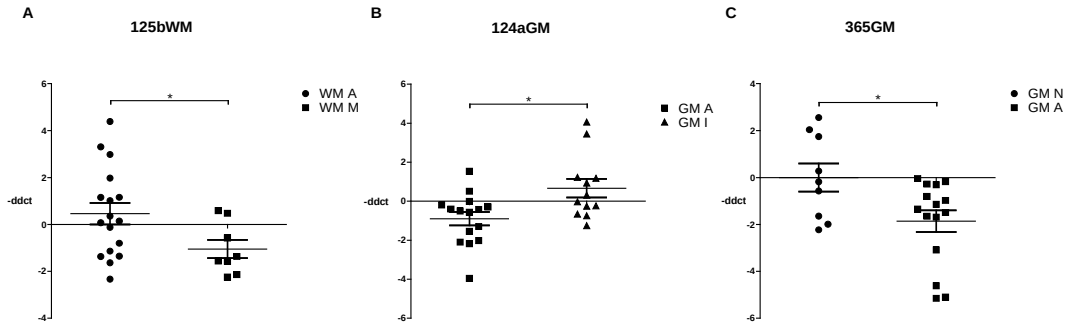


Figure 3. MiRNA expression in astrocytes captured from white matter or gray matter with T test (Chi-squared test $p > 0.5$ in GLMM). Expression of miR-125b in white matter (A), miR-124a in gray matter (B), and miR-365 in gray matter (C). WM = white matter; GM = gray matter; N = normal control; A = active lesion; M = mixed lesion; I = inactive lesion; -ddct = negative ΔΔ cycle time; *$p < 0.05$

