MicroRNA-210 Regulates the Metabolic and Inflammatory Status of Primary Human Astrocytes

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April 2021

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of science

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Abstract

Astrocytes are the most numerous glial cell type with important roles in maintaining homeostasis and responding to diseases. Astrocyte function is subject to modulation by microRNAs (miRs), which are short nucleotides that regulate protein expression in a post-transcriptional manner. Investigations into the miR expression profile of astrocytes in different diseases provides insight into the cellular stresses present and may uncover novel therapeutic targets. Here, we measured miR expression levels in astrocytes around human ischemic stroke lesions and observed increased expression of miR-210 in white matter chronic stroke astrocytes compared to astrocytes from control tissue. We aimed to understand the role of miR-210 expression in primary human fetal astrocytes by developing an *in vitro* assay of hypoxic, metabolic and inflammatory stresses. Combined induction of hypoxic and inflammatory stresses was observed to upregulate miR-210 expression. We found that transfection with miR-210-mimic (210M) increases glycolysis, enhances lactate export, and promotes an anti-inflammatory transcriptional and translational signature in astrocytes. Additionally, 210M transfection resulted in decreased expression of *C3* and *Sema5b*, indicative of a more neuroprotective astrocytic phenotype. We conclude that miR-210 expression in human astrocytes is modulated in response to hypoxic stress both *in vivo* and *in vitro*, corroborating the hypothesis that miR-210 is an indicator of ischemic stress in the human brain. In addition, our results show that miR-210 upregulation in stressed astrocytes is part of a protective cellular mechanism that results in a potentially neuroprotective astrocytic phenotype.

Résumé

Les astrocytes sont les cellules gliales les plus nombreuses du cerveau. Ils jouent un rôle important dans le maintien de l'homéostasie et réagissent aux situations anormales engendrées par des maladies. Les activités des astrocytes sont sujettes à des modulations par des micro-ARN (miR), des nucléotides de courtes séquences qui contrôlent l'expression de gènes de façon post-transcriptionnelle. L'étude du profil d'expression des miRs chez les astrocytes dans diverses maladies du cerveau pourrait permettre de mieux comprendre les stresses auxquels les cellules sont soumises, ainsi que la découverte de nouvelles cibles thérapeutiques. Dans la présente étude, nous avons mesuré l'expression de miR chez les astrocytes humains de lésions ischémiques et avons identifié l'expression de miR-210 comme étant régulé à la hausse dans la matière blanche d'ischémie cérébrale chronique. Nous avons élucidé le rôle du miR-210 chez les astrocytes humains fœtaux en développant des essais *in vitro* qui modèlent des conditions de stress hypoxique, métabolique et inflammatoire. Nous avons observé que l'induction combinée de stress hypoxique et inflammatoire mène à une augmentation de l'expression du miR-210 chez les astrocytes en culture. La surexpression du miR-210 par la transfection des cellules avec une mimique du miR-210 cause une augmentation de la glycolyse, l'exportation du lactate et l'expression de gènes anti-inflammatoires. De plus, une baisse de l'expression du *C3* et du *Sema5b* était indicateur d'un phénotype neuroprotecteur des astrocytes transfectés avec la mimique du miR-210. Nous concluons que l'expression du miR-210 chez les astrocytes sont modulés en réponse à un stress hypoxique tant *in vitro* qu'*in vivo*, confirmant que le miR-210 est un indicateur de stress ischémique dans le cerveau humain. Nos résultats montrent également que l'augmentation du miR-210 chez les astrocytes fait partie d'un mécanisme protecteur qui promue un phénotype neuroprotecteur des astrocytes.

Acknowledgements

I would like to begin by expressing my gratitude to my two supervisors, Dr. Luke Healy and Dr. Jack Antel. From the onset of my time in the lab, Dr. Healy went above and beyond to help me excel; he sat with me in the culture room hood to teach me how to culture stem cell-derived cells, he told me of his own experiences in academia and in pharma – focusing on what he had learned and how he had grown as a result – and he importantly listened to my own ideas with an open ear. Whether I was ranting about school, talking about my plans for my project, or simply recounting a funny story, Dr. Healy always had some witty Irish response. Dr. Jack Antel, although not physically next to me in the culture room hood, similarly provided me with unending project support and general mentorship. Additionally, Dr. Antel's stories about his own journey into medicine and science always put a smile on my face amid the countless difficulties and failures I experienced throughout my journey. Altogether, I want to thank Dr. Healy and Dr. Antel for their guidance and enthusiasm.

I also want to thank all members of my lab, both those that directly contributed to my Master's thesis and those that created the supportive and fun environment which gave me the motivation to keep coming in to lab every day. From our 8:30am lab meetings to the cramped birthday parties in the lunch room (pre-COVID), I am thankful for all of the memories that I was able to forge with such amazing people.

Finally, I want to thank my family and friends. Every Sunday evening, I Skyped my mom, dad, brother, and sister to rant about lab work, hear about the wild events occurring in Yardley, Pennsylvania, and (of course) see my dogs, all of which were all invaluable in maintaining my

sanity over the past few years. Similarly, the support from my girlfriend, Nicole, helped me enormously in completing my lab work and in making it through the stressful med school application process that we thankfully got to work through together. Additionally, all of my friends from Montreal, though many have left the city at this point, helped me develop the skills and passions that have led me to where I am today.

Contribution of authors

Rahul Suresh: Performed Western blots and analyzed Western data

Marie-France Dorion: Provided technical instruction and assisted with data collection

Manon Blain: Assisted with RNA isolation, qPCRs, ELISAs, Western Blots, and primary human astrocyte isolation

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Roberto Diaz: Provided supervision for Western blot experiments

Joshua Sonnen: Provided histopatological expertise and assisted in the creation of figures and tables

Samuel Ludwin: Provided histopathological expertise and assisted in the design of the research project

Jack Antel: Provided general guidance and direction for my project, and assisted in editing the thesis and manuscript

Luke Healy: Provided hands-on and hands-off support, assisted with experimental design, data interpretation, and provided significant assistance with the manuscript prior to submission.

Chapter I: Introduction

1.1 Astrocytes: A key cellular player in the Central Nervous System

The human brain consists of hundreds of billions of cells that interact with each other to create the most complex organ in the body. The two most dominant cell types are neurons and glia. Neurons propagate electrical and chemical impulses to direct the actions of the entire body (Aamodt, 2007). Glia, originating from the Latin term for "glue," were historically thought to act only as physical support for the neurons, lacking any complex roles that would affect brain health. Today, we understand that glia perform a vast array of functions that are necessary for neuronal and overall brain function. Glial cells can be classified into multiple different subtypes, the primary of which are microglia, oligodendroglia, and astroglia. Microglia act as the immune system of the central nervous system (CNS), which is critically important because macrophages and other innate immune cells are largely blocked from accessing the CNS. Microglia are also involved in a number of neurological processes, like synaptic pruning, which assists in learning and in brain development (Colonna & Butovsky, 2017). Oligodendroglia, often referred to as oligodendrocytes, ensheathe neurons in a fatty myelin coat that increases the efficiency of electrical signals sent down neuronal axons. Without proper myelin ensheathment, signals are often unable to reach their target destination, and thus patients are unable to adequately control their own body (Bradl & Lassmann, 2010). Finally, astroglia, similarly referred to as astrocytes, were the last of these primary glial cells to be properly recognized as critically important cells in proper neural function (Aamodt, 2007). Astrocytes are the most numerous glial cell type in the entire CNS (Brambilla, Martorana, & Rossi, 2013; Tower & Young, 1973), and the primary function of astrocytes has been defined as maintaining homeostasis in the brain. Each of these cells has important functions in brain function and health, but the role of astrocytes has only become appreciated since the turn of the

21st century. Therefore, the specific mechanisms by which astrocytes maintain brain health is still in the process of being elucidated.

1.1.1 Techniques Used to Study Astrocytes

The structure and function of all CNS cells, including astrocytes, have come to be understood through advances in *in vivo*, *in vitro*, and *in situ* experimental methods. Immunohistochemistry, used to identify astrocytes in tissue sections, have historically used glial fibrillary acidic protein (GFAP) to act as an identification marker for astrocytes, though more thorough experiments co-stain with S100B, as other glial cells have been shown to express either GFAP or S100B alone (Y. Chen & Swanson, 2003). These staining methods have led to a thorough understanding of how astrocyte morphology changes in different brain regions and disease states, but they have not provided much insight into the functional differences between astrocyte sub-populations. Over the past few decades, huge technological strides have been made that have increased our understanding of astrocytes. Two-photon imaging can provide a live snapshot of how astrocytes interact with other neuronal cells and how they respond to damage, electrophysiology experiments have led to a better understanding of how astrocytes are involved in glutamate and calcium signalling processes in brain tissue, induced pluripotent stem cell- (iPSC-) derived astrocytes allow for *in vitro* analysis of how human-like astrocytes respond to stress and different pharmacological interventions, and laser capture micro-dissection (LCM) is a way for researchers to capture astrocytes from *ex vivo* slices of brain tissue and subsequently investigate how astrocytes from different brain regions function (Almad & Maragakis, 2018; Rao et al., 2016). Together, these technologies have led to a greater understanding of different types of astrocytes throughout the brain and of their varying functions, both of which are described in the sections below.

1.1.2 Structure of Astrocyte Subtypes

Novel techniques are important in better defining the function of astrocytes, but staining techniques have provided researchers with a solid understanding of different astrocyte morphologies. There are three primary subtypes of astrocytes that have been identified: radial astrocytes, which surround ventricles, ; fibrous astrocytes, which are primarily located in the white matter and have a classical extended star-shaped morphology that allows the cells to provide metabolic support for numerous axons; and protoplasmic astrocytes, which are primary located in the gray matter and have a more compact, yet highly branched, morphology that allows for intimate connections with synaptic terminals (Kim, Park, & Choi, 2019; Ridet et al., 1996). Each of these astrocyte subtypes has its own specialized function, but they all share a number of key characteristics: they all associate closely with other neural cells including neurons, oligodendrocytes, and microglia; they all have key homeostatic functions (described in **section 1.1.3**); they all have end feet that protrude onto blood vessels to interact with molecules flowing through the blood; and they are all influenced by external cytokines which allows them to respond to different pathological states (described in **section 1.1.4**) (Kim et al., 2019).

1.1.3 Astrocytes in Homeostasis

Proper brain function is absolutely necessary for survival, and thus maintaining homeostasis is a critically important job. As the primary cell type responsible for maintaining homeostasis, astrocytes are understood to be some of the most important cells in the entire body. In order to promote health and prevent damage throughout the brain, astrocytes release antioxidants to mitigate oxidative stress, produce pro-/anti-inflammatory cytokines to respond to damage or

induce an immune response when necessary, and secrete neurotrophic and angiogenic factors when neurons are in need of additional nutrients. (Chung et al., 2013; Molofsky et al., 2014; Pentreath & Slamon, 2000; Sherwood et al., 2006; Tsai et al., 2012). The production of these molecules provisionally protects neurons from damage, but the role of astrocytes in maintaining homeostasis extends even beyond this. Astrocytes express their end feet against the blood brain barrier (BBB) to ultimately regulate which molecules have the ability to enter the brain (Alvarez, Katayama, & Prat, 2013). This association with the BBB and blood vessels allows astrocytes to take up glucose and oxygen directly from the bloodstream and shuttle these components directly to neurons. Further, astrocytes can break down their own stores of glucose into lactate and export it directly to neurons to act as a supplement energy source (Baltan, 2015). Astrocytes also form intimate connections with neurons to recycle neurotransmitters – which both increases efficiency of the metabolic demands of neurotransmitter production and prevents neurotransmitter-induced neurotoxic stress. Astrocytes are also active players in the growth/elimination of synapses, which is necessary for proper learning and involves their important interaction with microglia (Chung et al., 2013). The direct mechanism by which astrocytes regulate neurotransmitter recycling is through the formation of a tri-partite synapse, in which astrocytes extend their processes next to that of the pre- and post-synaptic terminals of two neurons and absorb excess neurotransmitters or modulate calcium signaling (Farhy-Tselnicker & Allen, 2018). Many of the functions of astrocytes described above of listed in **Figure 1.1**. The above findings have all been described only in the past 20 years, and though this has enormously expanded our understanding of the importance of astrocytes in health, much progress remains to be made to capitalize on using astrocytes to promote brain health.

1.1.4 Astrocytes in Disease

Up until the late 20th century, the general scientific consensus agreed that astrocytes had important homeostatic functions but were not involved in disease processes. Researchers believed that the morphological changes of astrocytes in disease was a nonspecific, relatively unimportant response to nearby neuronal damage. Even the formation of the glial scar, which is a hypertrophic response of astrocytes most frequently observed in ischemic stroke lesions that can easily be visualized histopathologically, was thought to be relatively unremarkable in its contribution to disease progression (Sofroniew & Vinters, 2010). Thus, the vast majority of researchers ignored astrocytes when trying to understand how neurological diseases progressed on a cellular and molecular levels. As the homeostatic functions described in **section 1.1.3** were being discovered, the role of astrocytes in disease was simultaneously being investigated. When astrocytic receptor aquaporin-4 (AQP4) was found to be the primary target of autoantibodies in NeuroMyelitis Optica (NMO), a disease that results in lesions very similar to that of multiple sclerosis (Lennon et al., 2004), researchers understood that loss of astrocyte function could indeed cause severe disease. This finding was around the same time that Alexander's disease, which causes severe brain damage, results in developmental disorders, and can often be fatal, was found to be caused by a mutation in the dominantly astrocytic protein GFAP (Li, Messing, Goldman, & Brenner, 2002). While there had been a growing belief that astrocytes may be involved in pathology, these seminal findings, along with other *in vitro* studies (Hinson et al., 2008), confirmed that astrocyte dysfunction can result in disease. Since then, continually emerging evidence suggests that astrocytes actively respond to a growing number of pathologies by altering their normal regulatory functions, adopting novel functions like expressing major histocompatibility complex class II (MHCII), and producing a broader array of cytokines than they do under non-pathological conditions. (Rostami et al., 2020;

Zeinstra, Wilczak, Streefland, & De Keyser, 2000). In major neurological and neurodegenerative diseases including stroke, multiple sclerosis (MS), Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS), astrocytes are phenotypically and functionally abnormal (Gonzalez-Reyes, Nava-Mesa, Vargas-Sanchez, Ariza-Salamanca, & Mora-Munoz, 2017; H. Phatnani & Maniatis, 2015; H. P. Phatnani et al., 2013; Zamanian et al., 2012). While astrocytes are clearly involved in disease, there remains contradicting evidence that astrocytes can both protect from disease and also further worsen disease status. Neuronal death can be inhibited with the addition of either live astrocytes or astrocyte-conditioned media (Gao et al., 2015; Lu et al., 2015), but neurotoxic astrocytes have also been documented that contribute to damage in disease (Liddelow et al., 2017). Astrocytes have also been found to produce both neurotoxic and neuroprotective cytokines in response to stress (H. Phatnani & Maniatis, 2015). Some of the astrocytic functions in disease are included in **Figure 1.2**. Astrocytes, therefore, are evidently critically important in the disease process, but their specific contribution to disease progression/clearance remains an active point of contention.

1.1.4.1 Astrocytes in Ischemic Stroke and Multiple Sclerosis: An Introduction

The astrocytic response, though important in all neurological disorders, varies in different pathological conditions. The two broad categories of the astrocytic phenotype under stressful conditions are scar-forming astrocytes and hypertrophic astrocytes (Khakh & Sofroniew, 2015). Ischemic stroke, specifically, is most notably characterized by astrocytic processes that form the glial scar. Alzheimer's disease, on the other hand, generates primarily hypertrophic pro-inflammatory astrocytes (Gonzalez-Reyes et al., 2017), while Multiple Sclerosis (MS) has often been documented to contain both subtypes. The variable response of astrocytes is driven by the

specific cellular stresses present. To understand the response of astrocytes to particular disease conditions, we must fully understand the disease process in question. The role of astrocytes in two diseases, MS and stroke, is rapidly evolving and holds significant promise to contribute to therapeutic efforts. While the MS and stroke disease processes are distinct, they both involve hallmark stresses that contribute to overall damage and cell death. Their disease responses both include the formation of a glial scar, increased BBB permeability, and increased signs of ischemic damage associated with the release of reactive oxygen species (ROS) (Bar-Or, Lau, & Winkler, 2000; Hoftberger & Lassmann, 2017; Paterno & Chillon, 2018). Though these aspects of disease are not an exhaustive list of either MS or ischemic stroke, they are some of the hallmark findings, and astrocytes are thus crucially involved in their pathogeneses.

