The Role of Microglia in Amyotrophic Lateral Sclerosis: Analysis of MicroRNAs

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Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive adult onset neurodegenerative disease characterized by selective death of the upper and lower motor neurons of the brain and spinal cord. Neuromuscular synapses are lost leading to paralysis and ultimately death. Non-neuronal cells, such as astrocytes, oligodendrocytes, and microglia, have been shown to contribute to ALS disease progression in mouse models. Microglia, the innate immune cells of the central nervous system, have been shown to be activated in ALS and contribute to disease progression. Hundreds of mRNAs have shown to be dysregulated in a variety of ALS cell types and tissues, including total spinal cord, acutely isolated microglia, and *in vitro* differentiated motor neurons. These mRNAs can be regulated post-transcriptionally by microRNAs (miRNAs), which are small endogenous non-coding RNAs with important regulatory roles in a wide range of cellular processes. This dissertation examines the contribution of miRNAs to ALS disease progression in microglia.

I acutely isolated primary microglia from the spinal cords of transgenic mice overexpressing human wild type (WT) SOD1 and human G93A SOD1. I used small RNA sequencing to profile the miRNAs that are expressed during disease progression, and identified miRNAs that are differentially expressed. I confirmed these results by quantitative PCR and examined the expression changes of predicted targets in a microglia RNA-seq dataset. Here I show that miRNAs are dysregulated in acutely isolated microglia from SOD1 G93A transgenic

mice, and that miR-155, a pro-inflammatory miRNA, and miR-210, a hypoxia-inducible miRNA, are significantly upregulated during disease progression. In addition, miR-1198-5p, miR-182, miR-503, and miR-668 are also dysregulated, and predicted mRNA targets of all six of these miRNAs are differentially expressed during disease progression.

To my knowledge, this is the first analysis of miRNA expression in microglia during ALS disease progression. This work contributes to the understanding of the contribution of a non-neuronal cell type to ALS disease progression and serves as a paradigm for studies in other non-neuronal cell types, such as astrocytes and oligodendrocytes, and other ALS mouse models.

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Chapter 1.

Introduction

Neurodegeneration

Neurodegenerative disorders such as Huntington's disease, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) are pathologies characterized by the progressive loss of neuronal structure and function and ultimately neuronal death. These disorders can occur either sporadically or in an inherited, familial manner. Recent advances in neurodegenerative research have identified common mechanisms involved in many of these diseases, suggesting there may be common dysfunctional pathways, which could be targeted for the development of therapeutics.

Autophagy, the degradation of a cell's own components in lysosomes, is a highly regulated process involved in normal cell growth, development, and homeostasis that is essential for maintaining the balance between synthesis, degradation, and turnover of various cellular components. Although autophagy has been studied for decades, its importance in the central nervous system has been recognized only recently (*1*). Quality control through autophagy is of particular importance in neurons where damaged organelles and misfolded proteins accumulate during disease progression. If these damaged components are not identified and removed, their accumulation can lead to neurotoxicity (*2, 3*). The ability of autophagosomes to function efficiently depends on their ability to navigate the unique architecture of the neuron, which contains long processes and dynamic traffic of vesicles and signaling molecules. Autophagic dysfunction has been identified in many human disorders, including a growing number of neurodegenerative disorders (*2-9*).

In addition to being the source of energy in the cell, mitochondria are critical regulators of cellular metabolism and play an important role in cell death pathways. Mitochondrial dysfunction and damage are important in many human diseases, including the pathology of many

neurodegenerative diseases (*10*). Due to high energy demands and increased sensitivity to apoptosis and reactive oxygen species, muscle and neurons are particularly susceptible to mitochondrial dysfunction. Mitochondrial dysfunction has been studied the most extensively in Alzheimer's and Parkinson's diseases. Examples of dysfunction include mitochondrial DNA mutations, mutations in nuclear-encoded mitochondrial proteins, and mutated proteins localized to incorrect intracellular compartments. In Alzheimer's disease, the accumulation of neurofibrillary tangles and plaques is observed, which are composed of the amyloid-β peptide (Aβ) in two conformations. Aβ appears to inhibit mitochondrial enzymes, such as cytochrome c oxidase and succinate-cytochrome reductase, directly interacts with mitochondrial proteins, and affects mitochondrial morphology and axonal transport of mitochondria (*11*). Despite these many links between Aβ and mitochondria, it is unknown how these different mechanisms contribute to disease pathogenesis. In Parkinson's disease, however, mitochondrial dysfunction is a direct causal factor for the disease. For example, two genes associated with autosomal recessive Parkinson's disease, Parkin and PINK1, interact to maintain mitochondrial function (*12*). Loss-of-function of either gene affects mitochondrial morphology and ATP production, and their expression suppresses mitochondrial fragmentation.

The expansion of trinucleotide repeat sequences within mRNAs is a prominent motif in at least 16 neurological disorders including Huntington's disease and fragile X syndrome (*13-15*). Expansion of these repeats beyond the normal threshold can have dramatic effects on the expression and stability of the host mRNA, leading to the disruption of cell and protein function, resulting in neurodegeneration. The varying number of repeats also helps explain the variability in phenotypes observed, and the larger the expansion, the earlier the onset and severity of disease. In addition, the expansions display germline instability, and thus subsequent

generations often have larger expansions, resulting in earlier disease onset and more severe disease progression. There are three primary types of diseases caused by repeat expansions: 1) Diseases caused by a loss of protein function, including fragile X syndrome in which there is a loss of FMRP. 2) Diseases caused by RNA-mediated gain-of-function, including myotonic dystrophy type 1 resulting from a repeat expansion in the 3'UTR of the kinase DMPK, which reduces its own expression and induces chromatin changes that alter the expression of neighboring genes (*16*). 3) Diseases caused by a gain-of-function, including Huntington's disease in which an expansion in the N-terminus of huntingtin causes protein aggregation (*13, 17*).

Tau, a microtubule-associated protein, is a neuronal phosphoprotein that has been shown to promote and stabilize microtubule formation *in vitro* and is involved in the development of axonal morphology (*18*). Many neurodegenerative diseases also exhibit tau pathology, including Alzheimer's disease, ALS, and Creutzfeldt-Jakob disease. These tauopathies are characterized by large aggregates of hyperphosphorylated tau protein in neurofibrillary tangles. Tau aggregates are associated with synapse and neuronal loss, and tau conformational changes, and thus neurodegeneration, are caused by alterations in tau splice forms (*19, 20*), phosphorylation (*21*), fibrillization (*22-24*), and association with Aβ (*25, 26*).

Inflammation is associated with many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, ALS, and multiple sclerosis. The immune system is critical for the maintenance of tissue homeostasis and the response to infection and injury. Microglia are the immune cells of the nervous system and reside in the brain and spinal cord. They survey the microenvironment and produce factors that are either toxic or protective towards neighboring neurons and astrocytes, which are glial cells of the brain and spinal cord. In a normal

physiological situation, microglia are in a resting state and produce anti-inflammatory and neurotrophic factors. However, in response to a pathogen or tissue damage, as observed in many neurodegenerative diseases, microglia switch to an activated state that is generally inflammatory leading to further activation of the immune system, initiation of tissue repair, and phagocytosis (*27*). An acute injury or insult may trigger oxidative and nitrosative stress, but is unlikely to be detrimental to long-term neuronal survival. However, chronic neuroinflammation, which occurs during neurodegeneration, results in long-term activation of microglia and sustained release of pro-inflammatory factors (*28*). Neuroinflammation will be discussed in greater detail below.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in adults and was first described in 1869 by the French neurobiologist and physician Jean-Martin Charcot (*29*). ALS is a progressive neurodegenerative disease characterized by selective death of the upper and lower motor neurons of the brain and spinal cord, leading to muscle atrophy and paralysis of voluntary muscles (*30*). Disease onset of ALS is typically between 40-70 years of age, with a median age of onset of 50, and the disease is generally fatal within 1-5 years of onset (*31*). There are ~30,000 American ALS patients at a given time.

The majority of ALS cases are sporadic with no known cause, and only 10% of cases are inherited (referred to as familial ALS). In both sporadic and familial ALS, there is progressive dysfunction of lower motor neurons and cortical motor neurons (*32*). In 1993, autosomal dominant mutations in the gene encoding the ubiquitous cytosolic Cu/Zn superoxide dismutase (SOD1) protein were shown to cause ALS (*33*). SOD1 is a homodimeric metalloenzyme that catalyzes the conversion of the toxic superoxide anion, a byproduct of oxidative phosphorylation

in the mitochodria, to hydrogen peroxide. Approximately 20-25% of familial ALS cases are caused by mutations in SOD1 (*34*). More than 114 disease-causing mutations have been identified that span all five exons of the gene (*34*). It is important to note that ALS is not a consequence of the loss of function of any of the enzymatic activities of SOD1 (*31*). Rather, the common feature of all of the characterized ALS-causing mutations is the presence of ubiquitinated SOD1 aggregates. SOD1 is a ubiquitous, highly abundant protein that appears to be highly susceptive to aggregation when mutated.

With the advent of more powerful genetic tools and whole exome or genome sequencing, many more ALS-causing genes have been identified (Table 1.1). For example, the nuclear protein TAR DNA-binding protein (TDP-43) was identified in sporadic ALS patients as a major component of ubiquitinated inclusions in motor neurons (*35-38*). More recently, a hexanucleotide expansion in *C9ORF72* was found to segregate with familial ALS in a Finnish population and is also present in 21% of sporadic cases (*39*).

A common feature of many ALS-causing mutant proteins is their tendency to form ubiquitinated protein aggregates. For example, TDP-43, a protein involved in transcription and alternative splicing, was found to be a major component of the ubiquitinated protein aggregates in patients with sporadic ALS and the most common form of frontotemporal dementia (*35, 36*). Aggregates were observed in the cytoplasm and nuclei of neurons and glia. In addition, brain and spinal cord samples from patients with these diseases showed abnormal ubiquitination of TDP-43. TDP-43 inclusions are recognized as a common characteristic in the majority of ALS patients, with the exception of patients with familial ALS caused by SOD1 mutations. In 2008, many groups reported additional dominant mutations in the TDP-43 gene (*37, 38*). These mutations were found in individuals with both sporadic and familial ALS. Most mutations are

Table 1.1. Common mutations in ALS.

Gene	% familial	% sporadic	Function
SOD1	20	10	Cu/Zn superoxide dismutase
TDP-43	5	2	transcription, splicing, transport, translation
FUS/TLS	4.5	rare	transcription, splicing, transport, translation
SETX	rare		DNA/RNA helicase
ANG	<1		promotes vascularization
OPTN	rare		inhibits activation of NFκB
VCP	1		protein degradation, autophagy
VAPB	rare		vesicle trafficking
FIG-4	1.5		vesicle trafficking
UBQLN2	rare		protein degradation
C9ORF72	45	20	unknown, expanded repeat

(*40*)

located primarily in the glycine-rich region (*41*), which has been shown to be highly prone to protein misfolding. Polymenidou *et al*. used antisense oligonucleotides to generate mice that lacked expression of TDP-43 in the striatum and observed that these mice were depleted of pre-mRNAs with very long introns and exhibited incorrect alternative splicing of some TDP-43 targets (*42*). However, it is currently unknown whether TDP-43 mutations lead to motor neuron degeneration through a toxic gain-of-function or a loss of normal function due to accumulation of the protein in cytoplasmic and nuclear inclusions.

In 2009, mutations in another DNA/RNA-binding protein, FUS/TLS, were found to cause familial ALS (*43, 44*). FUS/TLS binds to RNA, functions in diverse processes including transcription and splicing, and is normally localized to the nucleus. The mutations were located in the glycine-rich region and within the last 13 amino acids of the protein (*41*). FUS/TLS is both structurally and functionally highly similar to TDP-43. In ALS, FUS/TLS is found in neuronal cytoplasmic inclusions. Interestingly, TDP-43 inclusions are absent in ALS patients with FUS/TLS mutations, and thus, despite the functional similarity between TDP-43 and FUS/TLS, FUS/TLS mutations are independent of TDP-43 aggregation (*43*).

More recently, several new mutations have been identified that cause ALS. *Optineurin*, a gene that functions in autophagy and is the cause of a type of glaucoma, was found to carry several mutations that cause ALS (*45*). Optineurin has also been implicated in the NF-κB pathway, and missense and nonsense mutations in optineurin abolish the inhibition of NFκB activation, while another mutation causes altered cytoplasmic distribution of the protein. In addition, it was observed that TDP-43 and SOD1 inclusions in sporadic and familial ALS, respectively, also stained positive for optineurin.