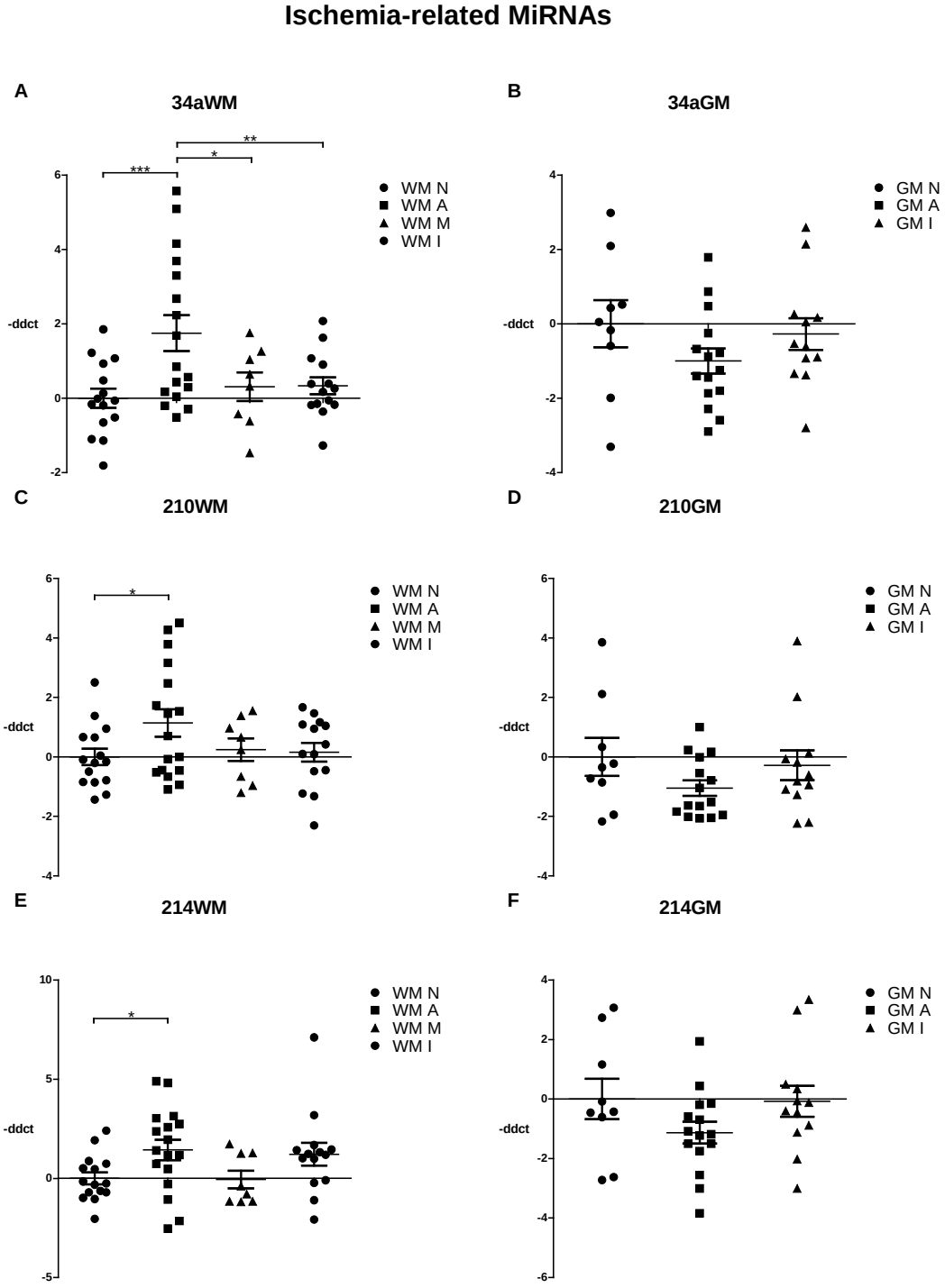


Figure 4. Ischemia-related miRNA expression. The expression of miR-34a, -210, and -214 in white matter (A)(C)(E) and in gray mater (B)(D)(F). WM = white matter; GM = gray matter; N = normal control; A = active lesion; M = mixed lesion; I = inactive lesion; -ddct = negative ΔΔ cycle time; *p < 0.05; **p < 0.01;

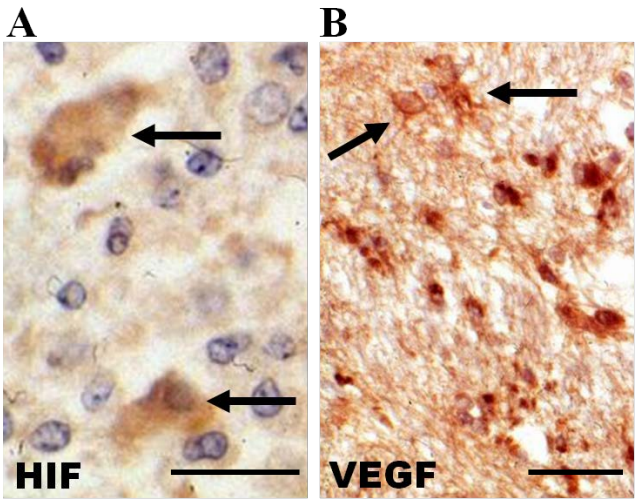


Figure 5. HIF-1 and VEGF IHC in white matter active MS lesions. HIF-1+ astrocytes in white matter active MS lesions (A) VEGF+ astrocytes in white matter active MS lesions (B). Approximate scale bar = 50µm