1.1.4.1.1 Multiple Sclerosis Etiology

Multiple Sclerosis is defined as an autoimmune disease, in which myelin-reactive immune cells infiltrate the brain and result in neuronal and oligodendrocyte loss (Kurtzke, 2005). Both genetic and environmental factors have been attributed to MS onset, as multiple HLA molecules are associated with disease risk, and lack of Vitamin D levels is similarly associated with disease onset. A number of other factors, including smoking, cancer, and even stroke itself increase the risk that one will develop MS. MS is also more common in women than in men, though the reason for this difference remains to be fully elucidated. Additionally, MS is most often diagnosed in people aged 15-45 years old (Kurtzke, 2005). As immunological understandings have progressed over the past few decades, it has become apparent that the immune system is overactive in the majority of MS patients. Immune cells can directly cause inflammation and cell-death, while also activating other cells in the brain – namely astrocytes and microglia – to increase their production of inflammatory

molecules and cause further cell death (Gandhi, Laroni, & Weiner, 2010). This internal damage and inflammation increases the permeability of the BBB, thereby increasing immune cell infiltration which amplifies the inflammation in the brain microenvironment (Liddelow et al., 2017). Whether this causes disease or is merely a response to another viral, genetic, or environmental factor, remains unknown.

1.1.4.1.2 Multiple Sclerosis Pathology and Symptoms

The etiology of MS remains elusive, but the pathogenesis of MS has been well defined. Patients are most commonly diagnosed with relapsing-remitting MS (RRMS), which involves symptoms of motor dysfunction, temporary vision loss, fatigue, and a multitude of others (Mount, 1973). Patients suffering from RRMS experience an episodic disease course, characterized by infrequent onset of symptoms, followed by extended periods of remission (Mount, 1973). For the vast majority of MS patients, however, this RRMS eventually evolves into a progressive form of the disease, termed secondary-progressive MS (SPMS), which is characterized by more muscle dysfunction, pain, and a continuous accumulation of disability in the absence of relapses (Goldenberg, 2012). The continuous damage of SPMS causes neuronal death, impairing signaling from the brain to the rest of the body. For many MS patients, this results in failed signalling from the CNS to the lungs, an inability to control the bladder that leads to bladder infections, and difficulty swallowing that can result in choking, any of which can ultimately lead to death (Sumelahti, Hakama, Elovaara, & Pukkala, 2010; Tzelepis & McCool, 2015).

1.1.4.1.3 Multiple Sclerosis Treatments

The early, neuroinflammatory portion of the disease is characterized by over-active immune cells and reactive glial cells, namely astrocytes and microglia, that produce inflammatory cytokines and reactive oxygen species (ROS) and damage surrounding tissue (Liddelow et al., 2017). These events during the RRMS portion of disease have been the target of the majority of MS therapeutics. Drugs include the use of anti-inflammatories such as interferon-beta, the effects of which result in lower MS morbidity, that likely acts through minimizing the effector potential of T and B cells (Goldenberg, 2012). Similarly, fingolimod is an MS therapeutic that retains adaptive immune cells in the lymph nodes to avoid their recruitment to the brain (Kowarik et al., 2011). Overall, these drugs aim to minimize brain inflammation by targeting immune cells (Chun & Hartung, 2010; Jacobs et al., 2000). These drugs successfully slow progression from RRMS to SPMS, but they have little to no impact on the progressive form of the disease (Chun & Hartung, 2010). Once the disease extends into the SPMS stage, anti-inflammatories fail to combat the damage that occurs, though the reason remains unclear. The lack of current drug efficacy suggests that a new approach to MS therapy may be required for substantial improvements. One relatively novel aspect of multiple sclerosis pathology is the ischemic stress that is present. Ischemia, or restricted blood flow that results in a lack of energy and oxygen availability, has been found to be present in cells in and around MS lesions (Aboul-Enein et al., 2003; Mahad, Ziabreva, Lassmann, & Turnbull, 2008). This type of stress is different from the inflammatory stress that is currently being targeted with approved MS therapeutics and holds promise as a novel treatment target for MS patients. Ways to mitigate ischemic stress include inducing angiogenesis (the growth of new blood vessels) through the expression of growth factors like vascular endothelial growth factor (VEGF). Other mechanisms include increasing metabolic efficiency of cells or decreasing the non-essential use of oxygen and glucose (Warlow, 2003).

1.1.4.1.4 Astrocytes in Multiple Sclerosis

Astrocytes are involved in the production and regulation of inflammatory cytokines in MS, and they can also shuttle nutrients (including glucose, lactate, and fatty acids) to nearby neurons and oligodendrocytes (Ludwin, Rao, Moore, & Antel, 2016). In addition to directly contributing to the inflammatory status of the brain in MS patients, they also regulate the permeability of the BBB and control which cells and factors are able to cross the BBB. Recently, the concept of "A1" and "A2" astrocytes has emerged, in which astrocytes have been found to be either neurotoxic (A1) or neuroprotective (A2) (Liddelow et al., 2017). MS patient brains have been examined for these factors, and multiple studies have concluded that patients appear to have more A1 than A2 astrocytes. The A1 astrocytes not only contribute to inflammatory molecule production, but they also produce more neurotoxic factors like Complement 3 (C3), which causes synaptic breakdown and neuronal cell death. Other groups challenge the existence of a "good" and "bad" astrocytic phenotype (Escartin et al., 2021), but what is clear is that astrocytes are functionally distinct in MS patient brains compared to brains of otherwise healthy individuals. The inflammatory contribution of astrocytes suggests that they are involved in the current treatment strategy used against RRMS, but their metabolic involvement with the rest of the brain highlights them as potential players in the more novel ischemic aspect of MS that may be treatable. Importantly, ischemic stress is not unique to MS lesions. The most pertinent ischemic disease is ischemic stroke.

1.1.4.2 Ischemic Stroke

Ischemic stroke is an incredibly complex and multifaceted disease that remains one of the world's leading causes of morbidity and mortality (Brouns & De Deyn, 2009). The World Health

Organization defines stroke as a cerebral deficit that occurs suddenly and which lasts over 24 hours, with no apparent cause other than a vascular blockage.

1.1.4.2.1 Ischemic Stroke Etiology

Ischemic stroke, although only one type of multiple subclassifications of stroke, accounts for the majority of all stroke cases and results from blood vessel occlusion that causes a lack of oxygen and nutrient delivery to local cells within the brain. The cause of this blockage can be from a vast number of factors, but one of the most common is heart disease, by which high blood pressure can result in thrombosis of a blood vessel (Warlow, Sudlow, Dennis, Wardlaw, & Sandercock, 2003). High blood pressure is associated with stroke even more closely than it is with ischemic heart disease (Lewington et al., 2002). Other factors, like cancer, diabetes, atrial fibrillation, and in fact MS can also increase risk of ischemic stroke (Krafft et al., 2012).

1.1.4.2.2 Ischemic Stroke Pathology

Ischemic stroke has a number of risk factors associated with human life that are not necessarily reproducible in a lab setting. The disease itself – a blockage that restricts oxygen and glucose access to the brain – however, is easily reproducible in animals. A common methodology used to study stroke has been the middle cerebral artery occlusion model, or MCAo, in mice. Because of this model, and because of imaging techniques in humans, we can appreciate many aspects of the ischemic stroke pathology. Following an ischemic stroke (or in the induced artery occlusion in animal models), oligodendrocytes, astrocytes, microglia, neurons, and endothelial cells adapt their cellular processes and energy metabolism to the new and stressful demands driven by the ischemic environment. Cells most directly affected by the ischemic lesions will experience the most cell

death, but the area surrounding the lesion, often referred to as the penumbra, has a variable size depending on the time- and severity-extent of the ischemic injury. The duration and significance of the occlusion determines the amount of damage that occurs, ranging from mild changes in cell function to cell death (**Figure 1.3)**. Additionally, the early stages of disease, termed the acute stages, which lasts for hours-days post-injury, involve severe metabolic, hypoxic, and – once immune cells begin responding to the damage – inflammatory and necrotic factors, all of which lead to cell death (Dirnagl, Iadecola, & Moskowitz, 1999). As the days progress since the initial injury, the cells respond to the longer-term changes, adapting their metabolism and cellular functions to the new environment, which often experiences less comparative blood flow. This chronic phase of the disease contains cells that are permanently altered post-stroke and study of these cells is an active area of interest (Kang, Latour, Chalela, Dambrosia, & Warach, 2004).

1.1.4.2.3 Ischemic Stroke Treatments

Clinical treatment of ischemic stroke is almost always focused on thrombolysis to remove the vessel occlusion, but for the majority of patients thrombus removal is not accomplished until some level of cell stress or cell death occurs (Meretoja et al., 2014). Because of this, post-thrombolytic therapies have aimed to promote repair and minimize damage in the more chronic stages of stroke disease. Neurons have been the primary focus of these targeted post-thrombolytic therapies, but over 1,000 neuron-targeted clinical trials have failed. Beyond these targeted-therapies, post-stroke treatment often involves aspirin, simply as a measure to minimize the chance of blood clotting that can result in repeated vessel occlusion. To treat patients after an ischemic stroke most effectively, novel therapies must be investigated.

1.1.4.2.4 Astrocytes in Ischemic Stroke

Due to how numerous astrocytes are in the CNS and their responsibility to maintain homeostasis, it is no surprise that astrocytes are involved in stroke. This was one of the first instances where the role of astrocytes was first appreciated, as they form a distinct glial scar directly around the most affected lesion sites. While this almost ensures the death of all cells directly around the lesion, it functions to block off as many of the necrotic, inflammatory factors from spreading around the rest of the brain parenchyma (L. Huang et al., 2014). In addition to formation of the glial scar, the homeostatic functions of astrocytes reviewed in **section 1.1.3** help explain why astrocytes are so critical for cell health in ischemic stroke patient brains. Astrocytes are directly involved in oxygen processing for neurons, can export lactate for an additional neuronal energy source, and regulate inflammatory cytokines to try to keep the neurons healthy (Baltan, 2015; Y. Chen & Swanson, 2003). Therefore, astrocytes represent a potential therapeutic target for stroke. Their role in maintaining the blood brain barrier allows them to directly access any drugs that attempt to enter the parenchyma, and they may use these treatments to then promote health in the brain after injury. The difficulty, however, is finding a way to promote astrocytic – and by proxy, neuronal – health, past the confines of the blood brain barrier and surpassing the difficulty of promoting health during extreme cellular stress.

1.2 microRNAs (miRs)

Astrocytes in different brain regions and in different diseases, including MS and stroke, are unique to astrocytes located elsewhere in the brain (**section 1.1**). There are many ways that astrocytes can alter their homeostatic functions in response to disease, but one method is by modulating their microRNA (miR) expression profile. miRs are ~22 nucleotide strands of RNA that post-

transcriptionally regulate protein expression (Saliminejad, Khorram Khorshid, Soleymani Fard, & Ghaffari, 2019).

1.2.1 miR Discovery and Function

MicroRNAs were discovered in 1993 when a gene in *C. elegans* was found not to code for a protein, but instead to produce the lin-4 miR that functions by binding the 3' untranslated region (3'UTR) of its target mRNA lin-14 to regulate larval development (Lee, Feinbaum, & Ambros, 1993). The function of lin-4, though unclear at first, was found to inhibit the translation of lin-14 through this binding to a non-coding portion of the lin-14 transcript. This discovery not only paved the way for our understanding of miRs, but also revolutionized the entire field of molecular biology, as non-coding portions of RNA were finally appreciated. Today, the complex cycle of miR transcription, processing, and target degradation/inhibition is well understood. Briefly, microRNAs encoded in the genome are transcribed into pri-miRs with a hairpin structure. Pri-miRs are cleaved into pre-miRs that associate with a protein DROSHA and the DROSHA-pre-miR complex is exported to the cytoplasm of the cell and is cleaved with DICER to produce two strands of nucleotides that are not physically bound by a hairpin structure. One of these two strands then gets loaded into the RISC protein complex, which closely associates with the target mRNA that has sequence complementarity with the miR (Y. Huang et al., 2011) (**Figure 1.4**). If this binding results in perfect sequence complementarity, the mRNA is degraded, whereas if there is incomplete complementarity, the mRNA is not translated but remains intact. Approximately one third of the entire human genome is thought to be regulated by at least one miR (Urbich, Kuehbacher, & Dimmeler, 2008). The majority of this binding is determined by the seed region of the miR, which is a sequence of 8 nucleotides towards one end of the miR (Hill, Jabbari,

Matyunina, & McDonald, 2014). Since their discovery almost 30 years ago, miRs have developed into a great therapeutic interest, as their ability to post-transcriptionally regulate many mRNAs enables them to be involved in many homeostatic or pathologic functions.

1.2.1.1 Methods to Investigate miR Function: *In Silico*

miRs function by binding complementary nucleotides on corresponding mRNAs (Hill et al., 2014). Because of this mechanism of action, predictive algorithms have been created that calculate the likelihood that particular genes may be influenced by particular miRs. Gene ontology tools can expand these predicted genes into entire pathways. These algorithms are constantly expanding, using *in vitro* experiments to confirm or refute their predictions, which then leads to more refined searches. One of the newest and most comprehensive of these tools is MiRabel, a bioinformatic tool that pools multiple algorithms together to obtain the most reliable miR targeting predictions (Quillet et al., 2019). Using MiRabel, the functions of miRs can be predicted, though experimental validation with *in-vitro* techniques is still required to confirm that the predicted target is indeed under the control of a particular miR.

1.2.1.2 Methods to Investigate miR Function: *In Vitro*

In order to conduct experiments that investigate the role of a miR in particular situations or cell types, well-controlled, highly specific assays must be performed. miRs regulate the majority of the genome, and thus any modulation of the miR pathway will likely have non-specific effects that extend beyond the single miR of interest (Ohtsuka, Ling, Doki, Mori, & Calin, 2015). Additionally, miRs are often encoded in introns of genes, and thus knocking them out of the genome entirely must be done with caution to avoid affecting the closely located mRNA-coding piece of DNA.

Because of the very small size of miRs, the most effective overexpression and inhibition models are using miR-mimics and miR-inhibitors, respectively. miR mimics are double stranded RNA fragments that have the exact same sequence as endogenous miRs (Jin et al., 2015). They are often chemically modified to promote the use of one particular strand (either the -3p or -5p strand) for association with the RISC complex. Because the miR-mimic has the same nucleotide sequence as the endogenous miR, it effectively acts as an overexpression model (Jin et al., 2015). To inhibit miRs, single stranded DNA (for increased stability) can be transfected into cells, which will bind the miR-RISC complex and stop the endogenous miR from acting (Chabot et al., 2012). Because the miR modulation process (either with mimics or inhibitors) involves transfection with double stranded RNA or single stranded DNA, the appropriate RNA or DNA controls (in the correct buffer) must be used. Similarly, the dosage and timing controls must also be taken into account during optimization experiments. After transfection with miR-mimic/-inhibitor and the appropriate controls, the general impact on cellular function or activity of particular pathways can be examined by any assay. That is, the effects of miR modulation may have effects on inflammatory cytokine production, metabolism, survival, and other aspects of cells. To know the specific downstream targeting of miRs at a protein level, assays to investigate the very specific downregulation of miR targets are the best ways to demonstrate that the miR-mimic/inhibitor is having specific effects.