Mutations in the gene encoding the protein ubiquilin 2, a component of the ubiquitin/proteasome pathway, were also found to cause familial ALS (*46*). These mutations result in a general decrease in proteasome-dependent protein degradation. Ubiquilin 2 colocalizes with cytoplasmic TDP-43 associated with ALS. The most recently discovered ALS-linked mutation is a hexanucleotide (GGGGCC) repeat expansion in *C9ORF72*, a gene of unknown function (*39*). This repeat expansion was found to account for 46% of familial and 21% of sporadic ALS in a Finnish population, making it the most predominant mutation currently known. Based on a previously described repeat expansion implicated in the disease myotonic dystrophy (*16*), the *C9ORF72* expansion has been proposed to act as a sponge for RNA binding proteins, thus preventing them from carrying out their normal functions. In the case of myotonic dystrophy, the key RNA proteins involved have been identified, and at least one of them (muscleblind) can complement the repeat expansion mutation (*47*).

Because the clinical and pathological characteristics of sporadic and familial ALS are indistinguishable, it is assumed that studies examining ALS-causing gene mutations will also provide insights on sporadic ALS. As SOD1 was the first gene identified with ALS-linked mutations, the majority of animal models are based on these mutations (*33*). Transgenic mouse models of the disease have been generated for numerous SOD1 mutations in which the mutant human proteins are overexpressed. SOD1 transgenic mice and rats develop motor neuron disease with the same features as ALS, including progressive paralysis and death due to motor neuron degeneration (*48-51*). The most commonly used animal model is the SOD1 glycine 93 to alanine (G93A) transgenic mouse (*49*). As mentioned above, despite the prevalence of mutations and the importance of the SOD1 protein, there is no obvious correlation between enzymatic activity and disease severity and progression. Although most mutations reduce

dismutase activity, others retain full catalytic activity (*31*). Interestingly, mice completely deficient in SOD1 activity develop normally and do not display motor defects, indicating that SOD1 is not necessary for normal motor neuron development (*52*). In addition, when transgenic mice overexpressing the human G85R SOD1 mutation were mated to mice expressing wild type human SOD1 at six times the level of endogenous mouse SOD1 or mice lacking endogenous SOD1, there was no effect on onset, disease progression, or accumulation of mutant protein (*53*). Thus, it is more likely that the mutations cause the acquisition of one or more dominant gain-of-function toxic activities.

Numerous ALS disease mechanisms have been proposed, including mitochondrial dysfunction, aberrant chemistry and oxidative stress, protein instability and aggregation, altered axonal transport, apoptosis, excitotoxicity and glutamate transport, and neuroinflammation (*34, 54*). In addition, extensive work has been done to understand the effect of non-neuronal cells on motor neurons. A common feature of SOD1-associated ALS is the presence of immunoreactive SOD1 aggregates in motor neurons, the neuropil, and astrocytes of transgenic SOD1 rodents and some human ALS cases (*53, 55*). These aggregates are also positive for ubiquitin (*48, 53*). It is not yet understood how SOD1 aggregates directly affect motor neurons in disease (*56*), however, due to the ubiquitin-positive SOD1 aggregates, it is likely that protein misfolding, aggregation, and stability are involved. These observations suggest a unifying hypothesis for ALS: a life-time imbalance of protein aggregation and degradation (proteostasis) ultimately leads to the accumulation of protein aggregates capable of inducing multiple cellular pathways leading to motor neuron death (*54*).

An important recent advance was the realization that the effects of mutant SOD1 are not solely cell autonomous. Using mouse blastocyst chimeras that were mixtures of normal and

SOD1 mutant-expressing cells, Clement *et al*. showed that toxicity to motor neurons required damage from mutant SOD1 expressed in non-neuronal cells and that non-neuronal cells not expressing mutant SOD1 delayed motor neuron degeneration and extended survival (*57*). Even more striking, the selective expression of mutant SOD1 protein in motor neurons was not sufficient to cause motor impairment or disease despite high levels of mutant SOD1 present in motor neurons (*58, 59*). Jaarsma *et al*. did a similar study, and although they observed motor defects, onset and end-stage of the disease were much delayed and there was no hindlimb paralysis observed (*60*).

Efforts have also been made to understand the contributions of mutant SOD1 in non-neuronal cell types. Although retraction of motor axons from synaptic connections to muscle is among the earliest pre-symptomatic events, deletion of mutant SOD1 from muscle did not affect onset or survival (*61*). Expression of mutant SOD1 in oligodendrocytes was also found to not affect disease onset (*62*). Deletion of mutant SOD1 from microglia had little effect on onset but significantly delayed disease progression (*63*). Similarly, deletion of mutant SOD1 in astrocytes did not affect onset but delayed microglial activation and disease progression (*64*). Very recently, it was shown that astrocytes expressing mutant SOD1 can induce the degeneration of wild type motor neurons *in vivo* (*65*). These papers demonstrated the critical role of astrocytes and microglia in disease pathogenesis and illustrated the importance of non-neuronal cells. In addition, cell culture studies using primary glial cultures and either primary motor neurons or embryonic stem cell-derived motor neurons showed that mutant SOD1 astrocytes secrete a toxic factor that is able to kill motor neurons (*66, 67*). Thus, mutant SOD1 within motor neurons causes direct cell autonomous damage to motor neurons in the spinal cord, but disease

progression likely depends on the expression of the mutant protein in non-neuronal cells, such as microglia and astrocytes (*68*).

Microglia

Microglia, which comprise 5-20% of all glial cells in rodents, are the innate immune cells (i.e. the resident macrophages) of the brain and spinal cord (*69*). Microglia were first described by Pio del Rio-Hortega in 1919 as a cell type in the nervous system distinct from neurons and astrocytes (*70*). Rio-Hortega observed that microglia are critical to pathological processes in the central nervous system and are able to rapidly change their morphology from a branched, ramified morphology to an amoeboid morphology in response to various insults including physical trauma and infection (*71, 72*). In addition to a distinct morphology, microglia are distinct from other glial cells, such as astrocytes and oligodendrocytes, based on their origin, gene expression patterns, and functions.

Microglia are hematopoietic in origin and are most closely related to bone marrow-derived macrophages (*73*). Recent studies suggest that microglia originate from the yolk sac macrophages that migrate into the central nervous system during early embryogenesis and are distinct from cells that are generated by definitive hematopoiesis in the bone marrow and from circulating cells (*74, 75*). Microglia appear to represent a distinct type of macrophage that are long-lived and/or locally self-renewing (*73*). In a normal physiological state, resting microglia are highly dynamic and actively survey their environment with extremely motile processes and protrusions (*76-78*). They are highly ramified with an elaborate branch structure. Each microglia cell occupies a 30-50 μm-wide region of the brain, and an individual cell rarely crosses branches with its neighbors. Upon traumatic injury and disruption of the blood-brain barrier,

microglia processes rapidly converge on the site of injury to create a barrier between the injured tissue and healthy tissue (*77, 78*).

Microglia play important roles in the normal physiology of the brain (*79, 80*). The loss of neuronal cells is a critical event during the development of the nervous system and involves apoptosis followed by efficient removal of the remains of the neurons. The removal of dead cells is accomplished by phagocytes, such as microglia. In addition, it has recently been shown that microglia actually promote the death of Purkinje neurons engaged in synaptogenesis (*81*). The rapid release of reactive oxygen species by microglia induces neuronal death, and then, these dead cells are contacted by spreading microglial processes and engulfed. As described above, microglia survey their microenvironment. Using two-photon imaging of fluorescently-labeled neurons and microglia, it was recently shown that resting microglial processes make brief, direct contacts with synapses approximately once per hour, and these contacts are activity-dependent and decrease in frequency in response to reductions in neuronal activity (*82*). Following transient cerebral ischemia, the length of the microglia-synapse contacts is extended (to around an hour) and are often followed by the disappearance of the presynaptic bouton. Very recently, a protective role for ramified, resting microglia has been described by Vinet *et al*. (*83*). These authors found that resting microglia protect hippocampal neurons in response to excitotoxicity and therefore limit neuronal death (*84*). In addition, it has been shown in mice that microglia actively engulf synaptic material and play a major role in synaptic pruning during postnatal development (*85*).

As the immune cells of the central nervous system, microglia monitor the presence of pathogens and injury in the nervous system. Microglia are often the first cell type to respond to tissue damage in the central nervous system (*86*). As discussed above, resting microglia are

ramified, with thin processes that continuously sense and survey the cell's immediate microenvironment. This random scanning changes to an actively targeted movement when microglia sense infection, trauma, ischemia, neurodegeneration, or altered neuronal activity (*72, 87*). Once a disturbance in brain homeostasis has been sensed, microglia are activated and also undergo phenotypic changes; the cell body increases in size, and the processes retract to become an amoeboid cell. Microglia can then carry out a number of diverse functions. Local populations of microglia can proliferate to provide more cells to defend against infection and restore tissue homeostasis (*72*). Microglia respond to different types of stimuli to act in either a neuroprotective or neurotoxic manner. Activation is associated with increased production of potentially cytotoxic molecules, such as reactive oxygen species, nitric oxide, proteases, and pro-inflammatory cytokines such as interleukins and TNFα (*88, 89*). Microglia can also release chemoattractant molecules to recruit other immune cell populations to the central nervous system. In addition, microglia can be further stimulated by interferon-γ secreted by T cells and act as antigen-presenting cells to present antigenic compounds to T cells to mount an adaptive immune response (*90, 91*). Activation can also lead to some neuroprotective roles for microglia. A primary neuroprotective role of microglia is to phagocytose tissue debris and damaged cells, thus allowing healthy cells to survive (*92*). Microglia can also produce growth factors and extracellular matrix to encourage wound healing. In addition, microglia can upregulate insulin-like growth factor 1 (IGF1), which supports neuronal survival (*91*).

Microglia in neurodegenerative diseases

It is not yet entirely clear whether microglia are beneficial or harmful in neurodegenerative diseases (*91, 93*). In some diseases, inflammation of the central nervous

system directly leads to neuronal damage. Bacterial meningitis leads to neuronal apoptosis via activation of microglia through TLR- and MyD88-dependent pathways (*94*). As mentioned previously, aggregated β-amyloid is observed in neurodegenerative diseases. β-amyloid has been found to activate microglia resulting in neuronal apoptosis (*95, 96*). In addition, inhibition of microglia with minocycline, an anti-apoptotic compound, has neuroprotective effects in mouse models of Parkinson's disease (*97, 98*). Thus, microglia activation can clearly be harmful in some diseases of the central nervous system. However, there is also evidence for a neuroprotective role for microglia in other neuronal diseases. In a mouse model of stroke, microglia express the neuroprotective protein IGF1, and selective removal of proliferating resident microglia led to a decrease in IGF1 and more ischemic damage (*99*). It has also been observed that in Alzheimer's disease, microglia play an important role in the clearance of harmful Aβ aggregates via phagocytosis (*100*). Additionally, in the SOD1 mouse model, mutant microglia have been observed to upregulate IGF1 as well as other growth factors, suggesting an anti-inflammatory response in these cells (*101*).

Neuroinflammation occurs in nearly every disease of the nervous system (*86*). During infection there are mechanisms in place to suppress neuroinflammation, including activation-induced cell death and regulatory T cells (*102, 103*). Although necessary to combat pathogens, neuroinflammation can be deleterious to nearby neurons when not controlled or in chronic situations (*89*). Neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, and ALS are examples of chronic neuroinflammation and microglial activation (*79, 104*). In Alzheimer's disease, microglia display an activated phenotype, surround β-amyloid plaques, and participate in their clearance (*105, 106*). In a rat model of Parkinson's disease, extensive microglia activation was prominent in the substantia nigra and striatum and

preceded dopaminergic neuron degeneration (*107*). In Huntington's, inflammation appears peripherally and in the central nervous system during disease progression (*28*).

Microglia in ALS

Neuroinflammation is a prominent feature in the spinal cords of ALS patients and rodent models of ALS. Inflammation and microglial activation and proliferation in the spinal cord parallels motor neuron degeneration (*108, 109*). Adaptive immunity is also involved in disease progression, with active accumulation of T cells and deposition of antibodies within the spinal cord. Numerous pro-inflammatory cytokines are elevated in ALS and the levels of their expression correlate with disease progression (*110-112*). In addition, Chiu *et al*. found that T cells infiltrate the spinal cord during disease progression and potentiate neuroprotective inflammation in the G93A SOD1 model of ALS (*101*).