Inflammation-related MiRNAs

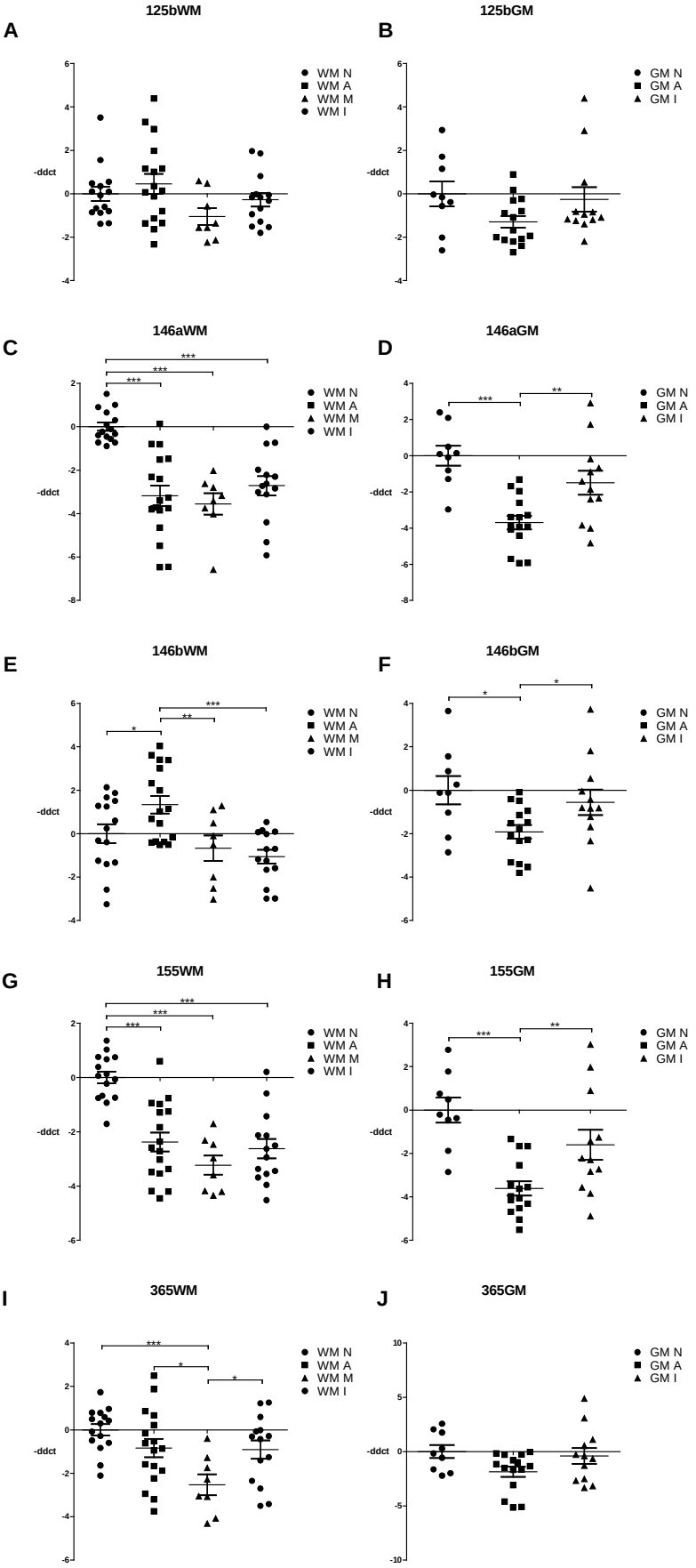


Figure 6. Inflammation-related miRNA expression. The expression of miR-125b, -146a, -146b, -155, and -181a in white matter (A)(C)(E)(G)(I) and in gray mater (B)(D)(F)(H)(J). WM = white matter; GM = gray matter; N = normal control; A = active lesion; M = mixed lesion; I = inactive lesion; -ddct = negative ΔΔ cycle time; *p < 0.05; **p < 0.01; ***p < 0.001.

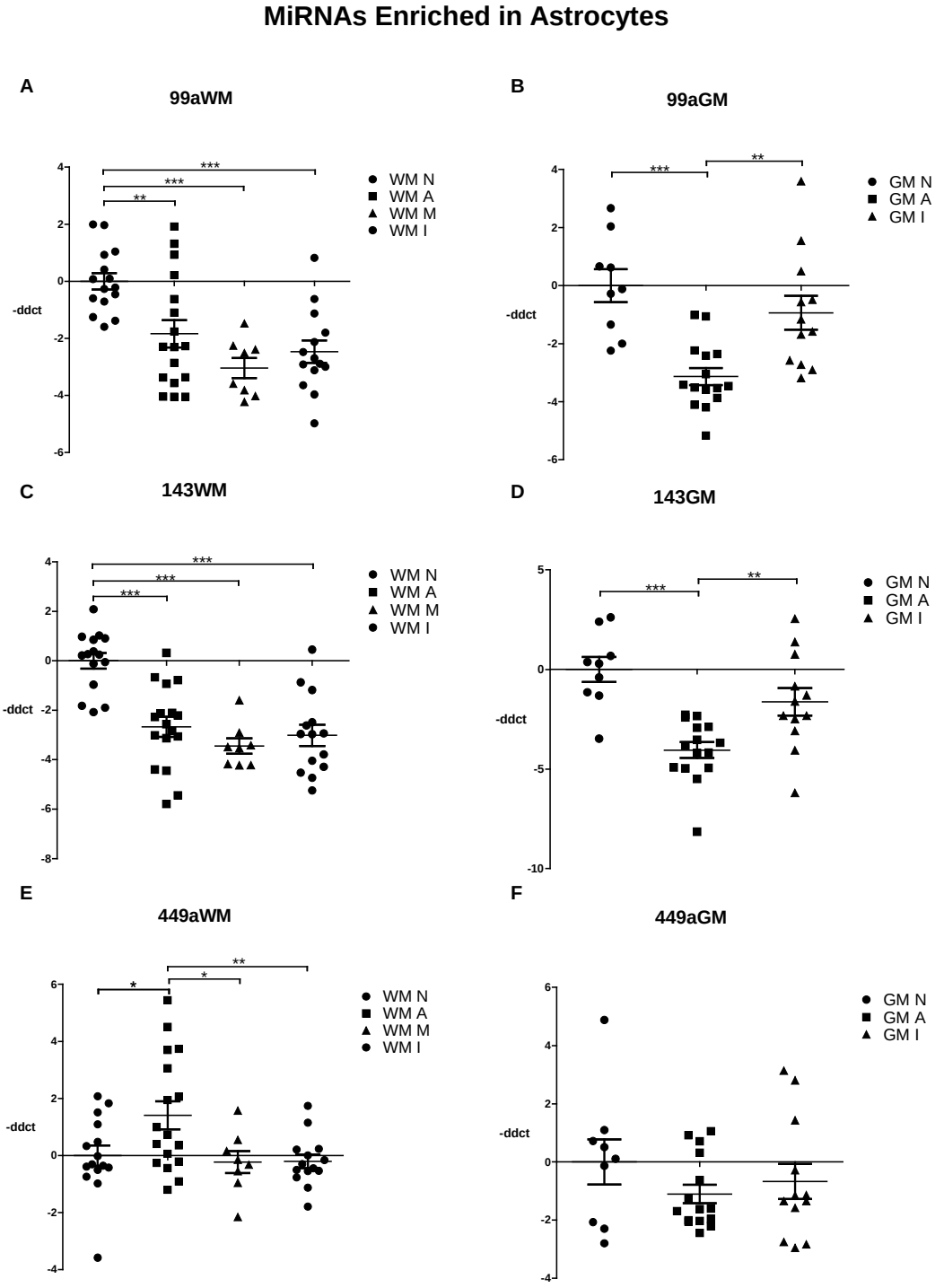


Figure 7. MiRNAs enriched in astrocytes. The expression of miR-99a, -143, and -449 in white matter (A)(C)(E) and in gray mater (B)(D)(F). WM = white matter; GM = gray matter; N = normal control; A = active lesion; M = mixed lesion; I = inactive lesion; -ddct = negative ΔΔ cycle time; *p < 0.05; **p < 0.01; ***p < 0.001