The techniques used to experimentally validate a miR-mRNA interaction can vary. They can include Western blotting or qPCR, showing that the protein or mRNA is downregulated/upregulated with overexpression/inhibition of the miR, but the most specific and thorough method is a dual-luminescence assay (Clement, Salone, & Rederstorff, 2015). The dual-luminescence assay is one in which a plasmid containing two luminescent signals, a Firefly signal

and a Renilla signal, is transfected into cells along with the miR-mimic. To determine the function of a miR, the Firefly signal has a genetic sequence inputted next to the Firefly Luminescent coding region that is thought to be targeted by a miR, whereas the Renilla signal has no such sequence. Therefore, if a miR targets the predicted sequence (which is that of an mRNA of interest), then the Firefly signal will be downregulated, whereas the Renilla signal will remain constant between transfected and non-transfected cells.

1.2.2 miRs in Disease

The transition from understanding the biology of microRNAs to fully realizing their role in disease states was not immediate and is still in the process of being fully elucidated for many diseases. The relationship between miRs and diseases became most obvious when single point mutations in the miR processing system were found to be associated with diseases like Fragile X Syndrome (Caudy, Myers, Hannon, & Hammond, 2002; Soifer, Rossi, & Saetrom, 2007). Since this discovery in 2002, miRs have been explored in other human diseases for which their direct processing mechanisms are not involved. Understanding the miR profile of diseases, either in particular cells or in the blood of patients, has proven useful for disease diagnosis, prognosis, treatment efficacy predictions, and for more defined classifications of the disease (de Planell-Saguer & Rodicio, 2011).

The disease most studied in regard to the role of miRs has been cancer. As a disease that thrives on mutations that result in the over-inhibition of tumor-suppressor genes or the over-activation of proto-oncogenes (Hanahan & Weinberg, 2000), cancer was an early candidate for which miRs may be important. Indeed, miRs have been found to be dysregulated in nearly every cancer type

ever discovered, as the vast inhibitory potential of miRs allows them to over- or under-inhibit pathways that may assist in tumor growth (McManus, 2003). The usefulness of miRs in disease extends beyond just their dysregulation in particular cells, as they have the potential to act as biomarkers for various diseases. The small size of miRs makes them relatively stable and capable of being packaged into exosomes, small vesicles that can travel throughout the body. Today, miRs have been used as biomarkers in various diseases, either as an indication of presence of disease, or as a correlation for disease severity. The role of miRs as biomarkers has been studied in cancer, multiple sclerosis, cardiac disease, renal disease, and other pathologies (de Planell-Saguer & Rodicio, 2011).

More recently, as technologies have allowed more precise analysis of microRNA expression levels in different tissues and cell types, researchers have begun to investigate the differential miR expression between normal and diseased tissues/cells beyond just cancer. In line with their ability to target a wide number of mRNAs, miRs appear to be involved in a number of disease process. miR dysregulation has been found in diseases such as neurological disorders and in respiratory cells in patients with chronic obstructive pulmonary disease (COPD) (Du et al., 2019). Further, miRs have recently been explored for their use as therapeutics. Promising therapies that promote survival of cells, prevent cancerous growth, or target the disease in a separate mechanism are being developed today. The two diseases which we spoke about previously are no different – miRs have the potential to be treatment targets for both MS and stroke. To do this, the miR expression profile of cells in these diseases must be fully characterized.

1.2.2.1 miRs in Multiple Sclerosis

MiRs have been found to alter inflammatory cytokine production, metabolism of cells, and production of neuroprotective/neurotoxic molecules (Z. Chen, Li, Zhang, Huang, & Luthra, 2010; He et al., 2019; Selvamani, Sathyan, Miranda, & Sohrabji, 2012), all three of which are implicated in MS pathogenesis (Hoftberger & Lassmann, 2017). MS is a disease that involves peripheral immune cells (T cells, B cells, macrophages, etc.), peripherally derived cytokines that can enter the CNS, and the local cells of the brain. Each of these components has been investigated to understand its contribution to MS pathology at a miR level. miRs that block B and T cell activation (miR-17 and miR20a) have been found to be decreased in MS patient blood samples (Cox et al., 2010). In peripheral blood mononuclear cells (PBMCs), precursors to immune cells in the body, expression of miR 146a, 146b, and 21 were increased in RRMS patients (Otaegui et al., 2009). The overall miR expression profile of immune cells appears to promote immune cell activation and block the production and activity of regulatory T cells (Otaegui et al., 2009). This miR expression thus regulates the activity of the peripheral immune cells and the cytokine production that effects disease progression (de Faria et al., 2012). Additionally, because miRs are essential for CNS function, including neurite outgrowth and oligodendrocyte differentiation (Krichevsky, Sonntag, Isacson, & Kosik, 2006; Vo et al., 2005), their dysregulation in CNS cells also contributes to neurological disease. In studies of MS lesions, about 50 miRs have been found to be upregulated in cells inside or in the rim of MS lesions, when compared to normal appearing white matter (NAWM), and about 30 miRs have been found to be downregulated in these same lesions (Junker et al., 2009; Noorbakhsh et al., 2011).

The miR expression of astrocytes in MS lesions has been investigated in two major studies. The first of these processed whole brain tissue of MS patients and normal patients and examined the

mRNA and miR expressions compared to normal tissue (Junker et al., 2009). Junker et al. reported that over 30 miRs were differentially expressed between cells around MS lesions compared to cells in normal tissue, and that astrocytes are capable of expressing a multitude of these miRs (Junker et al., 2009). Importantly, this study did not define the miR expression profile of astrocytes in particular, as it instead obtained the expression profile of cell clusters in white matter tissue. It was not until 2019 that the miR expression profile of astrocytes around MS lesions was specifically defined using laser capture micro-dissection (LCM) (Rao et al., 2019). The Rao et al. study identified a separate panel of differentially expressed miRs between MS and normal astrocytes compared to the Junker study, thereby pointing to the importance of investigating miR expression profiling in particular cell types (Rao et al., 2019). miR-210, which has previously been associated with hypoxia, was found to be upregulated in astrocytes around MS lesions (Rao et al., 2019). This finding supports the previous evidence that cells around MS lesions are actively responding to ischemic stress (Hoftberger & Lassmann, 2017). While the increased expression of miR-210 adds to the growing evidence that miRs are important regulators of cells in pathological conditions, the exact function of miRs - including miR-210 - in astrocytes around MS lesions remains unknown. *In silico* approaches exist to predict the targets of miRs, but the precise biological effects must be confirmed experimentally. It may be through changing their miR expression that astrocytes are able to quickly adapt their protein expression to the sudden cellular stresses of disease.

1.2.2.2 miRs in Ischemic Stroke

Similar to MS, miRs have been investigated in stroke patients and in MCAo animal model of stroke. Because stroke is generally a less chronic disease, there have been fewer human studies examining the miR expression profile in the periphery or in the CNS in the short time period

following an ischemic stroke. miR dysregulation previously discussed in **Section 1.2.2** finds that miRs are involved in cardiac disease, renal dysfunction, and other diseases that increase risk of an ischemic stroke, but miRs are also involved in the more direct pathophysiology of stroke, playing a role in the successful patient outcome and severity of disability following the event. miRs-21, -99a, and -497 miR-mimic treatment in rats with MCAo stroke models were found decrease ischemic stroke volume and lead to better outcomes. miR-210 overexpression has been found to increase angiogenesis and is currently undergoing investigation in mouse models of stroke. Other miRs, like miR 21, have been reported to inhibit apoptosis in stroke disease models, and may thus be useful in prolonging cell survival until other treatments can be used (Xu et al., 2018). Ultimately, the potential therapeutic effect of miRs is a relatively novel field that requires additional exploration. The promising findings that miRs are undoubtedly involved in stroke pathogenesis, and that particular miRs can promote cell survival and injury repair, gives hope that current research efforts will lead to a future treatment strategy.

1.3 Objectives

In this research project, we aimed to investigate the miR expression profile of astrocytes around ischemic stroke lesions and compare it to that of astrocytes around MS lesions (Rao et al., 2019). Upon discovery that miR-210 was upregulated in astrocytes around both MS and ischemic stroke lesions, we sought to elucidate the role of miR-210 in human astrocytes. To do this, we developed a stroke- and MS-relevant *in vitro* stress assay using primary human fetal astrocytes and used a miR-mimic transfection approach to model increased cellular miR-210 expression. With these models, we investigated miR-210-mediated modulation of human fetal astrocyte i) metabolism, ii) inflammatory cytokine production, and iii) neuroprotective molecule production. Specifically, we

measured oxygen consumption rate, glycolysis, lactate export, pro- and anti-inflammatory cytokine expression, and expression of neurotoxic/neuroprotective genes. Altogether, our research aimed to better understand the role of astrocytic miRs that are upregulated in disease conditions.

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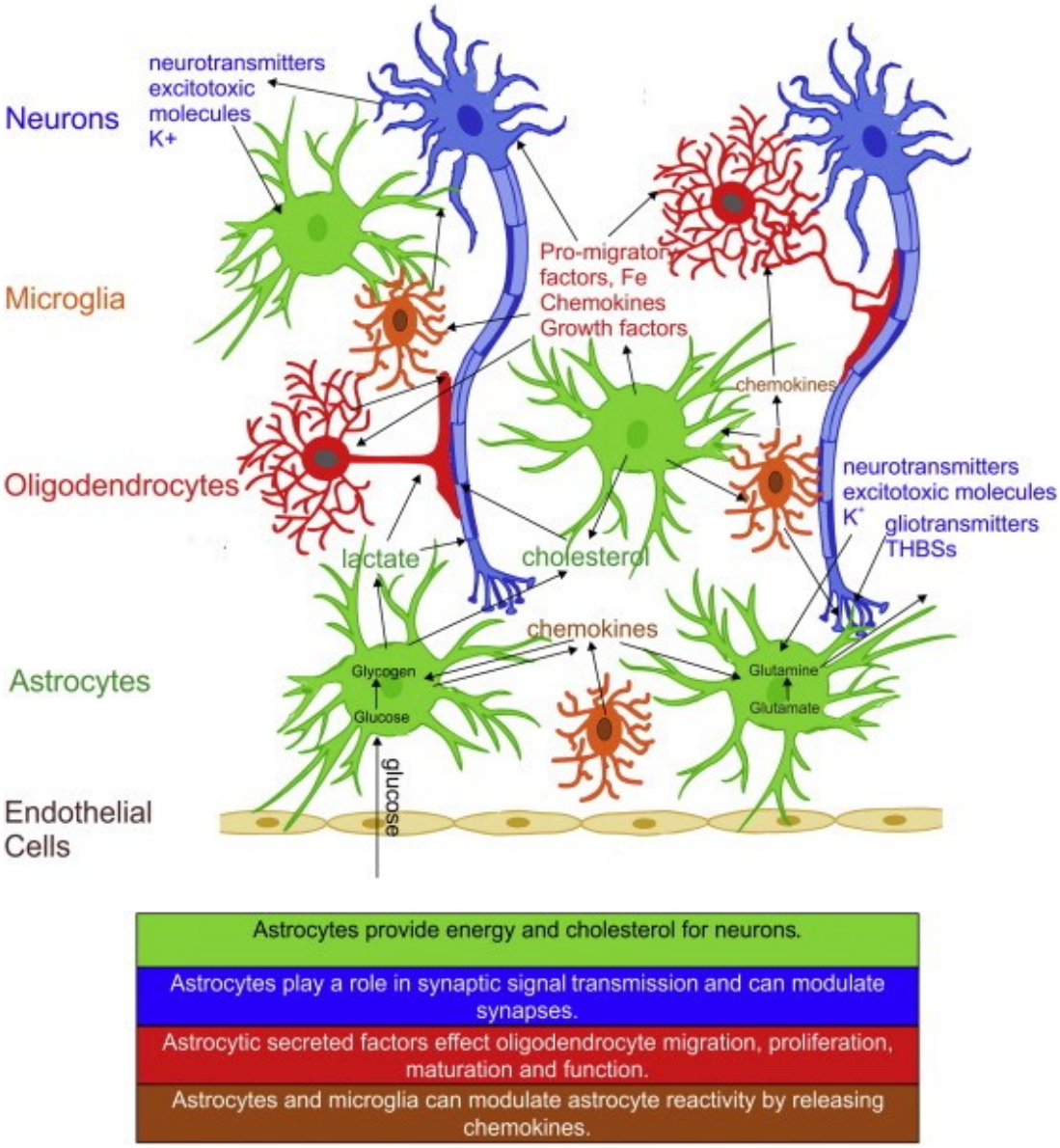
Figures:

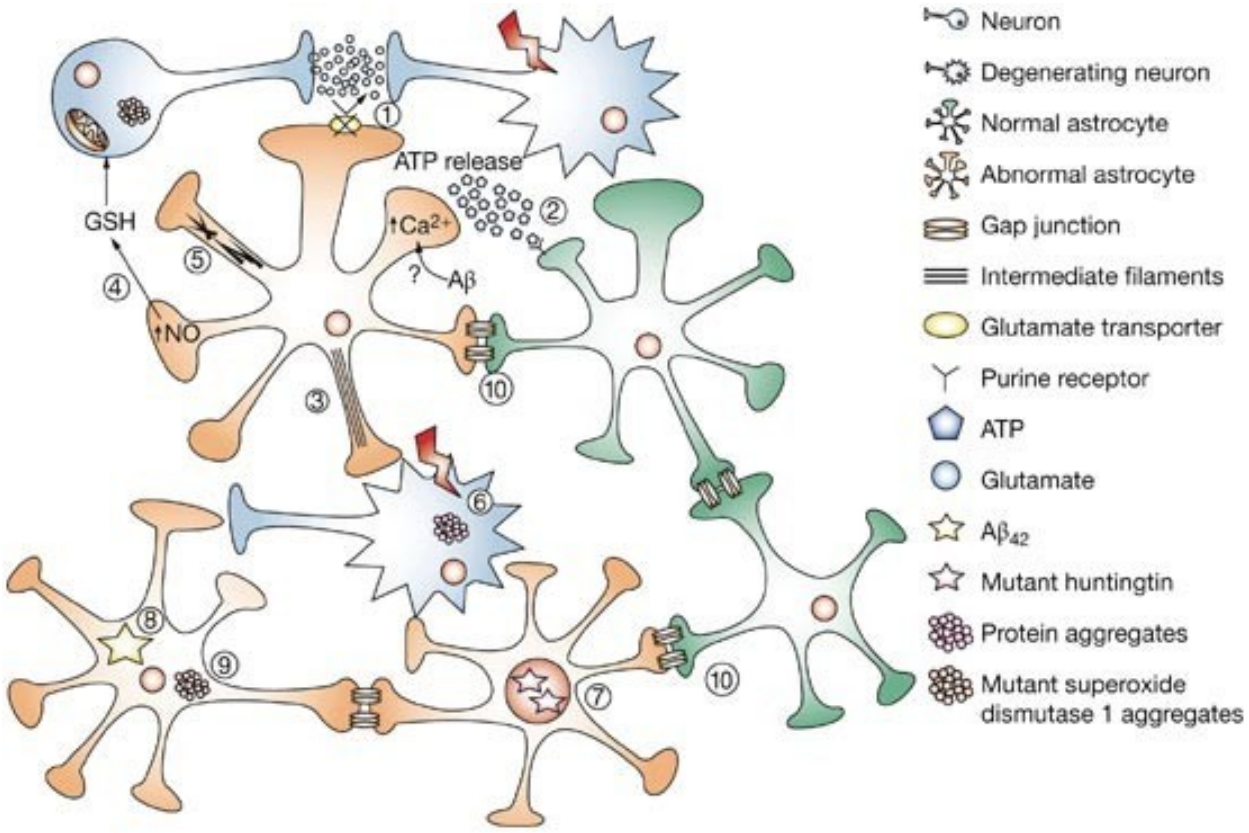
Figure 1.1:



(Kiray, Lindsay, Hosseinzadeh, & Barnett, 2016)

Figure 1.1: **Astrocytes perform numerous homeostatic functions, some of which require interactions with other cells**. Astrocytes (green) are shown interacting with numerous other cells to exert their homeostatic functions. From top to bottom, Astrocytes can take up neurotransmitters to prevent neurotoxicity, they can secrete chemokines and cytokines that impact oligodendrocytes (red) and neurons (blue), can secrete factors to microglia (brown) and also receive factors from microglia, and they can interact with endothelial cells to uptake glucose.