Several recent studies illustrate the deleterious effects of mutant SOD1 in microglia and suggest that mutant microglia contribute to the disease progression. Microglia activation is observed throughout the spinal cord and increases with disease progression (*104, 113*). Boillee *et al*. showed that selective depletion of mutant SOD1 from microglia and macrophages of transgenic mice led to an extension in lifespan (*63*). The deletion of mutant SOD1 had no affect on disease onset, but progression was dramatically retarded, suggesting the expression of mutant SOD1 in microglia had deleterious effects on disease progression. In addition, Beers *et al*. used SOD1 G93A mice that lack endogenous microglia to show that bone marrow transplantation of wild type microglia progenitors led to an extension of lifespan, whereas transplantation of mutant microglia progenitors did not affect lifespan (*114*). These studies illustrate the deleterious effects of mutant SOD1 in microglia and suggest that mutant microglia might be neurotoxic when

compared with wild type microglia (*115, 116*). Consistent with the involvement of SOD1, reactive oxygen species have been implicated as a mutant microglia-produced toxic factor. Exogenously supplied mutant SOD1 (G93A or G85R) activated microglial release of pro-inflammatory cytokines and free radicals (i.e., reactive oxygen species) (*117*). More specifically, deletion of *Nox2*, which encodes the inflammatory NADPH oxidase, a major source of reactive oxygen species in inflammation, slowed disease progression and improved survival (*97, 118*). Together, these data, along with other studies, indicate that microglia play a central role in ALS disease progression.

General miRNA background

MicroRNAs (miRNAs) are endogenous small, single-stranded non-coding RNAs ~21-25 nucleotides (nt) in length that are critical regulators of gene expression. miRNAs act by downregulating target mRNAs either by translational repression or mRNA degradation (*119-122*). miRNA genes are estimated to comprise 1-3% of all vertebrate genes and are highly conserved from flies to humans, suggesting that they play an important role in the regulation of gene expression (*123*). Since their discovery, miRNAs have been shown to function in diverse processes ranging from mammalian myoblast differentiation (*124*), cell death in *Drosophila* (*125*), and neuronal patterning in *C. elegans* (*126, 127*). Early studies in the mammalian nervous system showed that expression patterns of miRNAs change during development (*128-130*), suggesting that miRNAs are involved in the development and physiology of the nervous system. Many specific miRNAs have been examined in the central nervous system, an example of which is the mouse miR-124, one of the most highly expressed miRNAs in the mammalian brain. This miRNA has been shown to promote a neuronal-like transcriptional profile by lowering levels of

non-neuronal transcripts (*131*). Specifically, Makeyev *et al*. demonstrated that miR-124 targets PTBP1, a global repressor of alternative pre-mRNA splicing in non-neuronal cells, leading to neuron-specific alternative splicing (*132*).

Since the demonstration that the miRNAs let-7 and lin-4 control the timing of developmental events in *C. elegans* via complementarity to regions in the 3' untranslated region (UTR) of heterochronic genes (*133-136*), there has been substantial effort to identify how miRNs are processed and how they affect mRNA biogenesis. miRNA genes are located in intergenic regions or within introns of protein-coding genes. miRNAs located in intergenic regions are transcribed by RNA polymerase II to generate capped and polyadenylated primary miRNA transcripts (pri-miRNA) (*137, 138*). Alternatively, miRNAs encoded within introns are co-transcribed with the host mRNA and are subsequently processed post-transcriptionally (*123*). Pri-miRNAs have a stem-loop (hairpin) structure that contains the ~21-25 nt long mature miRNA. The stem-loop of the pri-miRNA is excised by the RNaseIII Drosha-DGCR Microprocessor complex to yield a ~60-80 nt precursor miRNA (pre-miRNA) (*139-143*). The pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5 (*144, 145*) and further processed by the RNaseIII Dicer to yield a miRNA duplex (*139, 146*). One strand of the duplex (the mature miRNA) is loaded into the miRNA RNA-induced silencing complex (miRISC) while the other strand is typically degraded. miRISC stabilizes the target strand and guides it to target mRNAs with sequences in the 3'UTRs complementary to the mature miRNA (*123*).

Following the targeting of miRISC to their cognate mRNAs, target mRNAs are then downregulated by either translational repression or mRNA degradation. There is evidence for translational repression at both initiation and post-initiation, although there is more evidence for repression occurring at initiation (*147*). In both *C. elegans* and mammalian cell culture

experiments, miRNAs were observed to associate with polysomes, suggesting their regulation occurred post-initiation (*148-152*). There exist numerous hypotheses for how this regulation occurs, including degradation of the growing polypeptide chain co-translationally (*151*) and premature ribosome dissociation from the mRNA (*152*). However, there are also studies that suggest repression occurs at translation initiation. In the presence of cognate miRNAs, mRNAs do not sediment with polysomes, but rather shift toward lighter fractions with fewer ribosomes or free messenger ribonucleoproteins (*153*). In addition, experiments performed using cell-free extracts have shown that miRNAs do not target mRNAs with an artificial cap structure and fail to silence transcripts driven by an IRES, which suggests that the silencing machinery targets the cap or interferes with the cap-binding complex (*154-158*).

There is also substantial evidence supporting the role of miRNAs in target degradation. Although it was initially thought that animal miRNAs acted primarily through translational repression, more recent studies provide evidence that animal miRNAs also act through target degradation. Much evidence for this target degradation comes from individual studies that examine specific miRNA:mRNA pairs in which the upregulation of a specific miRNA correlates with downregulation at the transcript level of specific targets (*147*). Transcriptome-wide studies examining the up or downregulation of a specific miRNA leads to corresponding down or upregulation of its predicted targets (*131, 159, 160*). In plant cells it is well established that miRNAs can lead directly to cleavage of cognate mRNAs (*161*). However, in animal cells it is more likely that miRNAs direct mRNAs toward mRNA decay by deadenylation of the mRNA (*162-166*). Several studies have also observed that the abundance of miRNA targets increases when decay factors are depleted corroborating their role in miRNA-induced mRNA decay (*162, 165, 166*). More recent quantitative mass spectrometry studies have measured the effect of

miRNAs at the proteome level (*159, 167*). The results of these studies show only modest inhibition in protein output, which is rarely more than four-fold. In addition, ribosome profiling to measure effects on protein production coupled with RNA-seq analysis to measure mRNAs revealed that decreased mRNAs were responsible for >84% of the observed decrease in protein production (*168*). This finding suggests that the destabilization of target mRNAs is the major reason for decreased levels observed at the protein level.

In metazoans, bioinformatic studies predict that individual miRNAs could potentially downregulate hundreds of mRNAs through partial complementarity to the 3' UTR of the target mRNA (*122, 131, 169*). It has been estimated that on average an individual *Drosophila* miRNA targets 54 transcripts and an individual human miRNA targets up to 200 transcripts (*170-172*). Lewis *et al*. predicted that up to 50% of mammalian mRNAs could be miRNA targets (*173*). However, due to the partial complementarity between miRNAs and mRNA targets, target prediction is computationally challenging. The miRNA region with the highest degree of complementarity is termed the miRNA seed and comprises nucleotides 2-8 from the 5' end (*174*). The seed is often flanked by an adenosine (*173*), and this region is usually conserved within miRNA families. The seed is critical for regulation, and sometimes sufficient for a miRNA to target an mRNA. More recent work has suggested that there are additional intricacies to a miRNA targeting an mRNA than simply the seed. There is evidence that, in the cases of less optimal pairing within the miRNA seed, there might be additional compensatory pairing between the 3' end of the miRNA and the mRNA (*175, 176*). Most UTRs contain more than one miRNA binding site, and these binding sites can exhibit different degrees of complementarity to the miRNA (*177*). mRNAs can also be targeted by more than one miRNA, suggesting combinatorial control (*174, 178*). Additionally, a single miRNA can bind an mRNA at more than one site and

act cooperatively for higher levels of downregulation than might typically be expected (*178-180*). There is a general view that individual mRNAs are likely regulated based on the number and nature of the miRNA target sequence, as well as the levels of miRNA present (*181*). The context of the target sites are also thought to play a role in the efficacy of a particular site (*182*). Grimson *et al*. suggested that close sites act synergistically and that being located within an AU-rich local sequence and near either end of the 3' UTR also contribute to targeting. The authors observed that sites within the first 15 nucleotides after the stop codon make poor target sites, perhaps due to interference with ribosomes (*175*).

Target site prediction is particularly challenging due to the necessity of as few as 6 complementary nucleotides for binding and recognition (*131*). Numerous target prediction tools are now available and typically identify targets based on the evolutionary conservation of seed matches within a given gene (*171, 173, 183*). Although various prediction tools, such as Targetscan, Pictar, and Miranda, are of great importance for the field, the algorithms generate different results and have high false-discovery rates (*167, 170, 184*). Recently a non-computational method called Argonaute (Ago) HITS-CLIP (high throughput sequencing of RNA isolated by crosslinking immunoprecipitation) was developed (*185, 186*). HITS-CLIP is a technique that allows the identification of *in vivo* protein-RNA interactions on a genome-wide scale. In brief, RNA-protein complexes that are in direct contact are covalently crosslinked using UV irradiation, and the RNAs bound by specific proteins can then be identified by high throughput sequencing. Ago HITS-CLIP enables the precise identification of mRNA targets that are bound to specific locations within 3'UTRs in a ternary complex with Ago, thus providing strong evidence of target recognition and subsequent downregulation.

Additionally, the 3' UTR provides additional opportunities for regulation through alternative processing. The 3' UTR contains many cis-acting regulatory elements that lead to a variety of events, including polyadenylation, decay, stability, subcellular localization, and translation (*187*). Single mutations can affect the regulation of a specific 3' UTR or a mutation or deficiency in a factor that interacts with the UTR could cause more widespread effects. Mayr *et al*. found that cancer cell lines often express higher levels of mRNAs with shorter 3' UTRs, due to alternative cleavage and polyadenylation. These shorter isoforms were observed to be more stable than the full-length isoforms and also produced much higher levels of protein (*188*). Additionally, the use of alterative 3' UTRs can affect the ability of a miRNA to target a cognate mRNA. In a recent study, the *Drosophila* Hox gene *Ultrabithorx* (Ubx) was found to generate mRNAs with variable 3' UTRs in different regions of the embryo (*189*). Ubx is targeted by two miRNAs, and the resulting alternative 3' UTRs carry different miRNA target sites, thus allowing the long isoform to be regulated by miRNAs. Mutations in the 3' UTR can also introduce or remove miRNA target sites. For example, Texel sheep are an extremely muscular breed due to lower levels of myostatin. Clop *et al*. identified a point mutation that caused a target site in the myostatin 3' UTR for two miRNAs that are highly expressed in skeletal muscle; thus, myostatin is translationally repressed leading to muscular hypertrophy of this breed of sheep (*190*).

miRNAs in the central nervous system

miRNAs are highly expressed in the nervous system and have been shown to be involved in the regulation of numerous processes ranging from patterning and cell specification to neuronal plasticity (*191, 192*). Some early studies examined the involvement of miRNAs in the nervous systems of invertebrates. Examples include the miRNA lsy-6, which controls left-right

neuronal asymmetry in *C. elegans* by targeting a homeobox gene, *cog-1 (126)*, and miR-9a, which was shown to ensure the specification of neuronal sensory organ precursor cells during *Drosophila* development (*193*). Many groups have also examined the expression patterns of miRNAs during mouse development (*128-130, 194*). These studies revealed many miRNAs that are specifically enriched in the brain, suggesting that these miRNAs could have specific functions in the central nervous system.

Early studies by Krichevsky *et al*. and Miska *et al*. demonstrated that various miRNAs exhibit precise temporal regulation during brain development, indicating they might be involved in development of the mammalian brain (*128, 130*). It has since been shown that miRNAs are indeed involved in the developing nervous system, and in processes including neural patterning, cell specification, axonal pathfinding, and apoptosis (*191*). miR-124 is a mammalian miRNA that has been extensively studied due to its high expression in mature neurons and its role in establishing and maintaining neuronal identity. A study in HeLa cells illustrated that overexpression of miR-124 causes these cells to express a more neuronal mRNA profile (*16, 131*). Similarly, in neural progenitor cells, miR-124 drives cells toward a more neuronal identity, perhaps by inhibiting gliogenesis (*195*). It was later shown by Makeyev *et al*. that miR-124 is able to promote neuronal differentiation by targeting PTBP1, a global repressor of alternative splicing in non-neuronal cells, thus triggering brain-specific alternative splicing (*132*). In addition, miRNAs are expressed in mature neurons and are involved in synaptic plasticity (*196*) and dendritic spine development (*197*).

miRNA in neurodegenerative diseases

The levels of mRNA transcripts are pathologically altered in most neurodegenerative diseases. As miRNAs are highly expressed in the nervous system, it has been hypothesized that miRNAs, which have the ability to regulate hundreds of mRNA targets, might be involved in neurodegeneration. miRNAs have been shown to be involved in processes that, if dysregulated, might contribute to degeneration, including supporting neuronal survival and cellular stress (*198-200*). In addition, deletion of Dicer from Purkinje cells (*201*) and motor neurons (*202*) leads to cerebellar and motor neuron degeneration, respectively. Progressive neurodegeneration due to the loss of Dicer suggested a potential role for miRNAs in neurodegeneration. Bilen *et al*. showed that in *Drosophila* and human cell line models of spinocerebellar ataxia, reductions in miRNA processing caused an enhancement of polyglutamine toxicity (*203*). In addition, it was shown that removal of Dicer in mouse adult forebrain neurons led to abnormal tau hyperphosphorylation and neurodegeneration (*204*).