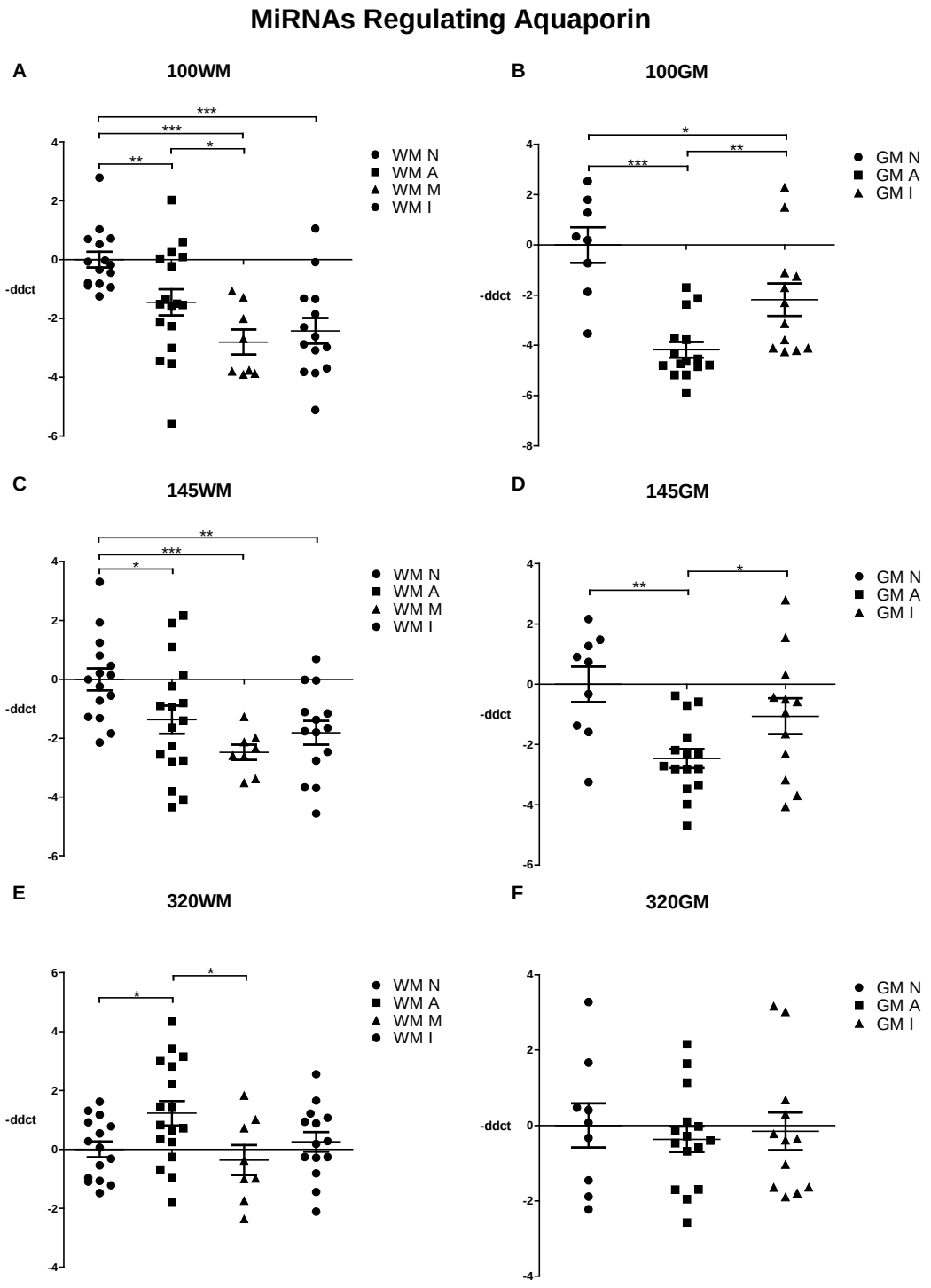


Figure 8. MiRNAs regulating aquaporin. The expression of miR-100, -145, and -320 in white matter (A)(C)(E) and in gray mater (B)(D)(F). WM = white matter; GM = gray matter; N = normal control; A = active lesion; M = mixed lesion; I = inactive lesion; -ddct = negative ΔΔ cycle time; *p < 0.05; **p < 0.01; ***p < 0.001