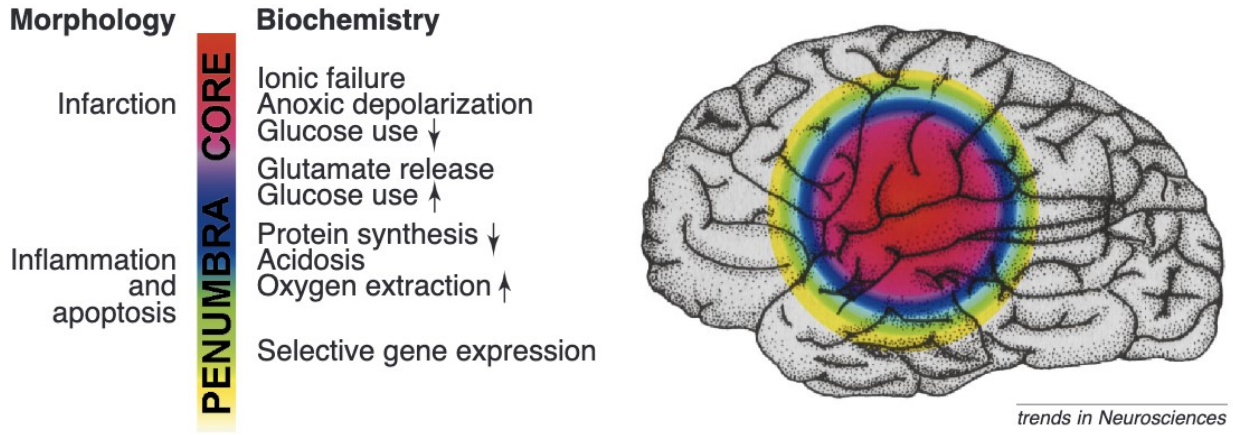
Figure 1.2:



(Maragakis & Rothstein, 2006)

Figure 1.2: **Astrocytes alter their functions in neurodegenerative diseases**. 1) Abnormal astrocytes (orange) fail to reuptake glutamate which is necessary to prevent neurotoxicity and is performed by normal astrocytes (green). 3) Astrocytes processes hypertrophy, extending outward through an increased production of glial fibrillary acidic protein (GFAP). 4) Abnormal astrocytes increase their production of antioxidants, decreasing neuronal oxidative damage from reactive oxygen species (ROS). 10) Astrocyte communication can be altered when in abnormal states, resulting in different cytokine and chemokine production. Although other numbers are included, only those discussed in the main text were described in the figure.

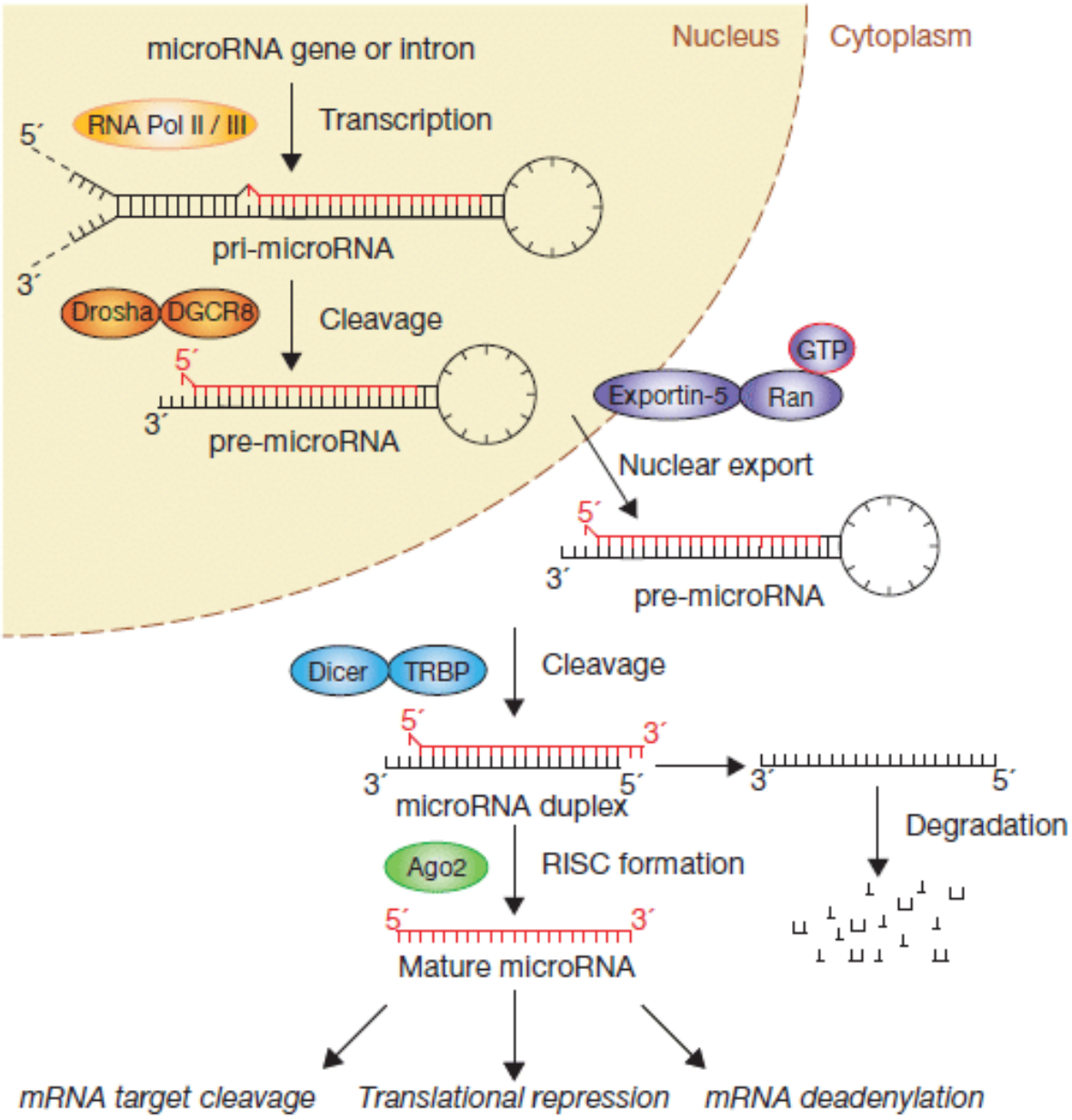
Figure 1.3:



(Dirnagl et al., 1999)

Figure 1.3 Ischemic Stroke results in various layers of disease severity. The left of the image describes the morphological and biochemical changes following a stroke. In the core of the ischemic lesion (purple-red), severe glucose drop and increase glucose usage for survival is exhibited, so many cells run out of the necessary glucose and die from necrosis. Further away from the core is the penumbra (blue-yellow), in which cells can more reasonably respond to the stress by decreasing protein synthesis, obtaining more oxygen from the blood, and expression survival genes, which partially causes inflammation and apoptosis. (Right) is a sample brain with the core and penumbra defined as being varying distances away from the ischemic site.

Figure 1.4:



(Tran et al., 2013)

Figure 1.4 MicroRNA Processing, from Transcription to Functionality. From top to bottom, a microRNA gene or the intron of a messenger RNA (mRNA) gene is transcribed into a pri-microRNA. Cleavage by DROSHA results in a pre-microRNA, which then exports the nucleus. The pre-microRNA's hairpin structure is cleaved by DICER to result in two complementarily bound strands of RNA. One of these strands is chosen by the Ago2 protein to create the RISC formation (red), while the other strand is left for degradation. The RISC-microRNA complex targets mRNAs and results in mRNA cleavage, translational repression, or mRNA deadenylation.

Chapter II: Manuscript

Title: MicroRNA-210 Regulates the Metabolic and Inflammatory Status of Primary Human Astrocytes

Short Running Title: The role of microRNA-210 in astrocytes.

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This manuscript was submitted to GLIA on February 28th, 2021

Abstract

Astrocytes are the most numerous glial cell type with important roles in maintaining homeostasis and responding to diseases in the brain. Astrocyte function is subject to modulation by microRNAs (miRs), which are short nucleotides that regulate protein expression in a post-transcriptional manner. Understanding the miR expression profile of astrocytes in disease settings provides insight into the cellular stresses present in the microenvironment and may uncover pathways of therapeutic interest. Here, we measured miR expression levels in astrocytes around human ischemic stroke lesions and observed increased expression of miR-210 in white matter chronic stroke astrocytes compared to astrocytes from healthy tissue. We aimed to understand the role of miR-210 expression in primary human fetal astrocytes by developing an *in vitro* assay of hypoxic, metabolic and inflammatory stresses. Combined induction of hypoxic and inflammatory stresses was observed to upregulate miR-210 expression. We found that transfection with miR-210-mimic (210M) increases glycolysis, enhances lactate export, and promotes an anti-inflammatory transcriptional and translational signature in astrocytes. Additionally, 210M transfection resulted in decreased expression of *C3* and *Sema5b*, indicative of a more neuroprotective astrocytic phenotype. We conclude that miR-210 expression in human astrocytes is modulated in response to hypoxic stress both *in vivo* and *in vitro*, corroborating the hypothesis that miR-210 is an indicator of ischemic stress in the human brain. In addition, our results show that miR-210 upregulation in stressed astrocytes is part of a protective cellular mechanism that results in a potentially neuroprotective astrocytic phenotype.

Keywords: Astrocyte, stroke, multiple sclerosis, microRNA-210, ischemia, hypoxia, inflammation.

Main Points

- Astrocytes around chronic white matter stroke lesions express increased levels of microRNA-210 (miR-210).
- Primary human fetal astrocytes transfected with miR-210-mimic (210M) increase glycolysis and lactate export, while decreasing expression of inflammatory cytokines.
- 210M induces a neuroprotective gene expression profile in primary human astrocytes.

Introduction

Astrocytes contribute to central nervous system (CNS) homeostasis through their critical roles in blood brain barrier (BBB) maintenance, production of pro- and anti-inflammatory cytokines, release of antioxidants, and provision of trophic factors to surrounding neurons and oligodendrocytes (Chung et al., 2013; Molofsky et al., 2014; Pentreath & Slamon, 2000; Sherwood et al., 2006; Tsai et al., 2012). Astrocytes contribute to the pathology of multiple neurological disorders, either through the loss of their physiologic functions and/or the production of disease-associated molecules (Gonzalez-Reyes, Nava-Mesa, Vargas-Sanchez, Ariza-Salamanca, & Mora-Munoz, 2017; H. Phatnani & Maniatis, 2015; H. P. Phatnani et al., 2013; Rostami et al., 2020; Zamanian et al., 2012; Zeinstra, Wilczak, Streefland, & De Keyser, 2000). Conversely, they can provide tissue protection and support repair through the export of lactate, which provides neurons with an additional energy source, and through the release of anti-inflammatory/neuroprotective factors, which promote neuronal regeneration and survival (Gao et al., 2015; Lu et al., 2015).

The dynamic properties of astrocytes in health and disease are regulated by their microRNA (miR) expression profile. miRs are ~22 nucleotide strands of RNA that post-transcriptionally regulate protein expression (Saliminejad, Khorram Khorshid, Soleymani Fard, & Ghaffari, 2019). Previously, we used laser capture micro-dissection (LCM) to reveal that the miR profile of astrocytes in human adult white matter (WM) differs from that of astrocytes in the gray matter (GM) (Rao et al., 2016). Our follow-up study showed a distinctive miR expression profile of astrocytes around multiple sclerosis (MS) lesions compared to astrocytes from normal appearing tissue (Rao et al., 2019). Of interest was our finding that miR-210 expression was increased in astrocytes around active MS lesions (Rao et al, 2019). miR-210 has previously been associated

with ischemia and is reportedly increased in the mouse brain subjected to the middle cerebral artery occlusion model of stroke (Jeyaseelan, Lim, & Armugam, 2008). In combination, these results are consistent with the concept of ongoing ischemic stress in MS lesions (Aboul-Enein et al., 2003; Mahad, Ziabreva, Lassmann, & Turnbull, 2008). However, neither the expression of miR-210 in human brain tissue undergoing confirmed ischemic injury, nor its functional role in glial cells, have been evaluated so far.

In this study, we determined the miR expression profile of astrocytes around human ischemic stroke lesions and in astrocytes isolated from normal tissue. We uncover increased miR-210 expression in astrocytes responding to ischemic stress *in-situ*. To understand the functional effect of increased miR-210 expression in human astrocytes, we developed an *in vitro* stress assay using primary human fetal astrocytes. We used a miR-mimic transfection approach to model increased cellular miR-210 expression and investigated miR-210-mediated modulation of human fetal astrocyte i) metabolism, ii) inflammatory cytokine production, and iii) neuroprotective molecule production. Specifically, we measured oxygen consumption rate, glycolysis, lactate export, pro- and anti-inflammatory cytokine expression, and expression of neurotoxic/neuroprotective genes. We show for the first time that miR-210 upregulation in human astrocytes may aid in the establishment of a protective astrocytic phenotype in response to hypoxic stress.

Materials and Methods

Neuropathological Identification of Infarcts in Human Brain Tissue

Sixteen adult human brains specimens aged 30-93 were obtained from the Montreal Neurological Institute and Hospital (Montréal, Québec, Canada) and Kingston General Hospital (Kingston,

Ontario, Canada). Formalin-fixed paraffin-embedded (FFPE) sections of cerebrum were evaluated by a board-certified neuropathologist (J.S.) using established criteria (Mena, et al. Acta Neuropathologica, 2004). Briefly, Hematoxylin and Eosin-stained sections were assessed for the presence of hyper-eosinophilic (acutely hypoxic) neurons, infiltrating neutrophils, alteration of parenchyma (rarefaction or cavitation), loss of hypoxia sensitive cells (neurons in GM and oligodendrocytes in WM), gliosis, mononuclear cell response (microgliosis or foamy macrophages), active angiogenesis and hemosiderin deposition in scavenger cells. Acute infarcts were primarily characterised by the presence of hyper-eosinophilic neurons with rarefaction or early cavitation, while chronic infarcts all demonstrated well developed gliosis and advanced cavitation. Tissues characterised as normal contained occasional hemosiderin deposition but no other changes.

Immunohistochemistry and Laser Capture Micro-Dissection

Laser capture micro-dissection (LCM) was performed as previously described (Rao et al., 2016). Briefly, FFPE sections were stained with an anti-GFAP (glial fibrillary acidic protein, Ventana cat. # 760-4345, Cell Marque, CA) antibody for astrocyte identification and cell capture. Cells were visualized using a secondary antibody conjugated to 3,3'-diaminobenzidine (DAB). For stroke samples, GFAP-positive astrocytes located around lesions, avoiding cells inside or distal from the lesion itself, were identified. Once the cells were identified, LCM was performed using a PALM-LCM (Montréal, Québec, Canada). Approximately 35 cells were captured from each slide, after which the cells were lysed in TRIzol and stored at -80˚C until further use. For slides with both WM and GM stroke lesions, captures were performed from both regions.

RNA Extraction and Quantitative Polymerase Chain Reaction for LCM

RNA extraction and quantitative polymerase chain reaction (qPCR) were carried out as described previously (Rao et al., 2016). Briefly, RNA was extracted using a standard TRIzol protocol. The miR profile chosen for investigation was based on a previous study that identified miRs which were expressed by astrocytes and which had predicted associations with neurodegenerative diseases (Rao et al, 2019). Reverse transcription was performed with Taqman primers specific to each miR of interest (ThermoFisher, USA,). A pre-amplification step was performed on the complementary DNA (cDNA) prior to qPCR (Rao et al., 2016). qPCR with Taqman probes was then used to measure the expression of each miR of interest. Data is presented relative to the mean cycle threshold (CT) value of all miRs measured, which has previously been shown to be an effective method of normalization (Mestdagh et al., 2009). CT values > 37 were discounted. miR expression is provided as the -ΔΔCT relative to the expression of each miR in the control tissue. Astrocytes captured from the WM around stroke lesions were compared to astrocytes captured from normal WM tissue, while those from GM stroke lesions were compared to cells from normal GM. -ΔΔCT was calculated using the previously defined method and is equivalent to the Log_2 (fold change) (Livak & Schmittgen, 2001).