Subsequent papers examined the role of miRNAs in neurodegeneration more specifically. Karres *et al*. found that in *Drosophila*, miR-8 acts to tune atrophin levels to prevent neurodegeneration (*205*). In human cells expressing *ataxin1*, a gene which when containing an expanded polyglutamine repeat causes spinocerebellar ataxia type 1, it was found that three miRNAs co-regulate ataxin1 levels and inhibition of these miRNAs enhance toxicity of the expansion (*206*). Various miRNAs are up or downregulated in models of different neurodegenerative diseases. For example, the bifunctional miR-9/miR-9* is downregulated in Huntington's disease (*207*). Some studies performed on post-mortum tissue from individuals with neurodegenerative diseases have shown aberrant miRNA expression, including the downregulation of miR-29a in the brains of Alzheimer's patients (*208*).

miRNAs have not been thoroughly examined in ALS, and to date only three papers have been published that address motor neuron disease and miRNAs. Haramati *et al*. showed that the loss of miRNAs in motor neurons due to mutant Dicer causes progressive locomotor dysfunction, muscular atrophy, and a loss of motor neurons, thus concluding that mice lacking miRNAs in spinal motor neurons resemble the degeneration observed in spinal muscular atrophy (SMA) (*202*). The authors also observed incorrect stoichiometry of the neurofilament subunits, and that miR-9, which targets one of the neurofilament subunits, is downregulated in a mouse model of SMA. Williams *et al*. published the only study specifically examining the role of miRNAs in ALS (*209*). They observed that miR-206, a skeletal muscle-specific miRNA, is dramatically upregulated in the SOD1 G93A mouse model of ALS, and acts to delay ALS progression by promoting the regeneration of neuromuscular synapses in mice. This regeneration is accomplished by miR-206 repressing the production of histone deacetylase 4, which itself has an inhibitory effect on neuromuscular junction reinnervation. Finally, Buratti *et al*. knocked down TDP-43 in a cell culture line and found that there were specific miRNAs that were up or downregulated (*210*). let-7b and miR-663 are two of these miRNAs and were also found to directly bind TDP-43.

miRNAs and microglia

Only a few labs have examined miRNAs in microglia. Rom *et al*. showed that miR-146a, a miRNA known to be involved in the regulation of the innate immune response and increased in chronic inflammation, targets the chemokine CCL8/MCP-2 in HIV-1-infected human microglial cells (*211*). Ponomarev *et al*. observed that miR-124 promotes microglia quiescence and suppresses experimental autoimmune encephalomyelitis, a model of multiple sclerosis, by

deactivating macrophages through the C/EBPα-PU.1 pathway (*212*). In addition, they showed that expressing miR-124 in macrophages transformed these cells from an activated to a resting phenotype. Finally, Cardoso *et al*. showed that miR-155, a pro-inflammatory miRNA, modulates the immune response in a microglia cell line by downregulating SOCS-1, an inhibitor of the inflammatory response, and by promoting the production of cytokines and nitric oxide (*213*).

Overview of dissertation

Our lab is highly interested in the role of non-neuronal cells, particularly microglia and astrocytes, in ALS disease pathogenesis. The primary goal of my dissertation is to examine the role of microglia in ALS disease progression by analyzing miRNAs. In addition to the fact that this is the first time that miRNAs have been examined in the context of ALS disease progression, this is also the first global examination of miRNAs expressed in microglia. To this end, I isolated primary microglia from mice overexpressing human wild type or G93A SOD1 during disease progression (at time points corresponding to pre-symptomatic, symptom onset, and end-stage) and used these acutely isolated cells for numerous analyses. miRNAs expressed in these microglia were sequenced by our collaborators at the HudsonAlpha Institute for Biotechnology (Shawn Levy) and genomic alignment and statistics were performed by Mike Muratet (Myers Lab, HudsonAlpha). I analyzed and validated the sequencing data. In addition, a former post-doc from our lab, Isaac Chiu, performed RNA-seq analysis on acutely isolated microglia from both non-transgenic mice and mice overexpressing human G93A SOD1 mice during disease progression (unpublished data). I have used this dataset to examine a functional relationship between miRNAs and their targets.

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Chapter 2.

The role of microglia in ALS: Analysis of microRNAs

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by selective loss of motor neurons of the brain and spinal cord, leading to paralysis and death. Non-neuronal cells have been shown to contribute to ALS disease progression in mouse models. Microglia, the innate immune cells of the central nervous system, have been shown to be activated in ALS and contribute to disease progression. Here I show that miRNAs are dysregulated in acutely isolated microglia from SOD1 G93A transgenic mice, and that miR-155 and miR-210 are significantly upregulated with disease progression. In addition, miR-1198-5p, miR-182, miR-503, and miR-668 are also dysregulated, and predicted mRNA targets of all six of these miRNAs are differentially expressed with disease progression. To my knowledge, this is the first analysis of miRNA expression in microglia during ALS disease progression.

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset, progressive neurodegenerative disease characterized by selective death of the upper and lower motor neurons of the brain and spinal cord, leading to muscle atrophy and paralysis of voluntary muscles (*1*). Only 10% of ALS cases are inherited (called familial ALS), and of these 20-25% are caused by mutations in the Cu/Zn superoxide dismutase (SOD1) (*2*). Mice overexpressing human mutant SOD1 develop motor neuron disease with the same features as ALS including progressive paralysis and ultimately death due to motor neuron degeneration (*3-6*). The most commonly used animal model is the SOD1 glycine 93 to alanine (G93A) transgenic mouse (*4*).

Recently non-neuronal cells have been directly implicated in ALS pathogenesis. In human patients and mutant SOD1 transgenic mice, loss of motor neurons is accompanied by the accumulation of activated astrocytes and microglia (*7*). Mouse blastocyst chimeras that were mixtures of normal and SOD1 mutant-expressing cells showed that damage caused by mutant SOD1 expressed in non-neuronal cells is toxic to motor neurons (*8*). In addition, deletion of mutant SOD1 transgenes from astrocytes and microglia significantly delayed disease progression, but had no effect on disease onset (*9, 10*). Microglia toxicity in ALS is not fully understood, and the role of microglia in ALS disease progression appears to be complex with both protective and toxic effects (*11*).

microRNAs (miRNAs) are endogenous small, single-stranded non-coding RNAs ~21-25 nucleotides (nt) in length that are critical regulators of gene expression. They act by downregulating target mRNAs either by translational repression or mRNA degradation (*12-15*), and have been shown to function in diverse processes including myoblast differentiation (*16*), cell death (*17*), neuronal patterning (*18, 19*), and disease (*20, 21*). They have also been shown to

be important regulators in immune cells, including B and T cells (*22*).

Many mRNAs display alterations in expression levels in ALS. As numerous pathways are aberrantly regulated, it has been hypothesized that miRNAs might be involved in the observed dysregulation. miRNAs have not been thoroughly examined in ALS disease progression. To date, miR-206, a skeletal muscle-specific miRNA, is the only miRNA known to be dysregulated in ALS (*21*). miR-206 was found to be dramatically upregulated in the skeletal muscle of SOD1 G93A transgenic mice, and this miRNA acts to delay ALS progression by promoting the regeneration of neuromuscular synapses in mice.

In this study, I characterize the miRNAs expressed in acutely isolated G93A SOD1 microglia during ALS disease progression. Small RNA sequencing revealed that miRNAs are dysregulated in microglia during ALS disease progression. miR-155 and miR-210 are significantly upregulated with disease, and additional miRNAs, miR-1198-5p, miR-182, miR-503, and miR-668, are also dysregulated. Examination of predicted targets of these differentially expressed miRNAs reveals that mRNA targets are also differentially expressed with disease progression.

Materials and Methods

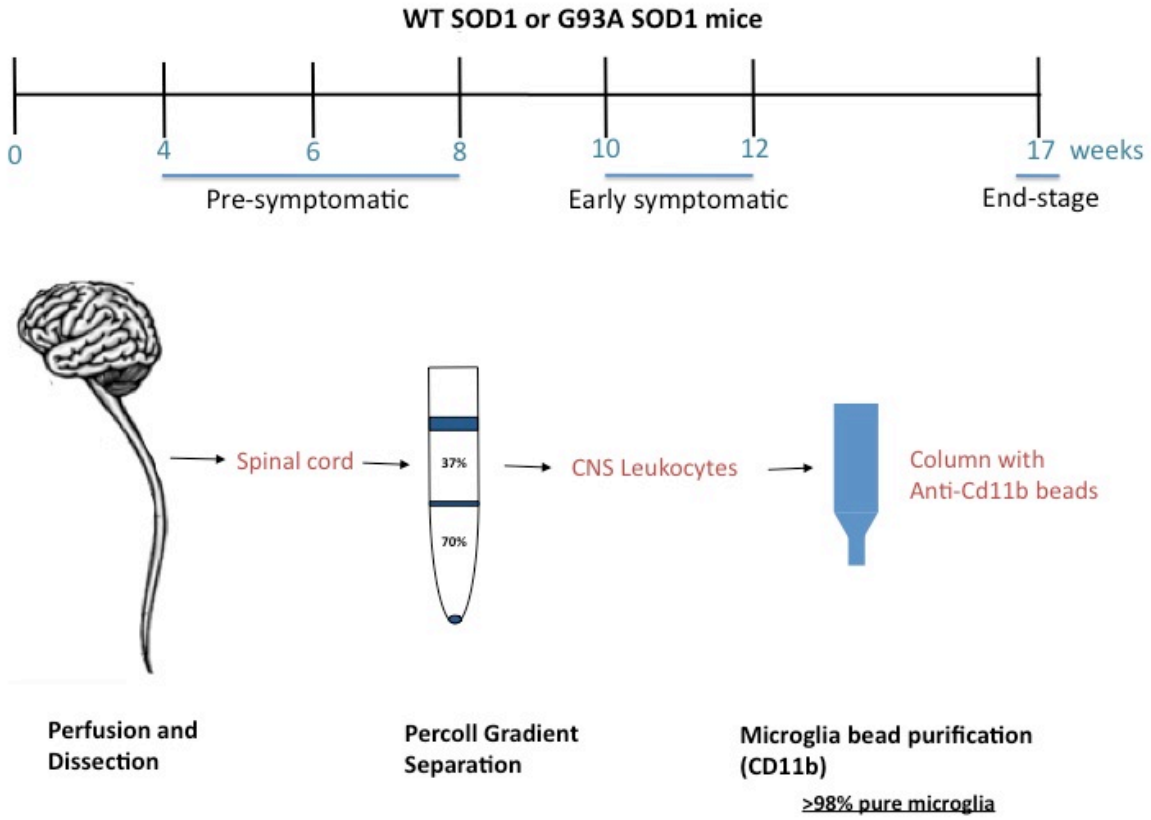
Mice

Mice were generated by crossing female B6SJLF1/J (Jackson Laboratory) with either male B6SJL-Tg(SOD1*G93A)1Gur/J (Jackson Laboratory) or male B6SJL/Tg(SOD1)2Gur/J (Jackson Laboratory). Mice were bred and maintained in barrier facilities at Columbia University Medical Center. F1 hybrids were used for all experiments. All studies were conducted according to institutional guidelines for animal use and care at Columbia University Medical Center.