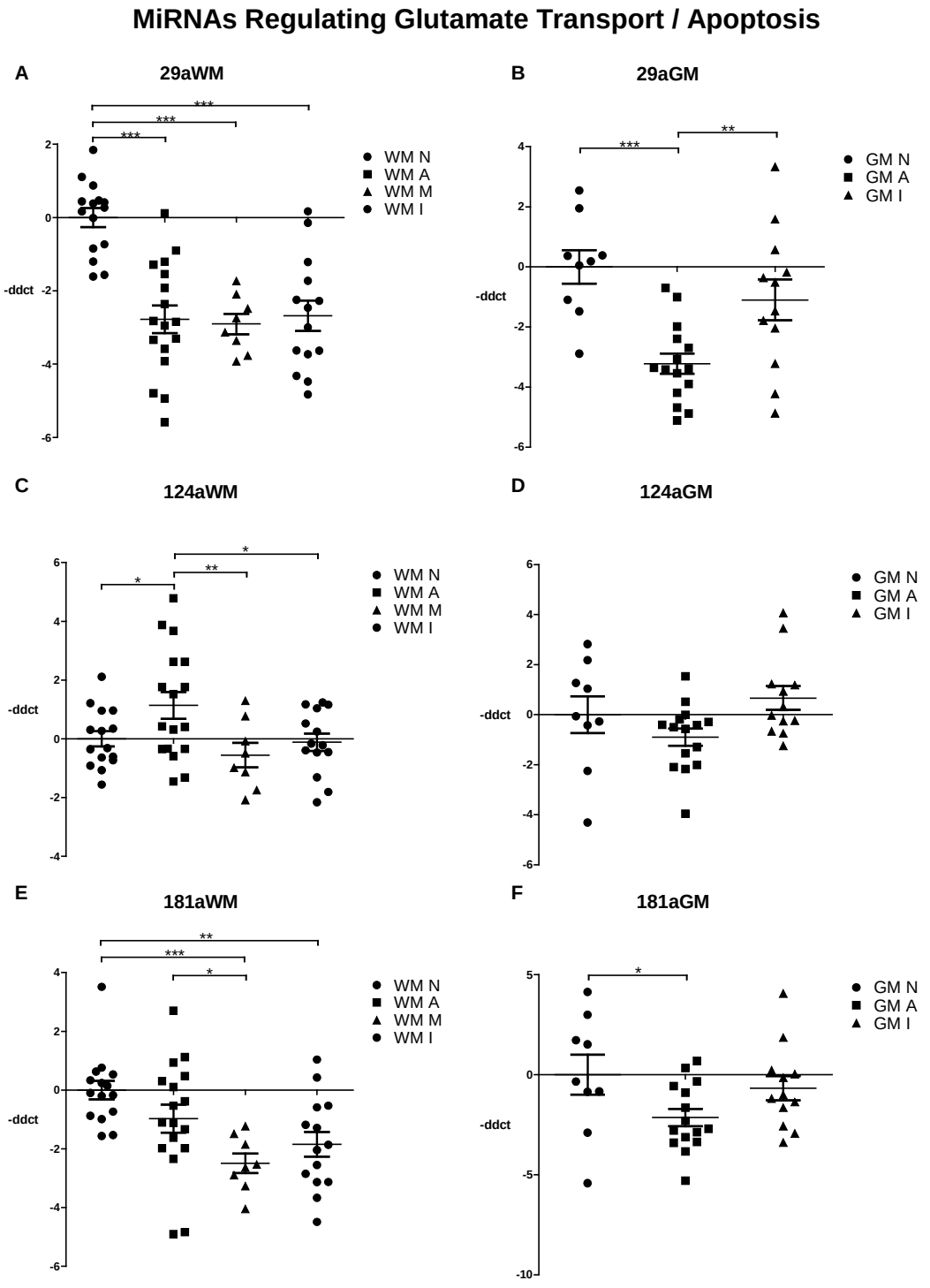


Figure 9. Glutamate transport and apoptosis-related miRNAs. The expression of miR-29a, -124, and -181a in white matter (A)(C)(E) and in gray mater (B)(D)(F). WM = white matter; GM = gray matter; N = normal control; A = active lesion; M = mixed lesion; I = inactive lesion; -ddct = negative ΔΔ cycle time; *p < 0.05; **p < 0.01; ***p < 0.001.

Connecting statement 2 (Chapters 2-3)

The following chapter presents the results from *in vitro* experiments studying the miRNA expression changes in human fetal brain derived astrocyte cultures under inflammatory and / or metabolic stress conditions, in response to IL1β. The relevance to our presented findings in MS tissues (Chapter 2) are discussed.

Chapter 3. Addendum: *In Vitro* Studies: *In vitro* miRNAs expression profiles of fetal astrocytes under inflammation or metabolic stress or under both inflammation-stress conditions

Background

Considering the microenvironmental changes that astrocytes undergo during the MS lesion development, we performed *in vitro* experiments aiming: (i) to address the fate of long term expression of miRNAs (shortlisted panel) after stimulation with IL-1β and (ii) to analyze the miRNAs expression changes in astrocytes undergoing metabolic stress with and without IL-1β stimulation.

Materials and methods

Long term expression of miRNAs: Primary fetal astrocytes from both Centre Hospitalier Universitaire Sainte-Justine and University of Washington, laboratory of developmental biology, were cultured in DMEM with 10% FCS. The effects of IL-1β on miRNA expression in astrocytes were assessed in human fetal astrocyte cultures at 48hr, 2 weeks, 3 weeks, and 4 weeks after IL-1β treatment. IL-1β (1ng/mL) was added to the medium. Cells after 4 weeks were confirmed to be viable (data not shown). A sub-panel of 5 miRNAs that are relevant to immune and injury responses was selected from our panel, including miR-21, miR-146a, miR-146b, miR-155, and miR-365. RNU48 and U47 were used as endogenous reference genes.