RNA Extraction and qPCR for In Vitro Samples

RNA extraction and reverse transcription were performed as described above with random hexaprimers used instead of specific primers during the reverse transcription (Jack et al., 2005). qPCR was performed with Taqman probes for each gene or miR. mRNA CT values were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)*, while miRNA CT values

were normalized to U47, a previously defined, stable miR (Hirschberger et al., 2019). Data is graphed as -ΔΔCT which is equivalent to the $\log_2$ (fold change).

Culturing of Primary Human Astrocytes and HeLa Cells

Primary human fetal astrocytes were isolated from 2nd trimester fetal tissue (17–23 weeks of gestation) obtained from the University of Washington Birth defects research laboratory (BDRL, project#5R24HD000836-51). As previously described (Jack et al., 2005), fetal CNS tissue was chemically dissociated with trypsin and DNase I and was subsequently mechanically dissociated with scalpels and washed with phosphate buffered saline (PBS) containing penicillin-streptomycin (PS) and fungizone. Cells were plated on poly-L-lysine coated flasks at $3x10^6$ cells/mL in T75 flasks using DMEM/F12 with 10% fetal bovine serum (FBS), 1% PS, 1% Glutamax, and glucose. Previous staining of cells isolated using this method has shown cultures to contain > 90% GFAP-positive cells (Jack et al., 2005). For *in vitro* stress conditions, astrocytes were treated for 24-hours with 100ng/mL interleukin-1beta (IL1β), glucose-free DMEM/F12 media, or 1% O_2 for inflammatory, metabolic, and hypoxic stress, respectively. For the control condition, the aspiration and PBS washing occurred alongside all treated wells, and fresh media was added for 24-hours before collection.

HeLa Cell Culturing, Treatment, and Collection

HeLa cells were cultured with DMEM/F12 with 10% FBS, 1% PS, 1% Glutamax, and glucose. Once the cells were 70% confluent, supernatant was aspirated, cells were washed with warm PBS, and fresh media was added. Cells were then put into the hypoxia chamber at 1% O_2 for 2-48 hours. For protein collection, cells were removed from the hypoxia chamber, and cells were lysed with

RIPA buffer and scraping within one minute of being removed from the hypoxia chamber. The cell lysate was then stored at -80˚C before use for Western blot.

Luminescence Assay

HeLa cells were cultured as described above and transfected with a dual-luminescent plasmid that had Firefly and Renilla luciferase signals. The Firefly signal was regulated by the publicly available iron sulfur cluster protein (*ISCU*) 3' untranslated region (3'UTR) that is expected to be targeted by miR-210. HeLa cells were transfected with a blank, mutant, or wildtype plasmid for 4-hours, after which the media was washed and cells were transfected with either 210S or 210M. 48-hours later, the luminescence was measured using the dual luminescence assay (Promega, USA, Cat# E2920) following manufacturer's instructions.

Transfection of Primary Human Astrocytes

Lipofectamine RNAiMax (ThermoFisher, USA, Cat# 13778075) was used for Alexa Fluor 555 BLOCK-iT control (ThermoFisher, USA, Cat# 14750-100) and for miR-mimic/seed transfections following the manufacturer's protocol. miR-210-mimic (210M) was created by annealing miR-210-3p and miR-210-5p strands, with the miR-210-3p strand as the functional strand. To generate the seed mutant (miR-210-seed, 210S) for use as highly specific control, the seed regions were transposed between the miR-210-3p and miR-210-5p strands, which has been shown to reduce the binding ability of miRs to their targets (Hill, Jabbari, Matyunina, & McDonald, 2014). The two strands of the mimic and of the seed-mutant were obtained from IDT using the publicly available sequence of miR-210. RNA strands were annealed following the IDT protocol for annealing oligonucleotides. Cells were transfected with 210M and 210S at a final concentration of 50nM.

For cells treated in hypoxia and inflammation conditions after transfection with 210M, cells were washed with warm PBS 48-hours following transfection, and then transferred into the appropriate stress condition.

Seahorse Analysis

Seahorse was used to measure the oxygen consumption rate (OCR) and extracellular acidification rate of primary human fetal astrocytes (ECAR), which measure cellular oxidative phosphorylation and glycolysis, respectively (TeSlaa & Teitell, 2014). Primary human astrocytes were plated on uncoated XFe-96 plates 48-hours before analysis at a density of 15,000 cells per well. The standard Seahorse guide was followed using a 3-0-3 split of mixing, waiting, and measuring after each injection. Basal Seahorse media was used, with the addition of glucose (17.5mM), glutamine (2mM), and pyruvate (1mM) for OCR. The media was only supplemented with glutamine (2mM) for ECAR. The pH of both medias was altered to 7.4 after the addition of the substrates. Oligomycin, FCCP, and Rotenone/antimycin-A were used for OCR at final concentrations of 1µM, 1µM, and 0.5µM, respectively. Glucose, oligomycin, and 2-deoxyglucose were used at final concentrations of 10mM, 1µM, and 50mM, respectively.

Western Blots

Cell lysates were obtained using RIPA lysis buffer with added protease inhibitor. Proteins were separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes were washed in TBS-T (100mM Tris-Cl, pH 8.1, 150mM NaCl, 0.1% Tween-20). Incubation with primary antibody in TBS-T with 1% non-fat milk was performed at 4°C overnight followed by incubation with

horseradish peroxidase conjugated secondary antibody for 1-hour and enhanced chemiluminescence detection. The following antibodies and dilutions were used: anti-MCT4 (Abcam Inc, Canada; ab74109) 1:1000, anti-GPD1L (ThermoFisher Scientific, USA; PA5-24216) 1:1000, 1:1000, anti-beta Tubulin (Abcam Inc, Canada; ab6046) 1:1000, and anti-HIF1 alpha (Abcam Inc, Canada; ab51608) 1:1000. An anti-rabbit IgG conjugated to horseradish peroxidase was used as a secondary antibody (New England Biolabs, USA; 7074) at a 1:500 to 1:20,000 dilution. Enhanced chemiluminescence detection on x-ray film was used to detect antibody signal. Equal protein loading was confirmed by re-probing blots for beta-Tubulin. Western blot densitometry values were calculated using FIJI.

Enzyme-Linked Immunosorbent Assays (ELISAs)

Cytokine secretion by primary human astrocytes was measured by ELISA. Forty-eight hours after transfection with 210M or 210S, media was aspirated and fresh media with the appropriate treatment (control or hypoxia and inflammation) was added. Cell supernatants were collected and measured for protein concentration 24 hours later. CXCL10 (IP10; BD Biosciences, USA, Cat# 550926), IL-6 (BD Biosciences, USA, Cat# 555220) and IGF-1 (R&D Systems, USA, Cat# DY291) ELISA kits were used.

Lactate Assay

L-Lactate assay kit (Eton Biosciences, USA, Cat# 1200014002) was used following manufacturer's instructions on sample supernatants. After the 48-hour transfection, media was aspirated, wells were washed with warm PBS, and serum-free, phenol red-free fresh media with

N1 supplementation (Sigma, Germany, N6530) was added to the cells. Following a 2-hour incubation, cell supernatants were collected for measurement of lactate concentration.

Statistical Analyses

All data are graphed as the mean ± SEM. One-way ANOVA, two-way ANOVA, or paired t tests were performed using Graphpad Prism 9, as noted in the figure legend. A p-value <0.05 was considered significant.

Results

miRs are differentially expressed in astrocytes from normal brain tissue compared to those surrounding stroke lesions.

We first aimed to define the miR expression profile of astrocytes around ischemic stroke lesions compared to astrocytes from normal brain tissue. Human brain tissue from both the white matter (WM) and gray matter (GM) of 16 control or stroke patients were obtained. LCM was performed on GFAP+ astrocytes from normal brain tissue and from around chronic and acute infarcts (Table 1, Figure 1A-B). The age, sex, location of cell capture, type of infarct, and histological characteristics are included for each patient in Table 1. 18 miRs that are known to be expressed by astrocytes and have *in silico* predicted, or experimentally validated, functions that relate to neuroinflammation or neurodegenerative disease processes were chosen as previously described (Rao et al., 2016) for qPCR assessment of their expression (Figure 1C, Supplementary Figure 1, Supplementary Table 1). miR-21, -100, -155, and -210, were significantly differentially expressed

in astrocytes surrounding infarcts compared to astrocytes from normal appearing tissue (Figure 1C-D). miR-21 was the only miR significantly upregulated around both WM chronic and acute infarcts, while miR-210 was the only miR with significant differential expression around both WM and GM infarcts. Expression of miR-210 was increased ~1.6 fold and decreased by ~0.6 fold in astrocytes around WM and GM chronic infarcts, respectively (Figure 1C-D).

In vitro hypoxic and inflammatory stress increases expression of miR-210 in primary human astrocytes.

We created an *in-vitro* stress paradigm in which we could investigate miR expression levels and subsequently modulate the expression of miRs of interest in primary human fetal astrocytes. Inflammation, metabolic stress, and hypoxia, as well as combination of these conditions, were induced using IL1β treatment, glucose-free medium and a 1% O_2 chamber, respectively (Figure 2A). After 24 hours, expression of canonical response genes were confirmed to be upregulated in cells subjected to inflammatory stress (*CXCL10* (Liu et al., 2011)), metabolic stress (*HMOX1* (Smid et al., 2018)), and hypoxic stress (*MCT4* (Saraswati, Guo, Atkinson, & Young, 2015)) (Figure 2B-D). Induction of a hypoxic response was further confirmed through measurement of HIF-1α protein expression in HeLa cells (Supplementary Figure 2A), (Seo et al., 2015). Following establishment of the *in vitro* stress system, the expression of the 18 miRs with functional relevance to astrocytes (Rao et al, 2016) was analyzed by qPCR (Figure 2E, Supplementary Figure 2B). miR-210 was significantly upregulated upon combined induction of hypoxia and inflammation (Figure 2F). Other miRs followed previously identified patterns, like miR-155, which was increased in the inflammatory conditions (Mahesh & Biswas, 2019).

Primary human astrocytes can be transfected with miR-210-mimic.

To elucidate the function of miR-210 in astrocytes, we used a miR mimic system. The miR mimic (210M) has the same sequence as endogenous miR-210 and therefore acts as an *in vitro* overexpression model. As a negative control, we generated a miR-210 construct with a transposed seed sequence between the miR-210-3p and miR-210-5p, hereafter referred to as 210S (Hill et al., 2014). To confirm that lipid-mediated transfection of primary human astrocytes is possible, we first verified efficient transfection of the cells with Alexa Fluor 555 BLOCK-iT, a fluorescently labelled short nucleotide sequence. GFAP-positive astrocytes showed an increase in Alexa Fluor 555 fluorescence signal after 48 hours compared to non-transfected cells (Figure 3A). Next, intracellular astrocytic miR-210 expression levels were measured by qPCR after transfection with 210M or 210S. Primary human astrocytes transfected with 210M had significantly higher levels of miR-210 relative to cells transfected with the control 210S (Figure 3B). To confirm the functionality of 210M, we measured the expression levels of known downstream targets of miR-210. MiRabel bioinformatic tool, which uses predictive algorithms to identify likely gene targets of a given miR (Quillet et al., 2019) was used to identify miR-210 targets (Figure 3C). From this target list, we measured the expression of cytoglobin (*CYGB)* and iron sulfur cluster protein (*ISCU)*, two experimentally validated targets of miR-210 (Chen, Li, Zhang, Huang, & Luthra, 2010; Du, Wei, Ma, Jiang, & Song, 2020). We found that mRNA levels of both *CYGB* and *ISCU* were significantly downregulated in astrocytes transfected with 210M relative to 210S (Figure 3D-E). We used a dual-luminescence assay to confirm the miR-210 dependent downregulation of ISCU. Three plasmids were generated: a Blank that had both Firefly and Renilla signals without any regulatory elements added, a mutant (MUT) that had the Firefly signal under the control of a mutated ISCU 3' untranslated region (UTR) and the Renilla signal unregulated, and a wildtype

(WT) plasmid that had the Firefly signal under the control of the wildtype ISCU 3' UTR (expected to be targeted by miR-210) and the Renilla signal unregulated. In all plasmids, the Renilla luminescence acts as an internal control, and thus the Firefly:Renilla ratio describes the amount of that the Firefly signal is regulated. After transfecting HeLa cells with one of the three plasmids, we then transfected them in triplicate with either 210S or 210M and observed the Firefly and Renilla luminescence. We found that astrocytes transfected with 210M had a lower Firefly:Renilla luminescent signal than 210S in all three plasmid transfections, suggesting some off-targeting effects of 210M. We also found a lower Firefly:Renilla signal in the WT plasmid when compared to the other luminescent signals. The Firefly:Renilla ratios for Blank, MUT, and WT plasmid transfections in the 210M transfected groups were 0.68, 0.65, and 0.39, respectively (Figure 3F). Altogether, these data demonstrate that primary human astrocytes are capable of being transfected with miR mimics using lipid-mediated delivery, and that transfecting astrocytes with 210M results in the downregulation of miR-210 target genes, suggesting that 210M is functionally active within these cells.

210M promotes glycolysis and lactate export in primary human astrocytes.

The bioinformatic software miRPathDB (Kehl et al., 2020) was used to predict pathways that could be regulated by miR-210. For miR-210-3p, the functional pathway with the 2nd most hits was the "regulation of metabolic process", and many of the other top pathways regulated by miR-210 were also related to metabolism (cellular aromatic compound, organic cyclic, and nitrogen compound metabolic processes) (Figure 4A). A Seahorse XF analyzer was therefore used to investigate the effect of miR-210 expression on primary human astrocyte metabolism. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which are respective proxies for oxidative

phosphorylation and glycolytic activity, were assessed. 24-hours post-transfection, there was no change in either the OCR or ECAR in cells transfected with 210M compared to those transfected with 210S (Supplementary Figure 3A-B). 48-hours post-transfection, there remained no significant change in OCR values between astrocytes transfected with 210M or 210S (Figure 4B). Conversely, ECAR values of basal glycolysis, normal glycolysis, and glycolytic capacity were significantly increased in astrocytes transfected with 210M (Figure 4C). The timepoint of 48 hours was therefore chosen to investigate the effect of 210M transfection on lactate export, a metabolite produced through glycolysis that has previously been shown to be critical for neuronal survival in stressful conditions (Muraleedharan et al., 2020). First, we measured the expression of monocarboxylate transporter 4 (MCT4, also known as SLC16A3), which is a monocarboxylate transport protein that exports lactate from astrocytes, expression of which has previously been shown to be critical for neuronal survival under states of oxygen and glucose deprivation (Gao et al., 2015). In astrocytes transfected with 210M, a ~2.2-fold increase in the expression of *MCT4* mRNA compared to those transfected with 210S was observed (Figure 4D). MCT4 protein expression in astrocytes transfected with 210M was also found to be increased by Western blot compared to astrocytes transfected with 210S, though this difference did not reach statistical significance (Supplementary Figure 3C). In addition, both mRNA and protein levels of GPD1L, which was previously shown to inhibit lactate production when overexpressed (Du et al., 2020), were decreased in astrocytes transfected with 210M compared to cells transfected with 210S (Figure 4E-F). Finally, to understand if the combined effect of increased MCT4 expression and decreased GPDL1 expression had an effect on the release of astrocytic lactate into the extracellular space, we evaluated lactate accumulation in the supernatant of astrocytes over a 2-hour period, 48 hours after transfection with 210M or 210S. We found that astrocytes transfected with 210M

exported ~1.3-fold more lactate than the 210S transfected cells (Figure 4G). Overall, 210M transfection promoted glycolysis in primary human astrocytes, resulting in increased lactate export.