Microglia isolation (Figure 2.1)

Mice overexpressing human wild type (WT) SOD1 or G93A (MT) SOD1 were sacrificed at 4, 6, 8, 10, 12, and 17 weeks of age. Mice were intracardially perfused with phosphate buffered saline (PBS) to remove blood contamination. Whole spinal cords were dissected into Hank's Balanced Salt Solution (HBSS, Gibco) and 1-2 spinal cords were pooled per sample. Spinal cords were minced in PBS, manually homogenized using a 7mL dounce with a loose-fitting pestle, and then passed through a 70 μm cell strainer (BD Biosciences). The homogenized tissue was spun for 10 min at 460 rcf, room temperature. The cell pellet was resuspended in 5mL of 70% Percoll (GE Healthcare) and 7mL of 37% Percoll was layered on top of cells. This was spun for 30 min at 1000 rcf, room temperature with no brake. The microglia and lymphocytes form a layer at the border between the two densities of Percoll (*23*). These interface cells were collected and diluted with buffer (0.5% BSA, 2mM EDTA in PBS) and spun for 15 min at 600 rcf, 10°C. The cells were transferred to an eppendorf tube for further pelleting (spun for 8 min, 350 rcf, 4°C). Cells were incubated with Cd11b magnetic beads (Miltenyi Biotek) and purified using MACS

Figure 2.1. Microglia isolation scheme.



Mice overexpressing human wild type SOD1 (WT) and human G93A SOD1(MT) were sacrificed at 4, 6, 8, 10, 12, and 17 weeks of age. Following perfusion with PBS, the spinal cord was dissected and manually homogenized using a dounce. After homogenization, the microglia and lymphocytes of the central nervous system were isolated using Percoll gradient centrifugation. These cells were further purified using beads conjugated to a microglia-specific antibody (Cd11b). Total RNA was extracted from these purified cells and used for further analyses.

separation columns according to the manufacturer's instructions and previous protocols (*24, 25*). The cells were then lysed in 250uL Trizol (Invitrogen). Trizol was stored at -80°C until RNA extraction.

LPS injection

Lipopolysaccharides from Escherichia coli 0111:B4 (LPS, Sigma) was resuspended in PBS pH 7.2 (Gibco) and stored at -20°C as a 1mg/mL stock. Mice were weighed and administered 5mg/kg LPS (*26*) by intraperitoneal injection. After 24 hours, mice were sacrificed, spinal cords removed, and microglia isolated as described above.

Total RNA extraction

Trizol samples were thawed and then pooled. RNA extraction was performed according to manufacturer's instructions with the following modifications. For the isopropanol precipitation, glycoblue (Ambion) was also added as a carrier, and this precipitation was carried out at -20°C, overnight. The next day ethanol precipitation was carried out. RNA quantity was determined by Nanodrop and quality was determined by Bioanalyzer (RNA Pico 6000, Agilent).

miRNA sequencing and analysis

Total RNA was sent to the Levy lab at the HudsonAlpha Institute for Biotechnology. The PureLink miRNA Isolate Kit (Invitrogen) was used to enrich the total RNA for small RNAs. Quality of the RNA was examined using a Bioanalyzer. Library construction was done using the ScriptMiner Small RNA-Seq Library Preparation Kit (Epicentre), and libraries were sequenced on Illumina sequencers. Alignment was performed by Mike Muratet (Myers lab,

HudsonAlpha) using the SHRiMP tool (Computational Biology Lab, University of Toronto) (*27*) and differential expression was calculated using the DESeq tool (Huber lab, EMBL) (*28*). Volcano plots were generated based on fold change and p-values calculated for each miRNA.

qPCR validation

Reverse transcription was carried out using 12 ng total RNA and the MicroRNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. miRNA-specific primers were used (Applied Biosystems). cDNA was diluted 1:10 with water. qPCRs were carried out using miRNA-specific Taqman assays and Universal Master Mix II, no UNG (Applied Biosystems), according to manufacturer's instructions. The only modification is that 2x the recommended amount of cDNA was used per qPCR reaction. qPCRs were done on 4-7 biological replicates. snoRNA 135 was used to normalize the qPCRs.

Target identification and examination

Predicted targets of individual miRNAs were determined using Targetscan (http://www.targetscan.org/mmu_60/) (*29*). Global trends in predicted targets were examined in the microglia RNA-seq dataset (unpublished data, Maniatis lab) using ExpressionPlot (*30*). For 2-way plot analysis, the following cut off parameters were used: p-value=1e-4, fold change=1.5, RPKM>2 for at least one condition. Individual mRNAs were then examined using the microglia dataset. Overlap of predicted targets for upregulated and downregulated miRNAs was determined using the Venny tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Functional categories of up and downregulated mRNA targets were analyzed using the web-based bioinformatic DAVID tool (Gene Ontology terms for Biological Process, Molecular

Function, and Cellular Component; Pathways from KEGG and BIOCARTA; and PIR Keywords, Sequence Features, and Protein Domains from INTERPRO and SMART) (*31, 32*).

Statistics

Student's t test was used to determine statistical significance.

Results

Identification of miRNAs that are constitutively expressed in microglia

Primary microglia were acutely isolated from transgenic mice overexpressing human wild type (WT) and G93A (MT) SOD1 at 4, 6, 8, 10, 12, and 17 weeks. Total RNA from these cells was examined by small RNA sequencing to identify the miRNAs expressed. By analyzing this sequencing data over this extended time course, I was able to identify the miRNAs that are constitutively highly and moderately expressed in primary spinal cord microglia irrespective of age or genotype (Figure 2.2). Although specific miRNAs have previously been examined in microglia (*33-35*), this reveals the signature steady state microglia miRNAs.

miRNAs are differentially expressed in G93A microglia compared to WT microglia during disease progression

As discussed above, primary microglia were acutely isolated from transgenic mice overexpressing human G93A SOD1 (MT) at 4, 6, 8, 10, 12, and 17 weeks (see Figure 2.1). These times points follow disease progression, with 4, 6, and 8 weeks being pre-symptomatic, 10 and 12 weeks are early symptomatic/symptom onset, and 17 weeks is end-stage. Total RNA from these microglia was examined by small RNA sequencing. Using the DESeq tool, I determined the expression patterns of miRNAs in G93A SOD1 microglia compared to WT SOD1 microglia. I found that a number of miRNAs are differentially expressed in the ALS microglia during disease progression, and that more changes are observed with disease progression (Figure 2.3). Upon closer examination of the sequencing results, I found that 3 miRNAs are upregulated and 3 miRNAs are downregulated at both 12 and 17 weeks, the time points corresponding to disease onset and end-stage (Figure 2.4). I chose to focus on these

Figure 2.2. miRNAs constitutively expressed at high and moderate levels in microglia.

miRNA sequencing was performed on total RNA isolated from microglia from WT or G93A SOD1 mice at 4, 6, 8, 10, 12, and 17 weeks. Alignment was performed using SHRiMP and relative expression levels were determined using the DESeq tool. I identified miRNAs that are constitutively highly (levels >16) and moderately (levels from 6.5-15) expressed using these relative expression levels. The highly expressed miRNAs are purple, and the moderately expressed miRNAs are blue.

Figure 2.2, continued.

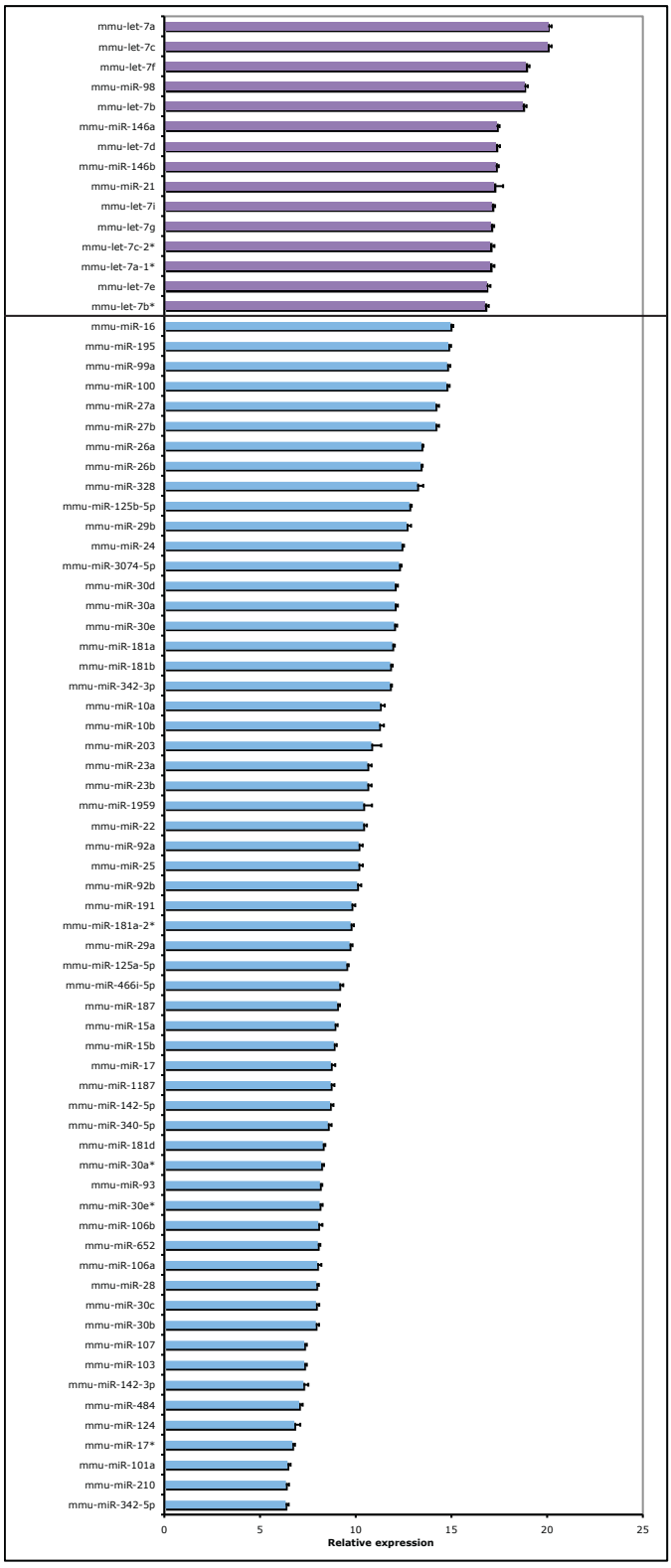
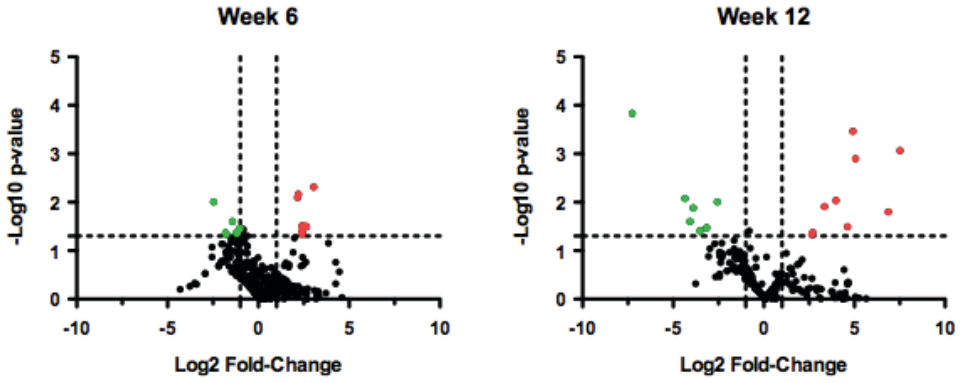


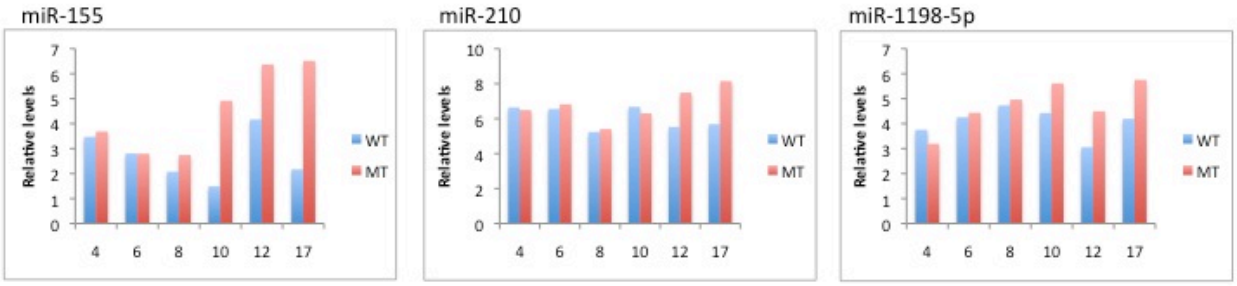
Figure 2.3. miRNAs are differentially expressed in G93A microglia compared to WT microglia.