Stress conditions: Four conditions were tested in this experiment. The control group was cultured with DMEM (4.5g/L glucose) + 10% FCS as described in the previous experiment. In –S-G group, astrocytes were cultured in DMEM without glucose and serum. IL-1β was added in 1ng/mL, and the cells were harvested after 24hr. RNA extraction, qPCR, and statistical analysis were performed as described previously. A sub-panel of 8 miRNAs that are relevant to immune and injury responses was selected from our panel, including miR-21, -34a,

-146a, -146b, -155, -210, -214, and -365. RNU48 and U47 were used as endogenous reference genes.

Statistical analysis: GLMM is applied as described in the earlier manuscript.

Results

Long term expression of miRNAs under IL-1β stimulation

Except for miR-146b, all miRNAs showed a robust increase of expression with IL-1β treatment after 48hr (Figure 1). Both miR-21 and miR-365 showed an increased expression with IL-1β treatment after 48hr but no difference was observed between control and treated groups after 2 weeks and later. For miR-146a, the increased expression sustained for 3 weeks but diminished after 4 weeks. For miR-155, the increased expression was sustained longer than 4 weeks. Comparing the miRNA expression within IL-1β treated group, we observed a decreasing trend over time for all the miRNAs.

Expression of miRNAs under metabolic stress conditions with and without IL-1β stimulation

We measured the expression of several inflammation-related and ischemia-related miRNAs. For the ischemia-related miRNAs, we observed a decreased expression of miR-34a in –S-G group compared to controls (Fig. 2A). For miR-210, although there was only a trend of increase in expression of the IL-1β treated group compared to the controls, we observed an increased expression in the IL-1β treated group compared to –S-G group and –S-G group treated with IL-1β (Fig. 2B). For the two inflammatory miRNAs: Mir-146a and miR-155, as previously shown, they were upregulated with IL-1β treatment. However, under the stress condition, the increased expression was diminished for the two miRNAs. MiR-21, miR-146b, miR-214, and miR-365 were not differentially expressed (Fig. 2E-H)

Discussion

Long term expression of miRNAs under IL-1β stimulation

The expression of miR-146a and miR-155 is of particular interest with their involvement

in NF-κB pathway (Iyer et al., 2012; Tarassishin et al., 2011). In astrocytes, an upregulation of miR-21, miR-146a, and miR-155 expression can be induced by IL-1β treatment after 24hr (van Scheppingen et al., 2016), which corresponds to our *in vitro* findings of the short term upregulation of miR-146a and miR-155 at 48hr. MiR-155, which is a potent inflammatory mediator, had a diminished expression trend after the robust increase at 48hr with IL-1β treatment, while the differential expression between untreated and treated groups remained after 4 weeks. On the other hand, miR-146a also had a similar diminished expression trend, but the differential expression between untreated and treated groups did not last up to 4 weeks. As for other two miRNAs, miR-21 and miR-365, the differential expression only showed up at 48hr but not further. Overall, this showed that IL-1β can affect the long term miRNA expression in human fetal astrocytes, with an increase of miR-146a and -155 expression up to 3 and 4 weeks respectively, while other miRNAs such as miR-21 and miR-365 were less affected in the long run.

Expression of miRNAs under metabolic stress conditions with and without IL-1β stimulation

In the stress response experiment, we did not observe an upregulation in –S-G groups compared to the controls for the ischemia-related miRNAs. We also noticed that the changes in ddct were marginal for these miRNAs. One explanation could be that the change in miRNA expression in ischemia requires time, and our treatment in 24hr is not sufficient to induce the ischemic-like changes in astrocytes. Even though –S-G condition models metabolic stress, it does not include hypoxic and anoxic aspect of ischemic pathology which may perhaps account for our expression observations. Interestingly, we found miR-210 to show similar trend of expression as miR-146a and miR-155 with an increased expression in the IL-1β treated group, which suggests that miR-210 may be an IL-1β-responsive miRNA. Comparing the two groups with IL-1β treatment and with or without glucose and serum, the diminished expression of miR-146a and miR-155 in the –S-G group indicates that the stress

condition attenuates the ability of astrocytes to react and respond to inflammatory stimuli, as demonstrated with IL-1β. This could be suggesting that the stressed astrocytes may be tuning down its inflammatory profile. As discussed earlier, reactive astrocytes can be categorized into A1 and A2 phenotype as described by Liddelow et al. (Liddelow et al., 2017). Although IL-1β alone is not sufficient to induce the inflammatory A1 phenotype of reactive astrocytes, ischemia-induced A2 phenotype has a distinct molecule profile from A1. We speculate that under the ischemia-like stress, astrocytes may not actively participate in inflammatory responses; astrocytes may just be reacting to stress or maintaining other essential functions over their participating in inflammatory responses.