210M induces an anti-inflammatory phenotype of primary human astrocytes.

miR-210-mediated increased lactate production by astrocytes may indicate the establishment of a neuroprotective astrocytic phenotype (Baltan, 2015). To further investigate this possibility, the effect of miR-210 overexpression on pro- (IL-6 and CXCL10) and anti-inflammatory (IGF-1) cytokine production in primary human astrocytes transfected with 210M was evaluated. As the expression of pro-inflammatory cytokines are low in astrocytes under basal conditions, cells were incubated with 210M or 210S for 48-hours and then stressed for 24-hours in combined hypoxic and inflammatory condition. Under this condition, decreased levels of CXCL10 mRNA and secreted protein (Figure 5A-B) were observed in 210M- compared to 210S-transfected cells. The expression of IL-6 mRNA also trended downwards with 210M transfection, while the cytokine level of IL-6 as measured by ELISA was significantly lower following 210M transfection compared to 210S transfection (Figure 5A-B). In addition to decreasing pro-inflammatory cytokine expression, astrocytes transfected with 210M were characterized by increased mRNA and secreted protein levels of the anti-inflammatory growth factor IGF-1 (Figure 5C-D). To further explore how increased miR-210 expression alters human astrocyte phenotype under stressed condition, we measured gene expression levels of complement 3 (*C3*), which has been found to be a driver of neuroinflammation associated with neuronal loss and the suppression of axonal growth (Peterson, Nguyen, Mendez, & Anderson, 2017). We found that *C3* mRNA levels were lower in cells transfected with 210M as compared to those transfected with 210S (Figure 5E). In line with

this potential neuroprotective function of miR-210, we found that *Sema5b*, which is a repressive cue for axonal growth (O'Connor et al., 2009), was decreased at the mRNA level with 210M transfection (Figure 5F). Overall, these findings confirm that miR-210 has anti-inflammatory effects in primary human astrocytes undergoing stroke-like stress, and that 210M transfection promotes the expression of neuroprotective markers.

Discussion

In ischemic stroke pathology, an initial acute pathological event is followed by an extended chronic phase of disease that is marked by gliosis and neuron loss. During the chronic phase, therapies used to treat the acute event (anti-inflammatory and anti-clotting agents) are ineffective at promoting long-term neuronal survival in the penumbra and preservation of homeostatic glia function. With recent advances in our understanding and ability to target miRs (Dasgupta & Chatterjee, 2021; Li & Rana, 2014), combined with the importance of astrocytes in disease, a deeper understanding of the role of astrocyte-expressed miRs is of considerable therapeutic interest. To advance the therapeutic potential of miRs, we must first better characterize miR expression in disease-state astrocytes, and more importantly, understand the functional roles of key miRs. In this study, we aimed to characterize the miR expression profile of astrocytes in normal brain tissue and of astrocytes around stroke lesions. Through this analysis, we found miR-210 to be significantly upregulated in astrocytes around WM chronic stroke lesions (Figure 1). We then created an *in vitro* stress paradigm for primary human fetal astrocytes using neurodegenerative-relevant stressors and found that miR-210 was upregulated in conditions that combine both hypoxia and inflammation (Figure 2). Through a 210M transfection system (Figure 3), we discovered that miR-210 promoted glycolysis and increased lactate export from primary

human astrocytes (Figure 4). Additionally, we found that 210M inhibits IL-6 and CXCL10 pro-inflammatory cytokine production, promotes expression of the anti-inflammatory factor IGF-1, and simultaneously promotes a potential neuroprotective phenotype through downregulation of *C3* and *Sema5b* mRNA in a stressed condition (Figure 5).

Laser capture micro-dissection (LCM) allows for the capture of astrocytes from specific locations around stroke lesions. While whole-tissue studies provide important insight into the biology of cells and tissues, our aim was to identify the miR expression profile of astrocytes around stroke lesions. LCM, in combination with immunohistochemistry, allowed us to identify and capture astrocytes without contaminating our sample with signal from other cell types. In our data, the majority of differences in the miR expression profile between normal appearing tissue and that around an infarct was present in the WM. Interestingly, the WM is more susceptible to stroke-induced injury than the GM, partly because of a smaller blood supply in the WM as compared to the GM (Wang et al., 2016). Astrocytes are also of higher importance in providing energy to neurons in the WM than in the GM because of the limited exposure of axonal surface area at the nodes of Ranvier (Baltan et al, 2015). Interestingly, we found miR-210 to be upregulated in the WM and downregulated in the GM, perhaps because the GM cells were not exposed to as severe stress conditions. Based on our finding that miR-210 increases lactate production and decreases inflammatory cytokine production in human astrocytes, it is possible that increased expression of miR-210 in astrocytes around WM stroke lesions may be a protective response designed to safeguard against excessive cell death.

In addition to observing significant changes in miR-210 expression in astrocytes around stroke lesions compared to those in normal tissue, our *in-situ* analysis uncovered other differentially expressed astrocytic miRs that we have not investigated further in the current study. For instance, miR-21 was significantly upregulated in astrocytes around WM chronic and acute infarcts, suggesting that it may play a role in the astrocytic response to disease in the WM. Likewise, miR-365 was upregulated in astrocytes around WM chronic and acute infarcts, however this finding did not reach statistical significance. Similar to miR-210, miR- 29a and -29b, two miRs that have been associated with neuroprotection (Hebert et al., 2008; Khanna et al., 2013), were upregulated in astrocytes around WM chronic infarcts, suggesting that high miR-210 levels may be indicative of an overall neuroprotective astrocyte signature.

In addition to comparing the miR expression profile of astrocytes between healthy and stroke patients, we studied the differences in miR expression between astrocytes surrounding chronic and acute lesions. Many of the miRs – miR-210 included – exhibited differential expression between the chronic and acute tissues. We believe that this differential expression profile is a result of astrocytes responding to the evolution of the local cellular stress environment over time. During the acute phase of an ischemic stroke, which includes the initial hours-to-days of damage, extensive cell death results from the onset of hypoxia, reduced glucose perfusion, increased levels of inflammatory cytokines. and the release of danger associated molecular patterns from apoptotic or necrotic cells. Chronic stroke, on the other hand, involves longer term evolution of changes to the cellular environment driven by gliosis and tissue remodeling (Chan et al., 2014). These two timepoints of disease (chronic and acute), therefore, contain their own unique cellular stress responses that may be responsible for the differential miR expression profile observed between

chronic and acute stroke lesions. In fact, astrocytes in chronic stroke lesions may not just be responding to present stresses but may have been influenced by previous occurrences to change their miR and protein expression profile. The concept of ischemic preconditioning, in which cells become more resistant to ischemic stresses after surviving an initial insult (Wegener et al., 2004), suggests a mechanistic framework to explain why astrocytes around chronic stroke lesions express increased levels of miR-210. We propose that chronic stroke is a primary indication for which neuroprotective therapeutic strategies may be successful. While identifying neuroprotective treatments of acute ischemic stroke to supplement existing thrombolytic therapies remains a public health imperative, disease modifying therapies for the chronic sequela of ischemic infarcts remain largely unexplored.

To investigate the functionality of miRs, we created an *in vitro* system that allowed for manipulation of miR expression levels. Inflammatory, metabolic, and hypoxic stresses are known to be involved in stroke pathogenesis and were thus the focus of our *in vitro* stress paradigm (Alfieri et al., 2020). This combination of stresses is not meant to perfectly recapitulate the complex stroke environment, as the crosstalk between cells alone is too complex to recreate using this reductionist *in vitro* approach. Instead, this system provides a method to investigate specific miRs of interest identified *in-situ*. For instance, miR-210 was robustly increased under combined hypoxic and inflammatory stress condition. While this particular condition does not recapitulate the glucose deprivation present in acute stroke tissue, it enables the investigation of miR-210 that may be important in the adaptation of astrocytes to hypoxic and inflammatory stress. The 210M transfection system provided us with a better understanding of miR-210's role in a stress condition that relates to that seen in stroke. The overexpression of miR-210 in astrocytes resulted in increased

glycolysis and anti-inflammatory cytokine production, while also decreasing the expression of genes related to neurotoxicity (Figure 4 and 5).

Increased lactate export from astrocytes has been shown to promote the survival of neurons *in vitro* and *in vivo* (Baltan, 2015). Blockade of the lactate exporter MCT4, which we observed to be increased in astrocytes transfected with 210M, has previously been shown to lead to decreased neuronal survival under stress conditions, providing additional evidence that astrocyte-derived lactate is crucial in supporting neuronal survival under stressful conditions (Gao et al., 2015; Hong et al., 2015). Conversely, other studies have shown that increased astrocyte-derived lactate can lead to acidosis-mediated-neurotoxicity (Xiang, Yuan, Hassen, Gampel, & Bergold, 2004). The relatively subtle increase of lactate export that we observe in this study following 210M transfection (~1.3-fold greater than control) is unlikely to cause neurotoxicity while still providing energy for surrounding cells.

Astrocytes are important contributors to the inflammatory response in the brain, primarily through the expression and secretion of inflammatory cytokines. Post-thrombolytic inflammation is one of the primary drivers of cell stress and death in stroke, and inflammation is also a central component of other neurodegenerative diseases like progressive MS (Nakase, Yamazaki, Ogura, Suzuki, & Nagata, 2008). Therefore, it is plausible that an increase in inflammatory mediators may abrogate any neuroprotective effect of increased lactate export. In general, as cells shift towards glycolysis as a means of energy production, this leads to increased production of pro-inflammatory cytokines (so-called Warburg effect) (Palsson-McDermott & O'Neill, 2020). Having observed a miR-210-mediated increase in glycolysis (Figure 4), we hypothesized that astrocytes transfected with 210M

would also increase production of pro-inflammatory cytokines. Interestingly we observed decreased expression of the pro-inflammatory cytokines IL-6 and CXCL10, and increased expression of the anti-inflammatory mediator IGF-1 (Figure 5). This supports previous evidence that miR-210 targets proteins involved in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway to inhibit inflammatory cascades (Qi et al., 2012). We also investigated the expression of previously defined "A1" or "A2" astrocytic markers that are thought to define neurotoxic or neuroprotective astrocyte phenotypes, respectively (Liddelow et al., 2017). However, we saw no changes in these markers (data not shown), with the exception of decreased *C3* expression when astrocytes were transfected with 210M. *C3* is defined as an 'A1 marker' but is more broadly associated with inflammatory cascades and which is implicated in mediating cytotoxicity. Additionally, expression of *Sema5b*, a molecule that represses axon guidance and outgrowth (O'Connor et al., 2009), was also inhibited following 210M transfection. Based on this data, we can speculate that decreased expression of Sema5b by astrocytes with a neuroprotective phenotype may serve to attract neurons in a post-damage environment.

Finally, a potential limitation of this study is that our *in-situ* investigation of miRs in astrocytes around stroke lesions was limited to a pre-defined list of miRs, as opposed to an unbiased approach. In our initial screening of the astrocytic miR profile, we chose to select a pre-determined list of miRs based on previous publications that have defined these 18 miRs to be involved in the astrocytic response to disease. Obtaining the necessary tissue to perform sequencing experiments and to subsequently confirm those findings at the PCR level would have not been feasible with the tissue available. Additionally, the small sample size for the *in-situ* experiment likely restricted the differential expression of certain miRs from reaching statistical significance. Despite this, our data

shows that many miRs displayed trends of increased or decreased expression around stroke lesions and should provide a basis for further study of astrocytic miRs and their functions. Finally, access to primary human astrocytes limited the breadth of *in vitro* experiments that could be performed. We focused on using 210M as an overexpression model of miR-210; however, as alternative sources of human astrocytes including induced pluripotent stem cell-derived astrocytes become more widely available, additional experiments using miR-210 inhibitors and miR-210 knock-out approaches could be used to explore this biology further. Notwithstanding these limitations, this study suggests that miR-210 is an important regulator of astrocytic function in ischemic tissue.

In summary, we found that human astrocytes around WM chronic stroke lesions have increased expression of miR-210 compared to astrocytes in normal brain tissue. Increased miR-210 expression boosts astrocytic glycolytic activity, enhances lactate production, and increases expression of anti-inflammatory molecules while inhibiting the expression of pro-inflammatory mediators. Overall, we propose a functional role for miR-210 in promoting beneficial functions of astrocytes that may be critical in promoting neuronal survival after stroke. While further research is required to confirm these findings, this study expands our knowledge of disease-associated astrocyte signatures and identifies miR-210 as a potentially modifiable mediator to promote a protective astrocytic phenotype.

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Table:

Table 1: Clinical Information of Human Brain Autopsy Used for Laser Capture Micro-Dissection of Stroke and Normal Astrocytes

Clinical Information and Classification						Histopathology					
Sample	Age (years)	Sex	Brain Region	Type	Eosinophilic Neurons	Tissue Cavitation	Gliosis	Macrophage infiltrate	Angiogenesis	Hemosiderin	Estimated interval
1	77	M	WM, GM	A	Present	Early	Gemistocytic	Confluent	Absent	Present	10 to 30 days
2	83	M	WM, GM	A	Present	Early	Gemistocytic	Confluent	Absent	Absent	2 to 7 days
3	35	M	WM, GM	A	Present	Early	Mild	None	Absent	Absent	24 hours
4	53	M	WM, GM	A	Present	Early	Mild	Rare	Present	Absent	7 to 10 days
5	75	F	WM, GM	A	Present	Early	Mild	Rare	Absent	Absent	24-48 hours
6	93	M	WM, GM	C	Absent	Advanced	Well developed	Rare	Absent	Present	Months to years
7	52	F	GM	C	Absent	Advanced	Well developed	Rare	Absent	Present	Months to years
8	63	M	WM, GM	C	Absent	Advanced	Well developed	Moderate	Absent	Present	Months to years
9	80	M	WM, GM	C	Absent	Advanced	Well developed	Rare	Absent	Present	Months to years
10	82	M	WM, GM	C	Absent	Advanced	Well developed	Rare	Absent	Present	Months to years
11	79	M	WM, GM	N							
12	50	M	WM, GM	N							
13	66	M	WM, GM	N							
14	62	M	WM, GM	N							
15	30	M	WM, GM	N							
16	74	F	WM	N							

Table 1 Key:

Legend	
M	Male
F	Female
WM	White Matter
GM	Grey Matter
A	Acute
C	Chronic
N	Normal

Figures:

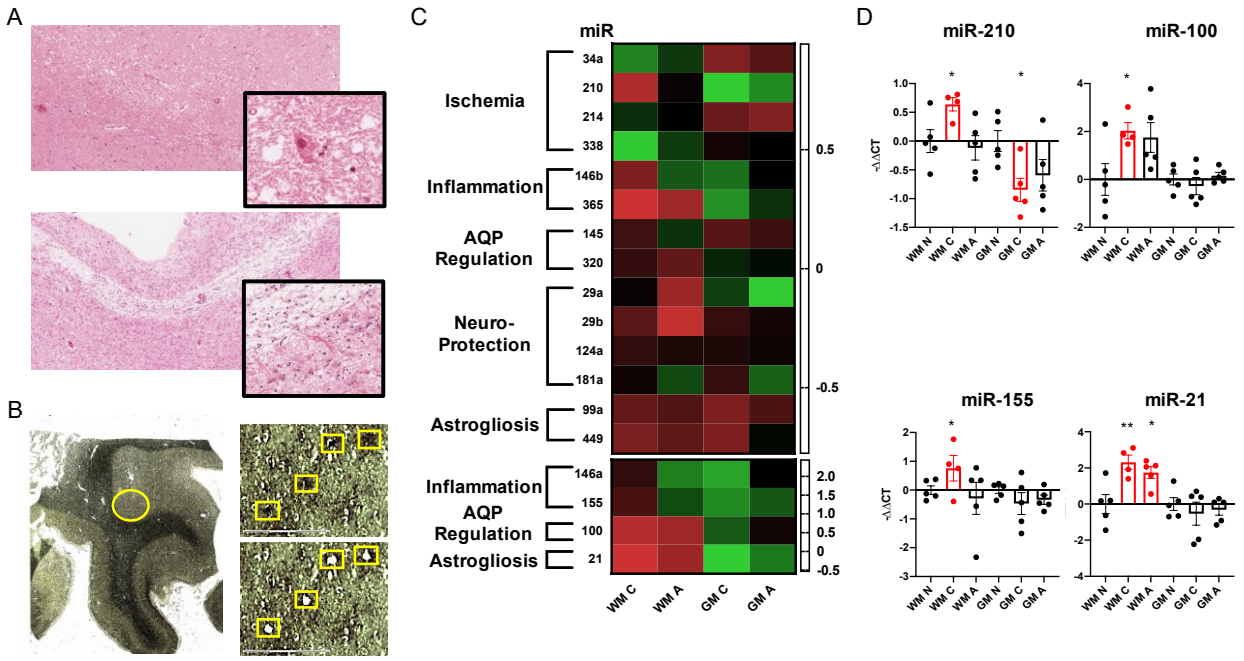


Figure 1: **microRNAs are differentially expressed in astrocytes from healthy brain tissue compared to astrocytes surrounding stroke lesions**. (A) Hematoxylin and eosin staining of early acute (upper panel) and late chronic (lower panel) infarcts. Subtle rarefaction/vacuolization of the parenchyma, loss of viable neurons, and acute ischemic, hypereosinophilic neurons (inset) are visible in the acute lesion. Cavitation of the parenchyma with a rim of well-developed astrocytosis (inset) is visible in chronic infarct lesions. (B-D) GFAP+ astrocytes were captured from white (WM) and grey (GM) matter of unaffected (normal, "N") brain tissue or around acute ("A")/chronic ("C") stroke lesions using laser capture micro-dissection (LCM). Around 35 cells were pooled from each slide for RT-qPCR assessment of microRNA expression. (B) Bright-field images showing a brain section before and after laser capture microdissection of astrocytes. (C) Heatmap of microRNA (miR) expression in GFAP+ astrocytes. Color code represents $\log_2$(fold change) in miR expression in lesioned WM and GM compared to respective normal brain tissues. miRs are grouped according to their associated function. (D) Histograms of miR-210, -100, -155, and -21 expression in GFAP+ astrocytes. Data are presented as mean ± SEM of n=5 donors, except for WM C for which n=4. One-way ANOVA was used for significance testing. miRs with statistically significant differences between normal and disease conditions are graphed in red in part D. *p<0.05; **p<0.01.