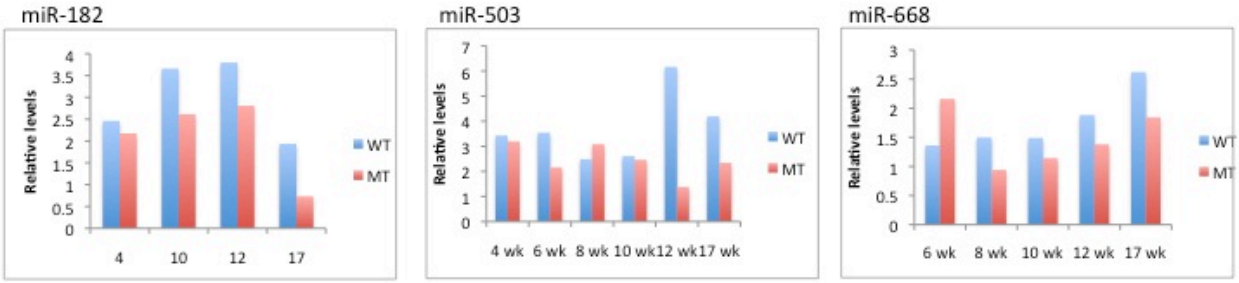
Graphpad Prism was used to plot log2(fold-change) vs -log10(p-value) for the miRNA sequencing data comparing the data from WT and G93A microglia at 4, 6, 8, 10, 12, and 17 weeks. miRNAs that correspond to $p<0.01$ and log(fold-change) >1 or <-1 are colored with red or green. The data for microglia isolated at 6 and 12 weeks is shown.

Figure 2.4. miRNAs are dysregulated in G93A microglia by small RNA sequencing.

A. miRNAs are upregulated at 12 and 17 weeks.



B. miRNAs are downregulated at 12 and 17 weeks.



miRNA sequencing was performed on total RNA isolated from wild type (WT) and G93A (MT) SOD1 microglia at 4, 6, 8, 10, 12, and 17 weeks. Alignment was performed using SHRiMP and relative expression levels were determined using the DESeq tool. I identified the miRNAs that were up (A) or downregulated (B) at both 12 and 17 weeks in MT compared to WT. Values are relative expression for a single replicate.

miRNAs for further analyses.

miR-155 is significantly upregulated in G93A microglia

miR-155 was upregulated in G93A microglia at 10, 12, and 17 weeks, which corresponds to early symptom onset through end-stage of the disease (Figure 2.4A). I confirmed these results by qPCR on biological replicates (Figure 2.5A). Indeed, miR-155 is upregulated ~1.6 fold at 8 weeks, 3.5 fold at 10 weeks, 7.3 fold at 12 weeks, and 8.6 fold at 17 weeks. Statistical analysis of the data revealed that the 10, 12, and 17 week changes are highly significant. In addition, I performed small RNA sequencing on microglia 24 hours after lipopolysaccharide (LPS) injection. LPS, a bacterial-derived sugar, is known to activate microglia, causing increased phagocytosis, secretion of cytokines, and induction of nitric oxide synthase (*36*). I found that miR-155 is consistently upregulated upon activation by LPS (Figure 2.5B). It is likely that the upregulation of miR-155 in the G93A microglia is due to their activation and might not be strictly a disease-specific response. A general theme in reactive gliosis is that astrocytes and microglia become activated in response to a variety of traumas, and these responses are context-dependent and graded (*37, 38*). Thus, the upregulation of miR-155 is a change common to more than one type of stimuli including acute LPS activation and chronic neurodegeneration.

miR-210 is significantly upregulated in G93A microglia

I found that miR-210 is upregulated only at 12 and 17 weeks (Figure 2.4A), and although the increase was small, it was confirmed by qPCR on biological replicates (Figure 2.6A). The observed upregulation of miR-210 is not as dramatic as for miR-155, however it is still upregulated 2.4 fold at 12 weeks and 3.5 fold at 17 weeks, both of which are significant changes.

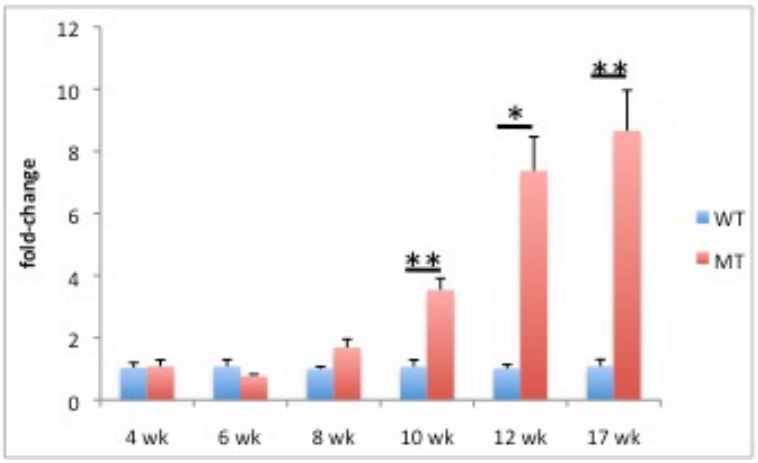
Figure 2.5. miR-155 expression in acutely isolated microglia.

A. miR-155 is upregulated in acutely isolated G93A microglia at 10, 12, and 17 weeks. Microglia were acutely isolated from the spinal cords of mice overexpressing human wild type (WT) and G93A (MT) SOD1 mice at 4, 6, 8, 10, 12, and 17 weeks. Total RNA was isolated from 5-8 spinal cords and pooled to yield a biological replicate. RNA quantity and quality were examined using a Nanodrop and Bioanalyzer, respectively. miR-155-specific reverse transcription and Taqman qPCR were performed. qPCR were normalized to snoRNA 135. * $p<0.01$, ** $p<0.002$, Student's t test, WT 4 (n=4), MT 4 (n=4), WT 6 (n=4), MT 6 (n=4), WT 8 (n=3), MT 8 (n=5), WT 10 (n=4), MT 10 (n=5), WT 12 (n=5), MT 12 (n=4), WT 17 (n=5), MT 17 (n=6). Values represent mean +/- SEM.

B. miR-155 is upregulated in response to LPS activation. Mice overexpressing human wild type SOD1 were injected with 5mg/kg LPS 24 hours before microglia isolation (WT LPS). Microglia were isolated at 4, 6, 8, 10, 12, and 17 weeks. Total RNA was isolated from 5-8 spinal cords and small RNA sequencing was performed. Alignment was performed using SHRiMP and relative expression levels were determined using the DESeq tool. WT LPS was compared to WT. Values are relative expression for a single replicate.

Figure 2.5, continued.

A. miR-155 is significantly upregulated in acutely isolated G93A microglia at 10, 12, and 17 weeks.



B. miR-155 is induced by LPS injection.

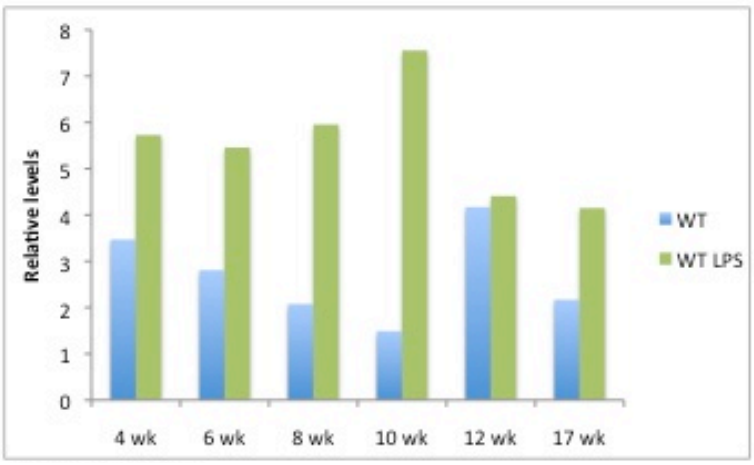


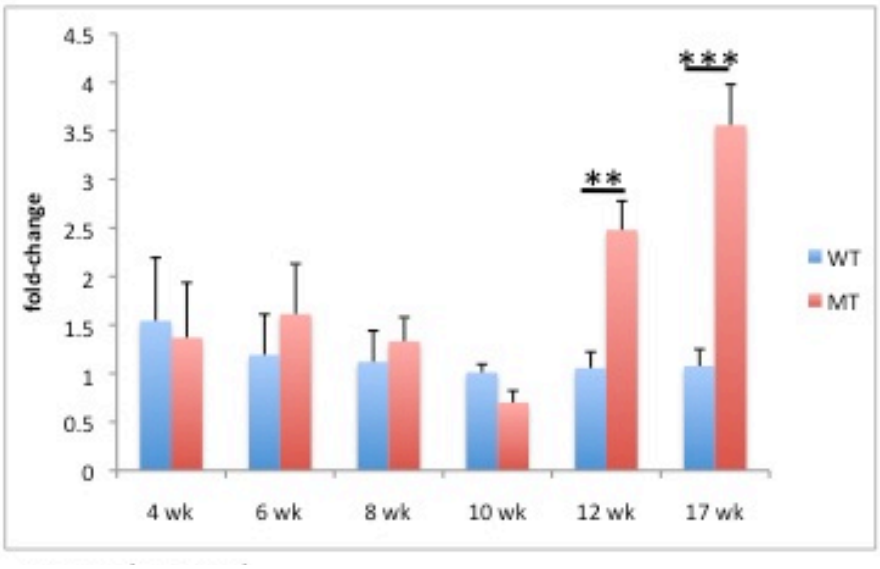
Figure 2.6. miR-210 expression in acutely isolated microglia.

A. miR-210 is upregulated in acutely isolated G93A microglia at 12 and 17 weeks. Microglia were acutely isolated from the spinal cords of mice overexpressing human wild type (WT) and G93A (MT) SOD1 mice at 4, 6, 8, 10, 12, and 17 weeks. Total RNA was isolated from 5-8 spinal cords and pooled to yield a biological replicate. RNA quantity and quality were examined using a Nanodrop and Bioanalyzer, respectively. miR-210-specific reverse transcription and Taqman qPCR were performed. qPCR were normalized to snoRNA 135. ** $p<0.006$, *** $p<0.0007$, Student's t test, WT 4 (n=4), MT 4 (n=4), WT 6 (n=4), MT 6 (n=4), WT 8 (n=3), MT 8 (n=5), WT 10 (n=4), MT 10 (n=5), WT 12 (n=6), MT 12 (n=5), WT 17 (n=7), MT 17 (n=7). Values represent mean +/- SEM.

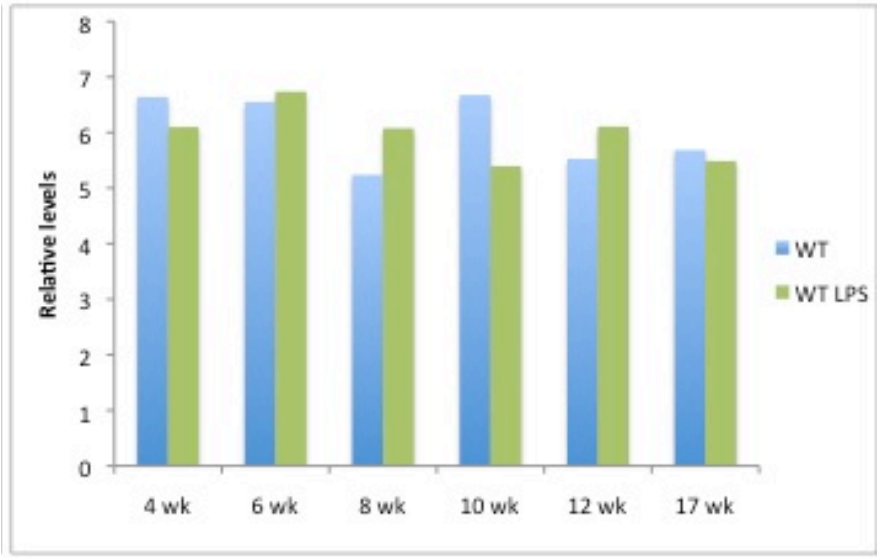
B. miR-210 expression is not changed in response to LPS. Mice overexpressing human wild type SOD1 were injected with 5mg/kg LPS 24 hours before microglia isolation (WT LPS). Microglia were isolated at 4, 6, 8, 10, 12, and 17 weeks. Total RNA was isolated from 5-8 spinal cords and small RNA sequencing was performed. Alignment was performed using SHRiMP and relative expression levels were determined using the DESeq tool. WT LPS was compared to WT. Values are relative expression for a single replicate.

Figure 2.6, continued.