Conclusion

In our experiments of IL-1β treatment on primary human fetal astrocytes, we found that there was an upregulation of many miRNAs including miR-146a and miR-155 with IL-1β treatment, which persisted for several weeks, showing a prolonged response after the IL-1β treatment. In our stress response experiment, we found the same two miRNAs were upregulated by IL-1β, but the increase was diminished by the stress condition when cultured without glucose and serum. It is not ideal to be comparing in situ profiles obtained under different pathological states with the in vitro profiles under inflammatory and stress conditions. However, we believe it will be of some interest to compare profiles from active lesions with those obtained in vitro under inflammatory and stress conditions (24-48hr). For example, we found miR-146a and -155 were downregulated in active lesions in situ. However, these two miRNAs were upregulated in fetal astrocytes upon IL-1β stimulation in vitro. Overall, not many miRNA expression profiles obtained in situ for the active lesions vs. normal controls were comparable to those from the in vitro studies. One can only speculate regarding these differences observed in situ vs. in vitro. It is not clear as to what degree IL-1β plays a role in MS pathology and how this affects the expression of astrocyte miRNAs, in

vivo. Also, it is quite possible that there exist many other factors that impact the lesion pathology. The overall miRNA expression of our *in situ* study indicates a less active role of astrocytes in inflammation during MS. The tendency of returning to normal level of expression in inactive lesions in several miRNAs also suggest that astrocytes try to maintain some normal functions after being stimulated in the early active stage.