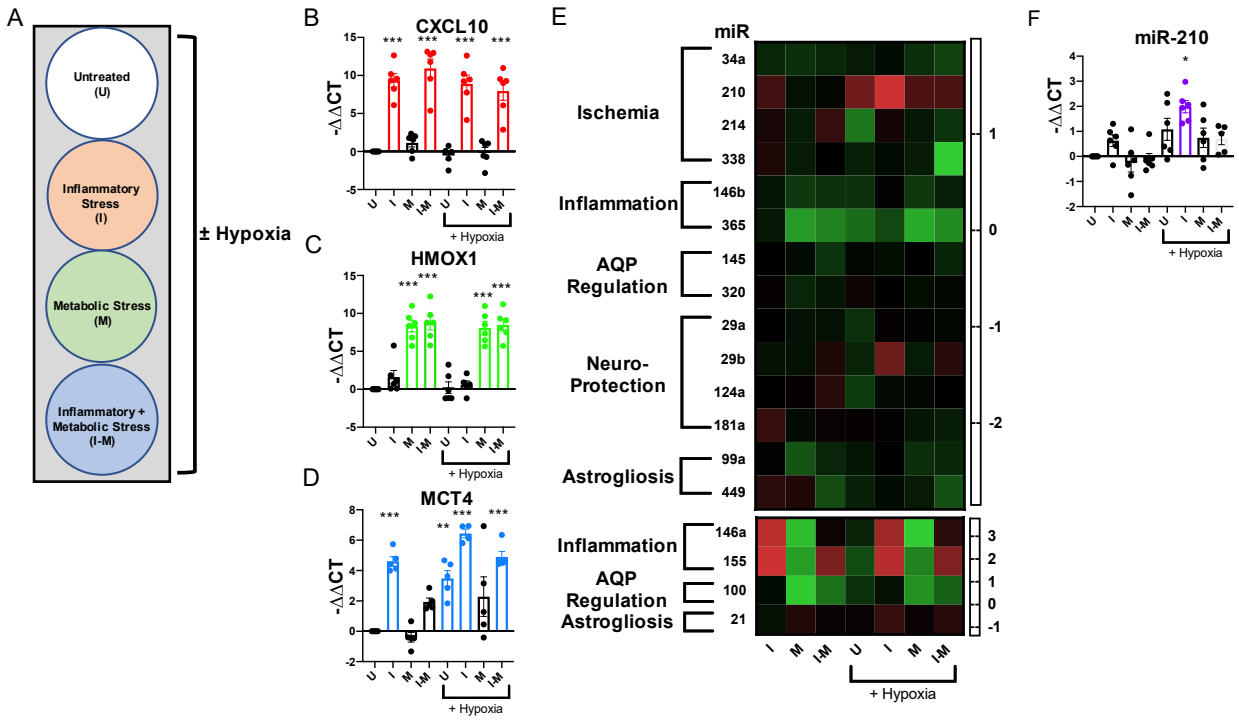


Figure 2: **microRNAs are differentially expressed in primary human astrocytes undergoing disease-associated stresses.** Primary human astrocytes were untreated ("U") or subjected to inflammatory ("I"), metabolic ("M"), and/or hypoxic ("H") *in vitro* stress conditions using Interleukin-1 beta (IL1b), glucose-free media, or 1% oxygen, respectively. (B-D) RT-qPCR assessment of *CXCL10*, *HMOX1*, and *MCT4* expression compared to untreated control. (E) Heatmap presenting the RT-qPCR assessment of microRNA expression in stress conditions relative to untreated control. (D) Histogram of miR-210 expression in stress conditions relative to untreated control. Data are presented as mean ± SEM of n=5-6 donors. One-way ANOVA with Sidak's multiple comparison correction was used for significance testing. *p<0.05; **p<0.01; ***p<0.001.

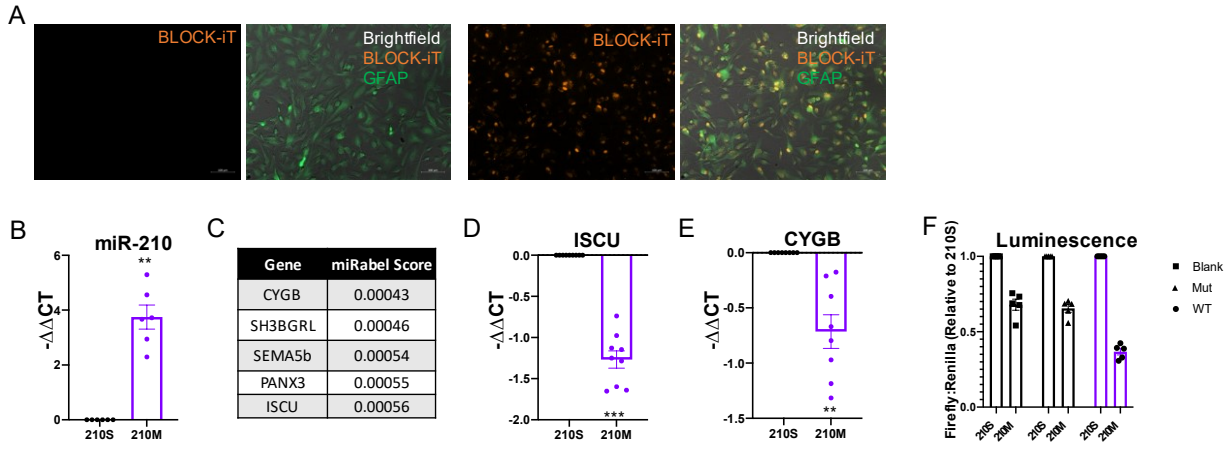


Figure 3: **Primary human astrocytes downregulate known targets of miR-210 following transfection with miR-210-Mimic.** (A) Primary human astrocytes were transfected with AlexaFluor BLOCK-iT as a positive control of transfection. Representative images of non-transfected (left) and transfected (right) cells immunostained for GFAP and overlayed with brightfield. (B) Primary human astrocytes were transfected with miR-210-Mimic (210M) or miR-210-Seed-mutant (210S) as a control. The expression of miR-210 in 210M-transfected relative to 210S-transfected cells was assessed by RT-qPCR. (C) MiRabel analysis of miR-210 shows the five most likely gene-targets of miR-210 based on sequence-specific predicted targeting, with lower MiRabel scores signifying a higher likelihood of being targeted by miR-210. (D) RT-qPCR assessment of microRNA-210 targets Cytoglobin (*CYGB*) and (E) Iron Sulfur Cluster Protein (*ISCU*) in 210M-transfected relative to 210S-transfected cells. (F) HeLa cells were transfected with a blank, mutant (Mut), or wildtype (WT) dual-luminescent plasmid with both Firefly and Renilla signals. Cells were then transfected with 210S or 210M and the luminescence ratio of Firefly:Renilla was measured. n=5. All data is graphed as mean ± SEM. Each dot represents a separate human sample, except in part F when HeLa cells were used. Paired t tests were performed for tests of significance. *p<0.05; **p<0.01; ***p<0.001

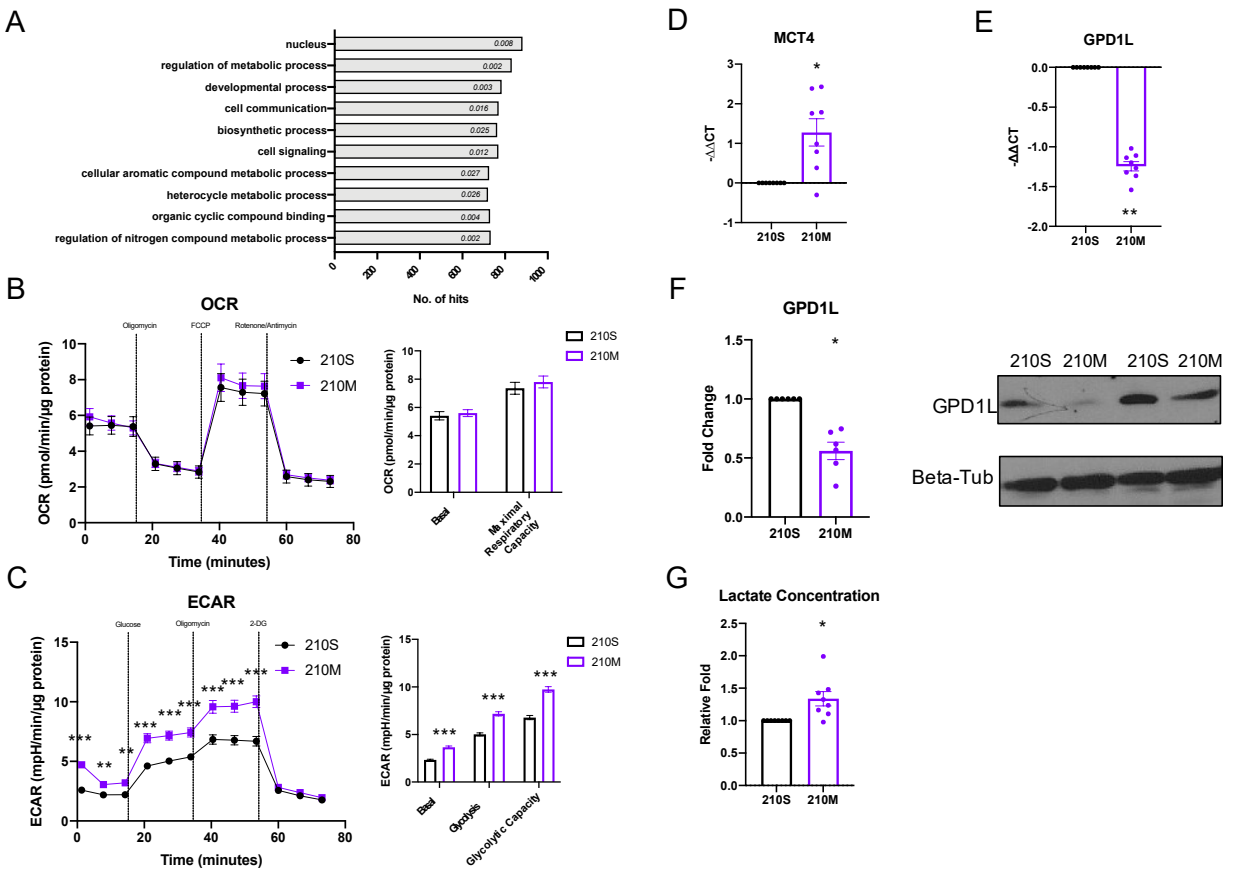


Figure 4: **210M induces glycolysis and lactate export of primary human astrocytes.** (A) miRPathDB was used to predict pathways targeted by microRNA-210. The pathways with the most hits are listed in descending order, and the p-value of each pathway is included inside the respective bar. (B) Oxygen Consumption Rate (OCR) was measured in primary human astrocytes 48-hours after transfection with 210S or 210M. The basal oxygen consumption (before oligomycin) and maximal respiratory capacity (between FCCP and Rotenone/Antimycin A) are presented in the histogram. N=3 with 10 technical replicates for OCR experiments (C) Extracellular Acidification Rate (ECAR) was measured in primary human astrocytes 48-hours after transfection with 210S or 210M. The basal (before glucose), normal glycolysis (between glucose and oligomycin) and glycolytic capacity (between oligomycin and 2-DG addition) are presented in the histogram. N=4 with 20 technical replicates for ECAR experiments. (D) RT-qPCR assessment of MCT4 in 210M-transfected cells relative to 210S-transfected cells 48 hours after transfection. Mean ± SEM of 8 donors. (E) RT-qPCR assessment of GPD1L in 210M-transfected cells relative to 210S-transfected cells 48 hours after transfection. Mean ± SEM of 8 donors. (F) Immunoblotted bands of GPD1L and Beta-tubulin (Beta-Tub) proteins and their quantification in 210M-transfected relative to 210S-transfected cells 48 hours after transfection. Mean ± SEM of 6 donors. (G) Primary human astrocytes were transfected with 210S or 210M. After 48 hours, cells were washed, and the concentration of lactate was measured in media that was incubated with astrocytes for 2 hours. Results are presented as fold change relative to 210S-transfected cells. Mean ± SEM of 8 donors. Statistical comparisons between Seahorse groups were made by a two-way ANOVA with Sidak's correction (B and C) or by a paired t-test (D to G). *p<0.05; **p<0.01; ***p<0.001.