A. miR-210 is significantly upregulated in acutely isolated microglia at 12 and 17 weeks.



B. miR-210 expression is not changed in response to LPS.



Interestingly, unlike miR-155, miR-210 does not respond to LPS stimulation (Figure 2.6B), so it is likely that this upregulation is specific to disease and is distinct from the general inflammatory response pathway.

Predicted targets of miR-155 and miR-210 are downregulated in G93A microglia

In addition to the microglia miRNA dataset, we also have RNA-seq data from acutely isolated microglia from G93A and non-transgenic mice at 9, 14, and 18 weeks, which corresponds to presymptomatic, symptom onset, and end-stage, respectively (unpublished data, Maniatis lab). I identified putative targets of miR-155 and miR-210 using the Targetscan software (*29*). These sets of mRNAs were globally examined in the microglia RNA-seq dataset using ExpressionPlot (Figure 2.7A, 2.8A) (*30*). As miR-155 and miR-210 were both upregulated, I focused on the predicted downregulated mRNA targets. I found that 10 miR-155 targets and 3 miR-210 targets were significantly downregulated during disease progression (Figure 2.7B, 2.8B). Interestingly, two of these mRNAs, Tppp and Etv5, are targeted by both miR-155 and miR-210. Tppp, tubulin polymerization-promoting protein, is a myelin protein that is reduced in demyelinated lesions that are enriched in the cerebrospinal fluid of multiple sclerosis patients (*39*).

Additional miRNAs are dysregulated at 17 weeks in G93A microglia compared to WT

By analyzing the small RNA sequencing, I also found that miR-1198-5p, miR-182, miR-503, and miR-668 were dysregulated at both 12 and 17 weeks (Figure 2.4). miR-1198-5p was upregulated, and miR-182, 503, and 668 were all downregulated. Although I was able to confirm these trends by qPCR on biological replicates, the qPCR changes were not statistically significant (Figure 2.9A, 2.9C). Similar to the trend observed for miR-155, miR-503 is

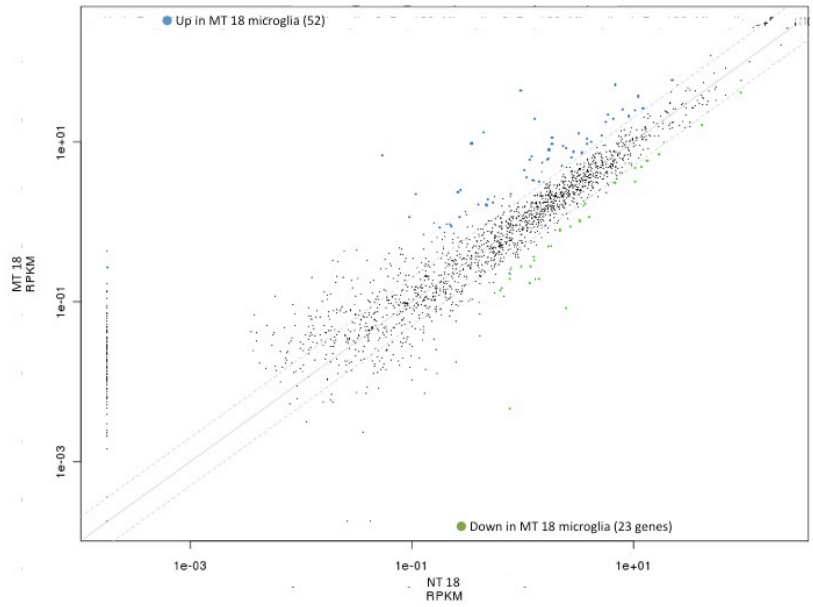
Figure 2.7. Predicted targets of miR-155 are differentially expressed in a microglia RNA-seq dataset.

A. Global view of trends in predicted targets of miR-155. Predicted targets of miR-155 were determined using Targetscan for mouse (http://www.targetscan.org/mmu_60/). These genes were then analyzed by 2-way plot using our acutely isolated microglia RNA-seq dataset and Expression Plot. The microglia RNA-seq was carried out using microglia isolated as described in Figure 2.1 from mice overexpressing human G93A (MT) and non-transgenic mice (NT) at 9, 14, and 18 weeks. Genes that were significantly (p=1e-4, fold-change=2) up or down regulated in MT microglia versus NT microglia are colored as blue or green dots, respectively.

B. Predicted targets of miR-155 are downregulated. The predicted targets that were downregulated in G93A (MT) microglia compared to non-transgenic (NT) were further examined in the RNA-seq data. mRNAs with RPKM below 2 for at least condition were filtered out. *p<0.05, **p<0.003, ***p<6e-5 Student's t test, NT 9, NT 14, NT 18 (n=3), MT 9 (n=5), MT 14 (n=7), MT 18 (n=9). Values represent mean +/- SEM.

Figure 2.7, continued.

A. Global view of trends in predicted targets of miR-155.



B. Predicted targets of miR-155 are downregulated.

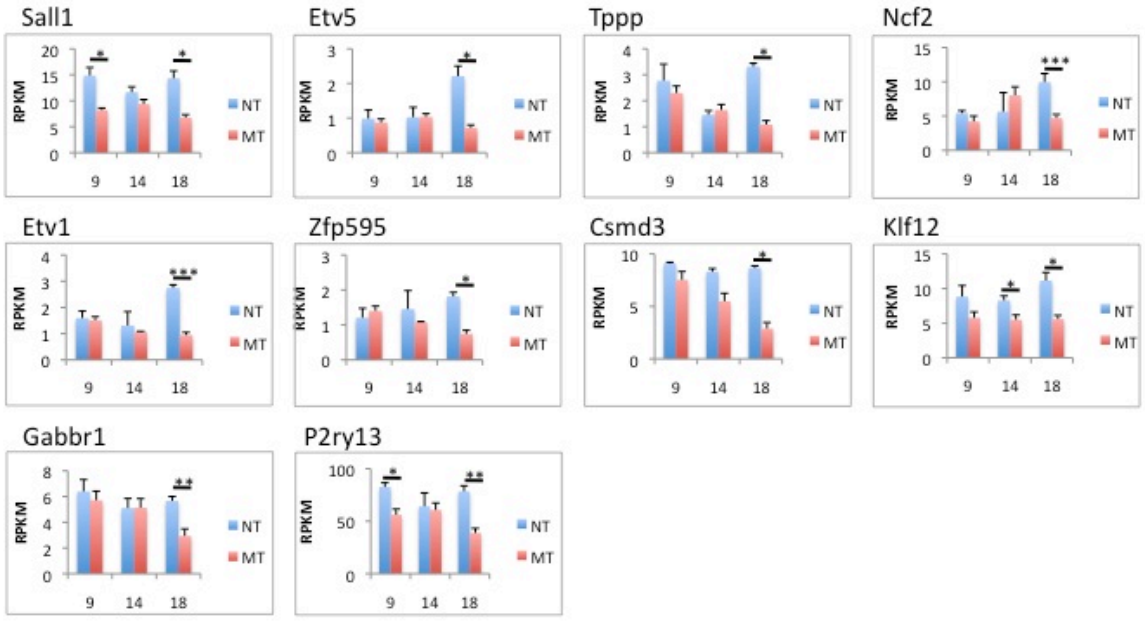


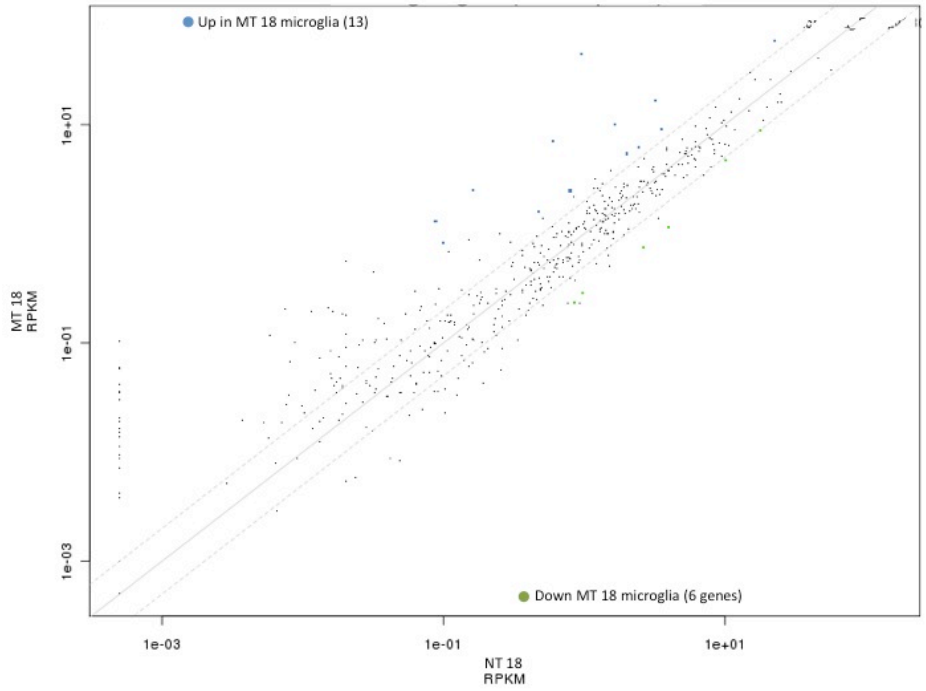
Figure 2.8. Predicted targets of miR-210 are differentially expressed in a microglia RNA-seq dataset.

A. Global view of trends in predicted targets of miR-210. Predicted targets of miR-210 were determined using Targetscan for mouse (http://www.targetscan.org/mmu_60/). These genes were then analyzed by 2-way plot using our acutely isolated microglia RNA-seq dataset and Expression Plot. The microglia RNA-seq was carried out using microglia isolated as described in Figure 2.1 from mice overexpressing human G93A (MT) and non-transgenic mice (NT) at 9, 14, and 18 weeks. Genes that were significantly (p=1e-4, fold-change=2) up or down regulated in MT microglia versus NT microglia are colored as blue or green dots, respectively.

B. Predicted targets of miR-210 are downregulated. The predicted targets that were downregulated in G93A microglia compared to non-transgenic (NT) were further examined in the RNA-seq data. mRNAs with RPKM below 2 for at least one condition were filtered out. *p<0.03, ***p<6e-6 Student's t test, NT 9, NT 14, NT 18 (n=3), MT 9 (n=5), MT 14 (n=7), MT 18 (n=9). Values represent mean +/- SEM.

Figure 2.8, continued.

A. Global view of trends in predicted targets of miR-210.



B. Predicted targets of miR-210 are downregulated.

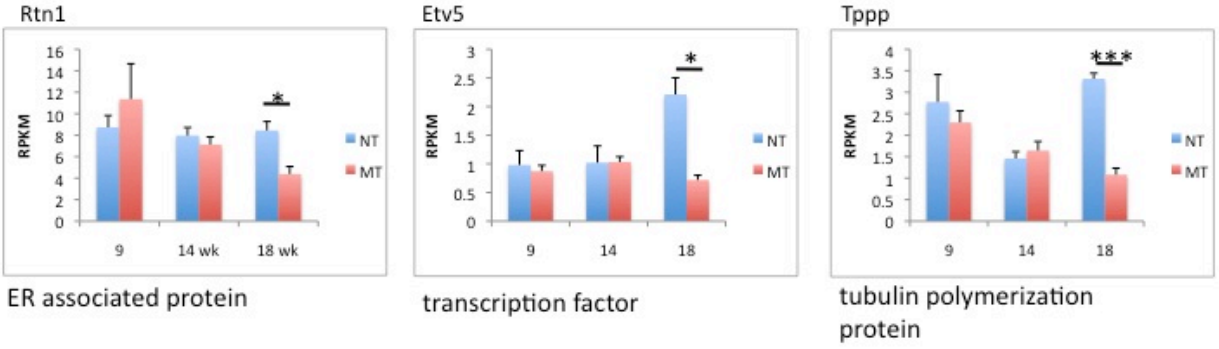


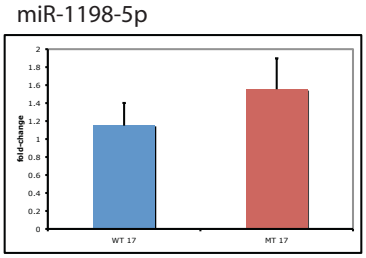
Figure 2.9. miRNAs are dysregulated at 17 weeks in G93A microglia compared to WT.