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Addendum Figures

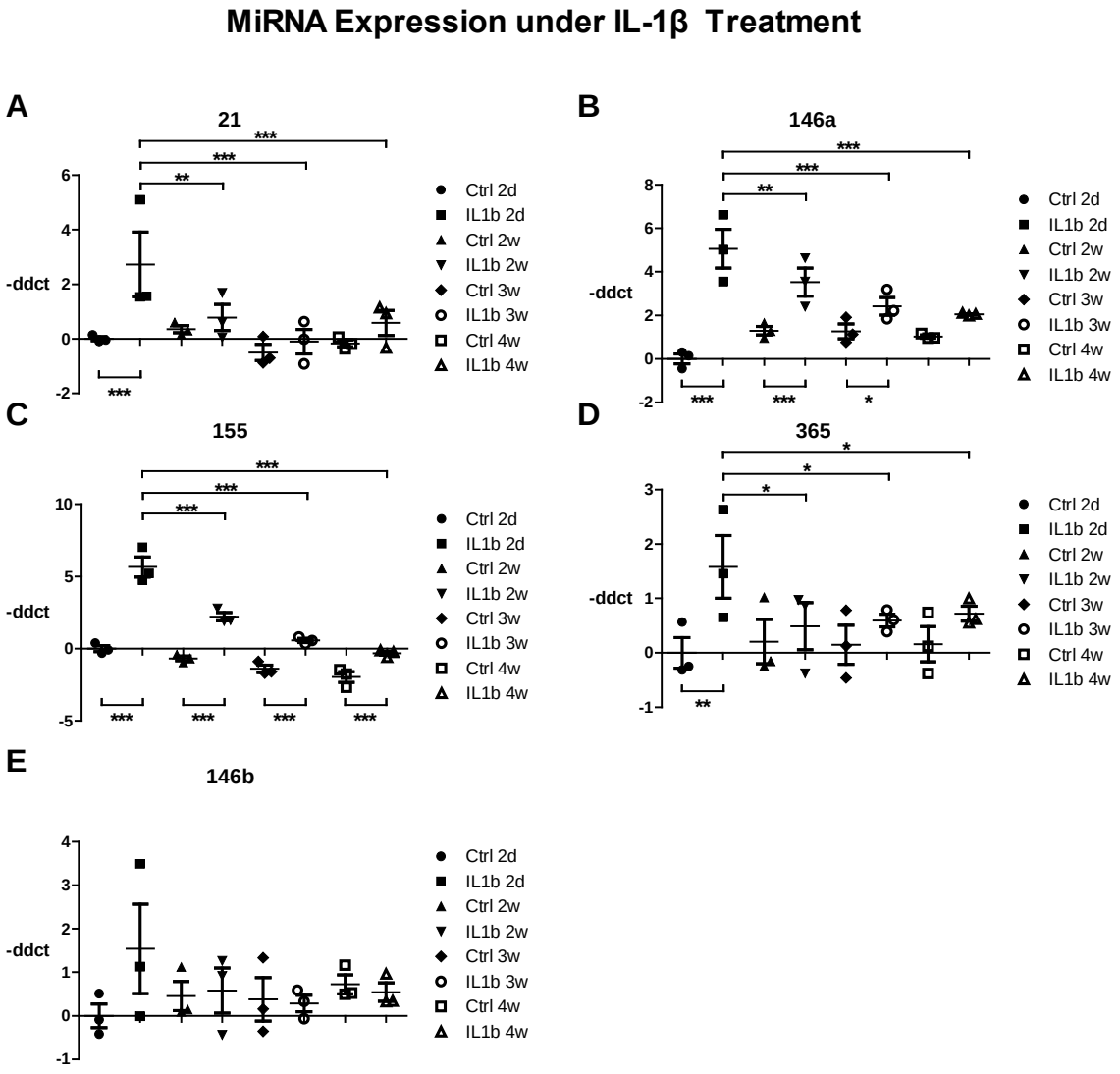

Figure 1. MiRNA Expression under IL-1β Treatment. The expression of miR-21 (A), -146a (B), -155 (C), -365 (D), and -146b (E) in cultured fetal astrocytes. Ctrl = untreated group; IL1b = IL-1β treated group; 2d = 48hr; 2w = 2 weeks; 3w = 3 weeks; 4w = 4weeks; -ddct = negative ΔΔ cycle time; *p < 0.05; **p < 0.01; ***p < 0.001

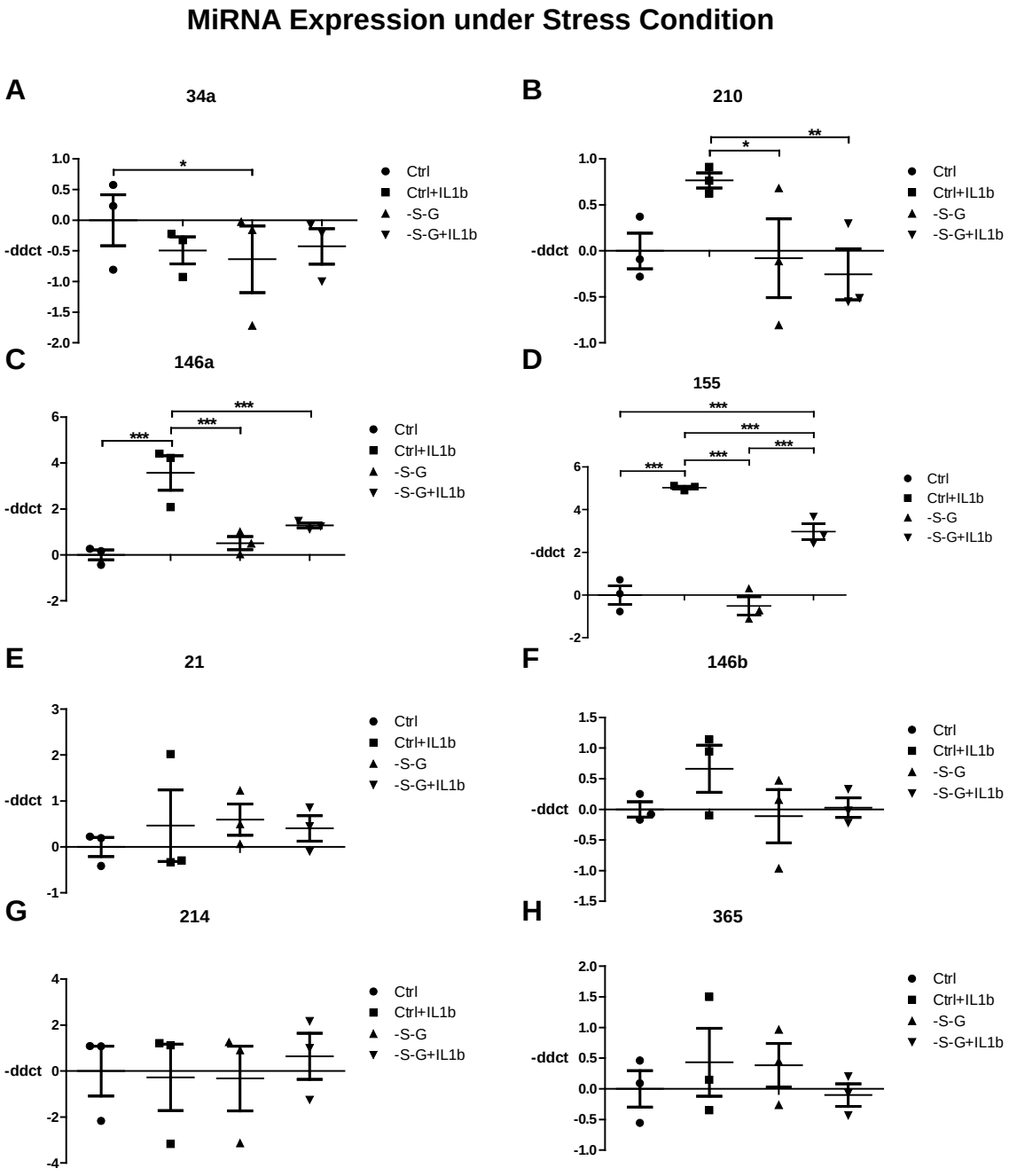

Figure 2. MiRNA expression in astrocytes under stress condition. Expression of miR-34a (A), -210 (B), -146a (C), -155 (D), -21 (E), -146b (F), -214 (G), and -365 (H) were shown in –ddct. Ctrl: DMEM with glucose and serum added. -S-G: DMEM without glucose and serum added. +IL-1b: With IL-1β added. -ddct = negative ΔΔ cycle time; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$