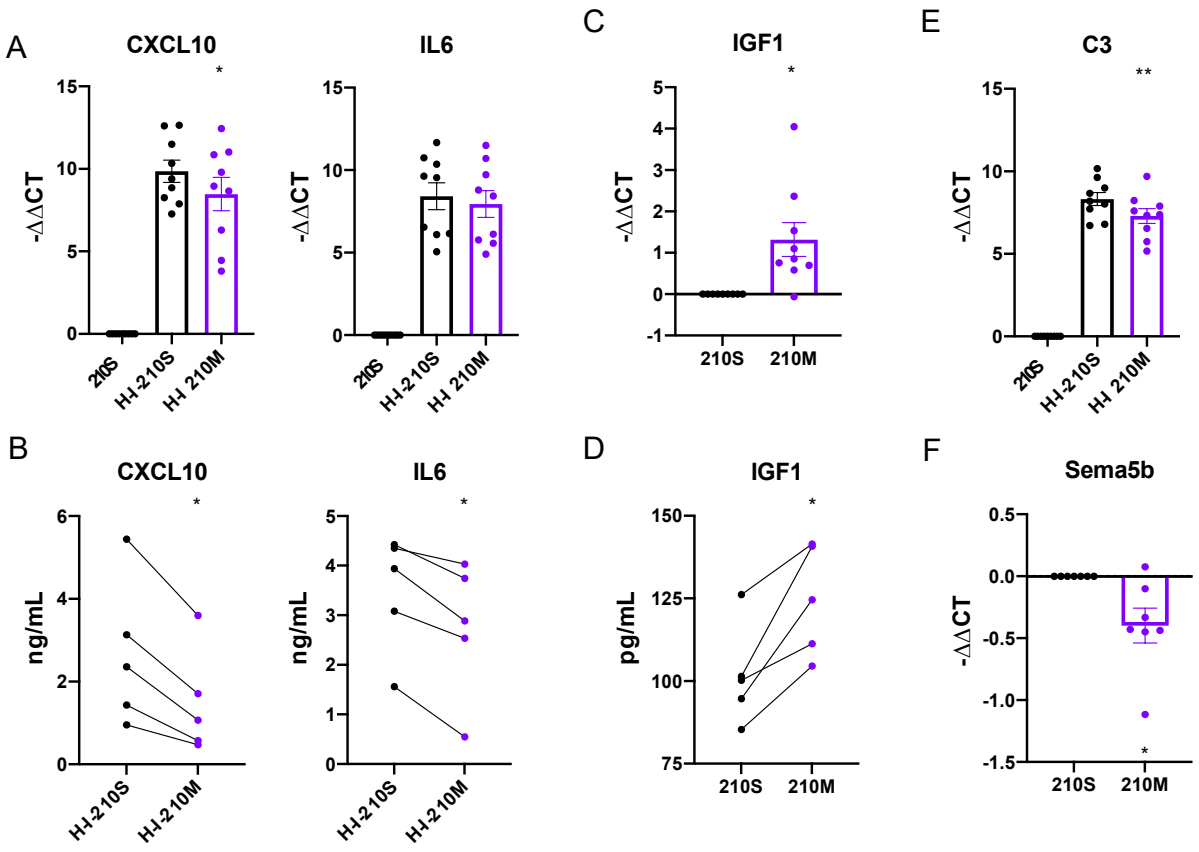
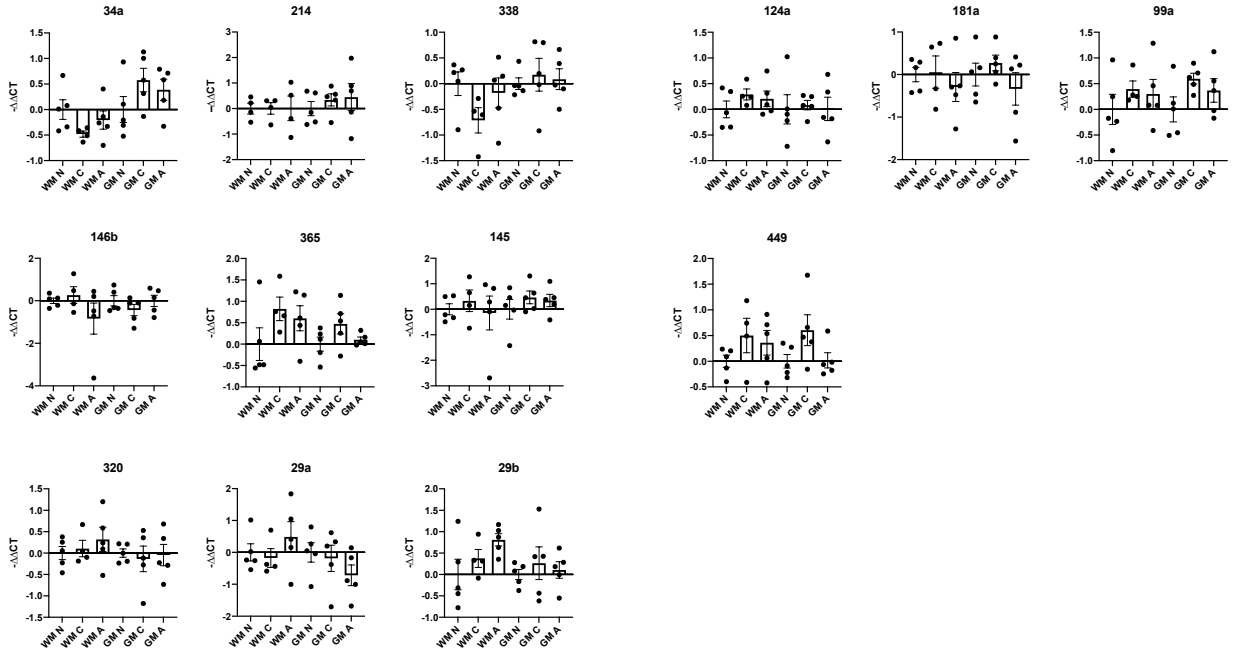
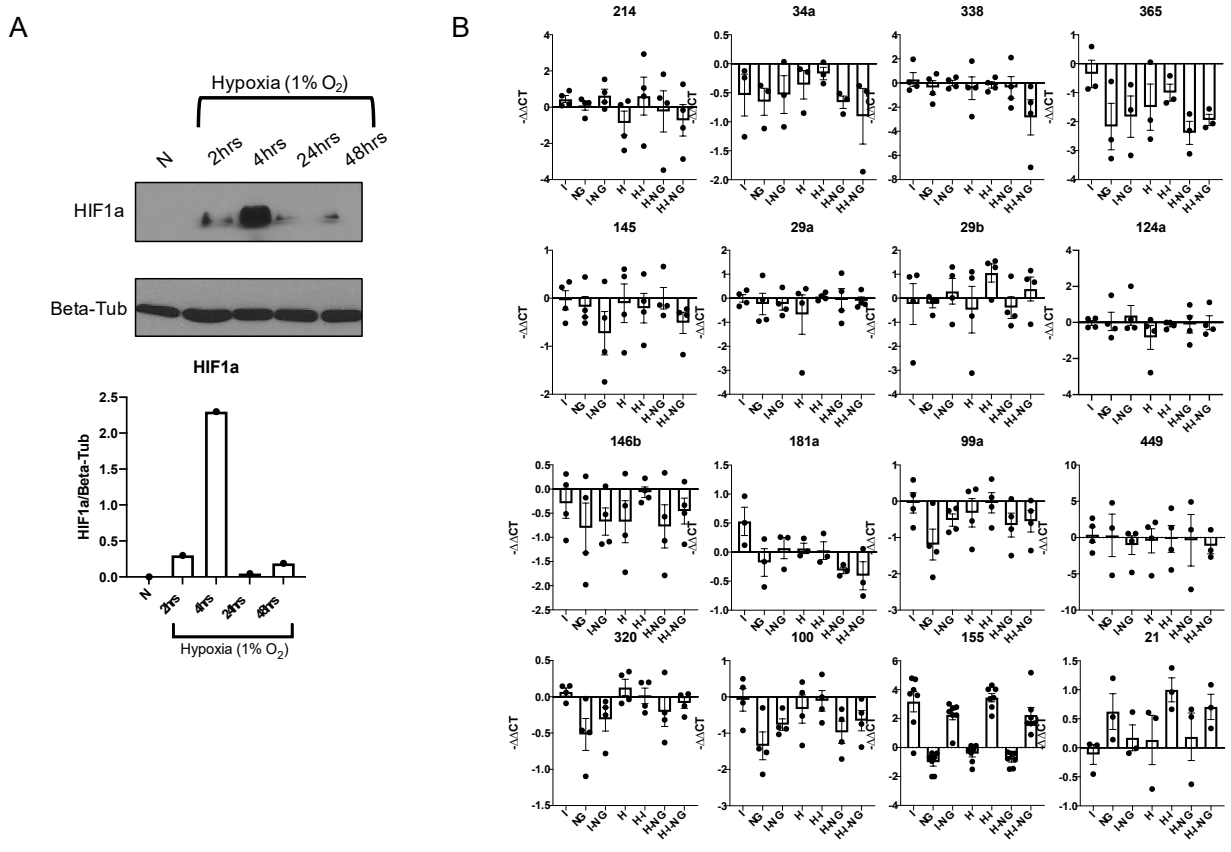


Figure 5: **210M transfected astrocytes exhibit an anti-inflammatory and neuroprotective phenotype.** Primary human astrocytes were transfected with 210S or 210M, with or without prior exposure to hypoxic and inflammatory stress ("H-I") using 1% oxygen and interleukin 1-beta, respectively. (A, C) RT-qPCR assessment of *CXCL10, IL6, IGF1* gene expression. (B, D) CXCL10, IL6, and IGF1 protein levels in cell supernatant measured by ELISA. (E,F) RT-qPCR assessment of *C3* and *Sema5b* gene expression. All data are presented as mean ± SEM of n=9 (A, C and E), 5 (B and D) or 7 (F) donors. Statistical comparisons between groups were made by a one-way ANOVA (A and E) or by a paired t-test (B, C, D and F). *p<0.05; **p<0.01.

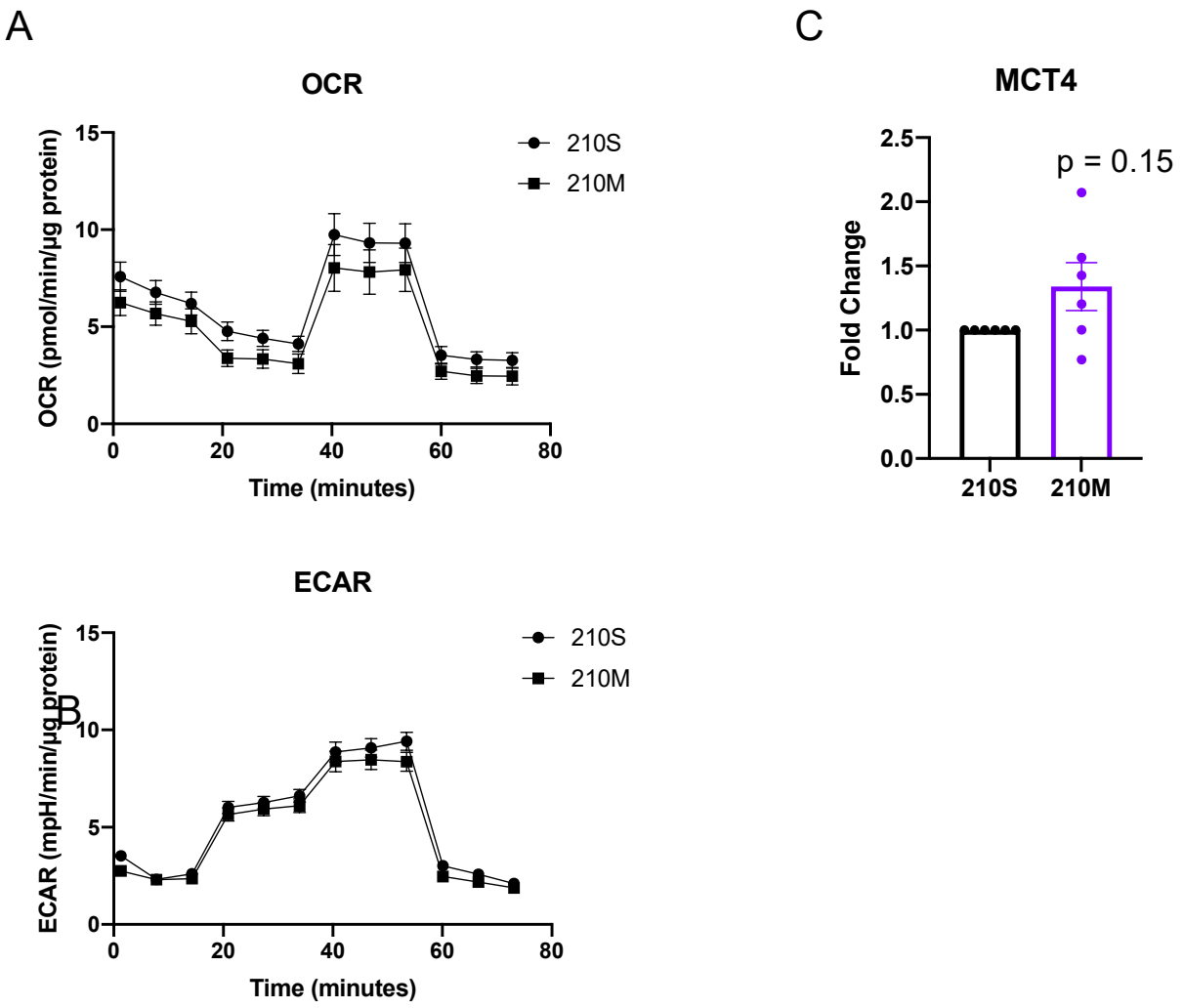
Supplementary Material



Supplementary Figure 1: **microRNA expression profile of astrocytes in normal tissue and around ischemic stroke lesions** GFAP+ astrocytes were captured from white (WM) and grey (GM) matter of unaffected (normal, "N") brain tissue or around acute ("A")/chronic ("C") stroke lesions using laser capture micro-dissection (LCM), and the expression levels of the listed miRs were then measured by RT-qPCR. Expression levels of white matter astrocytes are relative to the WM N condition, whereas expression levels of gray matter astrocytes are relative to the GM N condition. No graphs listed reached significance using one-way ANOVAs. n=4-5



Supplementary Figure 2: **Treatment in a hypoxia chamber induces HIF1a protein expression, and microRNAs are differentially expressed in primary human astrocytes undergoing different stresses.** (A) HeLa cells were left in normoxic (N) conditions for 48 hours or were treated in 1% O_2 (hypoxia, H) for the noted time period. After 2-48 hours, the cells were removed and were immediately lysed with RIPA buffer for subsequent Western blot. n=1. (B) Primary human astrocytes were untreated ("U") or subjected to inflammatory ("I"), metabolic ("M"), and/or hypoxic ("H") *in vitro* stress conditions using Interleukin-1 beta (IL1b), glucose-free media, or 1% oxygen, respectively. Histograms of miR expression in stress conditions relative to untreated control. Data are presented as mean ± SEM of n=5-6 donors.



Supplementary Figure 3: **Primary human astrocyte metabolism does not change after 24-hour transfection of 210M, and they do not significantly differentially express MCT4 protein levels after 48-hour transfection.** (A) Oxygen Consumption Rate (OCR) was measured in primary human astrocytes 24-hours after transfection of 210S or 210M. n=4 with 10 technical replicates for OCR experiments. (B) Extracellular Acidification Rate (ECAR) was measured in primary human astrocytes 24-hours after transfection of 210S or 210M. n=4 with 20 technical replicates for ECAR experiments. (C) Protein expression of MCT4 and Beta Tubulin (Beta-Tub) were measured by western blot in six separate human samples. Data is graphed as the fold change of MCT4 expression normalized to Beta-Tubulin expression and relative to the expression of 210S. For (C), each dot represents a separate human sample. Two-way ANOVE with Sidak's correction was performed for Seahorse experiments, whereas One-Way ANOVA was used for MCT4 expression.

Supplementary Table 1: **Mean Cycle Threshold Value of microRNAs from Human Brain Autopsy White Matter and Gray Matter Astrocytes**

miR-	WM Mean CT Value	GM Mean CT Value
34a	28.72	28.50
210	32.85	31.70
214	35.82	35.99
338	25.16	24.99
146b	33.49	32.42
365	30.73	30.35
145	28.83	28.31
320	31.50	30.83
29a	32.50	32.39
29b	32.57	32.73
124a	31.33	31.33
181a	31.43	30.97
99a	29.10	28.35
449	27.13	27.47
146a	30.24	29.59
155	33.49	33.70
100	29.32	27.34
21	32.41	31.88

Legend	
miR	microRNA
CT	Cycle Threshold
WM	White Matter
GM	Grey Matter

Thesis Conclusion

This study successfully discovered that miR-210, which has previously been shown to be upregulated in astrocytes around MS lesions (Rao et al., 2019), is also upregulated in astrocytes around ischemic stroke lesions. Further, we used primary human fetal astrocytes to elucidate the role of miR-210 in astrocytes and found that it promotes a potentially neuroprotective phenotype of the cells. This study therefore concludes that miR-210 is a promising candidate for further therapeutic studies in mouse models of disease. Recent advances of *in vivo* miR targeting techniques (Babar et al., 2012) will allow for studies that upregulate miR-210 specifically in animal astrocytes, which will provide additional insight into the neuroprotective ability of astrocytic miR-210 upregulation.