A, C. The microglia miRNA sequencing data was examined and the miRNAs that were changed in the same direction (i.e. upregulated or downregulated) at both 12 and 17 weeks were identified. Additional microglia were acutely isolated from the spinal cords of mice overexpressing human wild type (WT) and G93A (MT) SOD1 mice at 17 weeks. Total RNA was isolated from 5-8 spinal cords and pooled to yield a biological replicate. RNA quantity and quality were examined using a Nanodrop and Bioanalyzer, respectively. miR-1198-5p (A), miR-182 (C), miR-503 (C), and miR-668 (C)-specific Taqman assays were used. qPCR were normalized to snoRNA 135. $p<0.08$ (miR-503, miR-668), $p<0.38$ (miR-182, miR-1198-5p), Student's t test, WT 17 (n=7), MT 17 (n=7). Values represent mean +/- SEM.

B, D. Global view of trends in predicted targets for miR-1198-5p (B), miR-182 (D), miR-503 (D), and miR-668 (D). Predicted targets of specific miRNAs were determined using Targetscan for mouse (http://www.targetscan.org/mmu_60/). These genes were then analyzed by 2-way plot using our microglia RNA-seq dataset and Expression Plot. The microglia RNA-seq was carried out using microglia isolated as described in Figure 2.1 from mice overexpressing human G93A (MT) and non-transgenic mice (NT) at 9, 14, and 18 weeks. Genes that were significantly ($p=1e-4$, fold-change=2) up or down regulated in G93A microglia versus non-transgenic are colored as blue or green dots, respectively.

Figure 2.9, continued.

A. miR-1198-5p is upregulated at 17 weeks.



B. Global view of trends in predicted targets of miR-1198-5p.

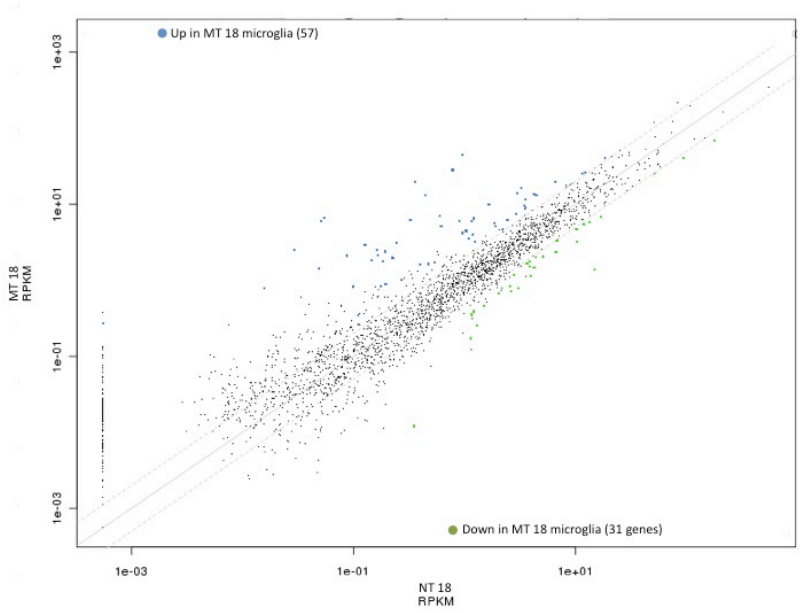
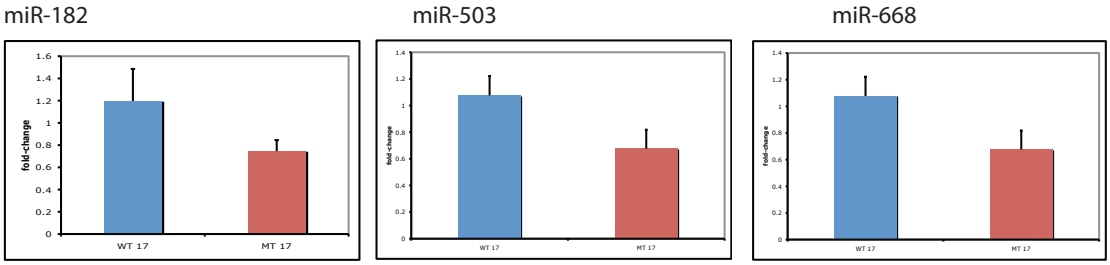
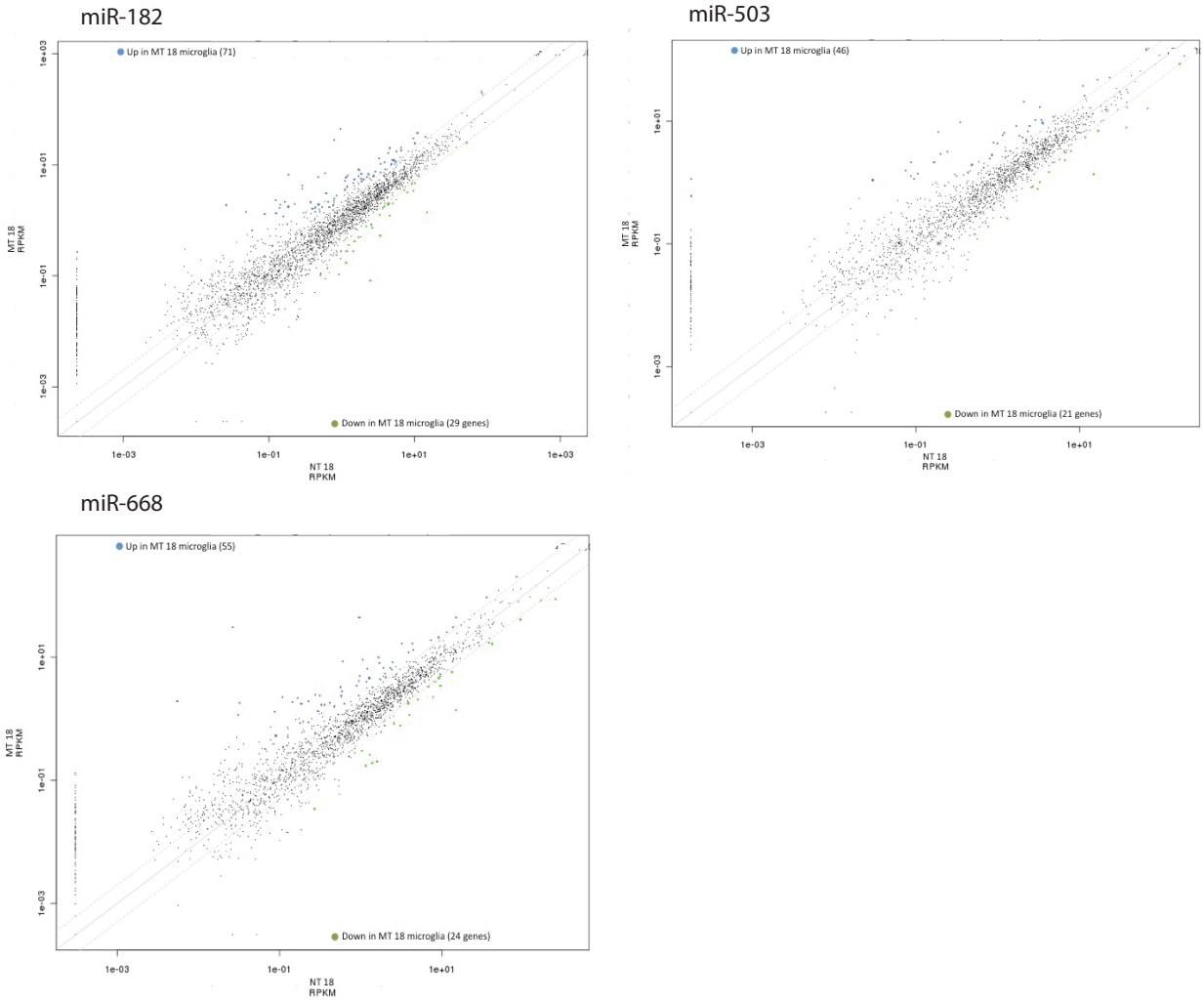


Figure 2.9, continued.

C. Several miRNAs are downregulated at 17 weeks.



D. Global view of trends of targets of downregulated miRNAs.



downregulated upon LPS injection at 12 and 17 weeks suggesting this downregulation might be a general result of activation (Figure 2.10). Predicted targets of these miRNAs were determined using Targetscan, and these mRNAs were examined in the microglia dataset. For all 4 miRNAs, there were predicted targets that changed in the expected direction, i.e. targets of upregulated genes were downregulated and targets of
downregulated genes were upregulated (Figure 2.9B, 2.9D). More targets are upregulated than downregulated, but this is likely due to the fact that in the G93A microglia, more mRNAs are upregulated than downregulated as a general trend (unpublished data, Maniatis lab).

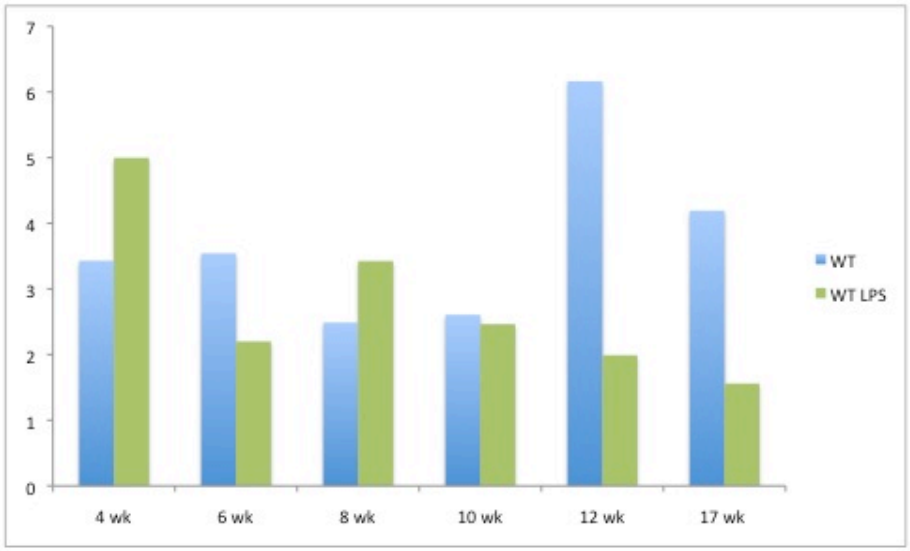
Common trends in predicted targets of dysregulated miRNAs

The predicted targets of the upregulated miRNAs (miR-155, miR-210, and miR-1198-5p) and downregulated miRNAs (miR-182, miR-503, and miR-668) were examined. The upregulated (Figure 2.11A) and downregulated targets of were compared (Figure 2.11B). There was overlap between all upregulated genes and all downregulated genes, suggesting common pathways might be affected.

Hypoxia-related genes are upregulated in G93A microglia

miR-210 is considered a hypoxamir and is induced in hypoxic conditions in response to upregulation of HIF-1α (*40*). HIF-1α directly binds the hypoxia responsive element on the miR-210 promoter (*41*). Thus, I examined HIF-1α levels in the microglia RNA-seq dataset. I observed that HIF-1α is significantly upregulated at 14 and 18 weeks (Figure 2.12A), which correlates with the observed upregulation of miR-210 (Figure 2.12B).

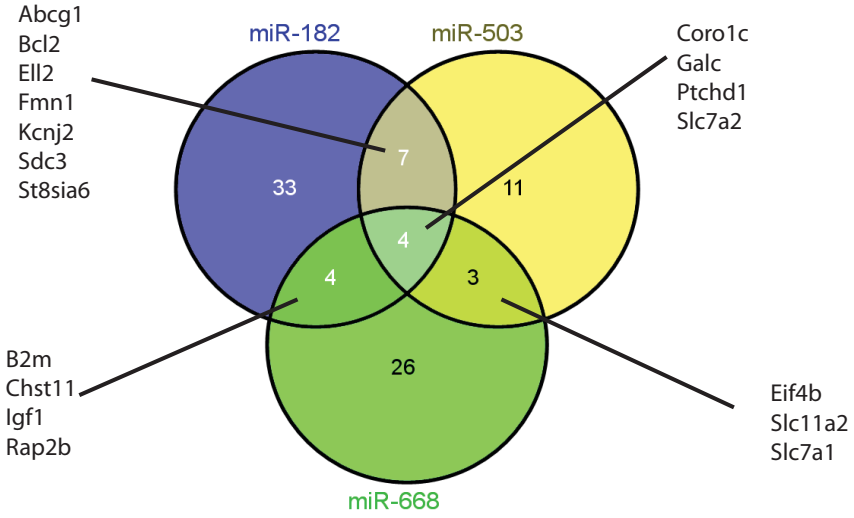
Figure 2.10. miR-503 is downregulated in response to LPS.



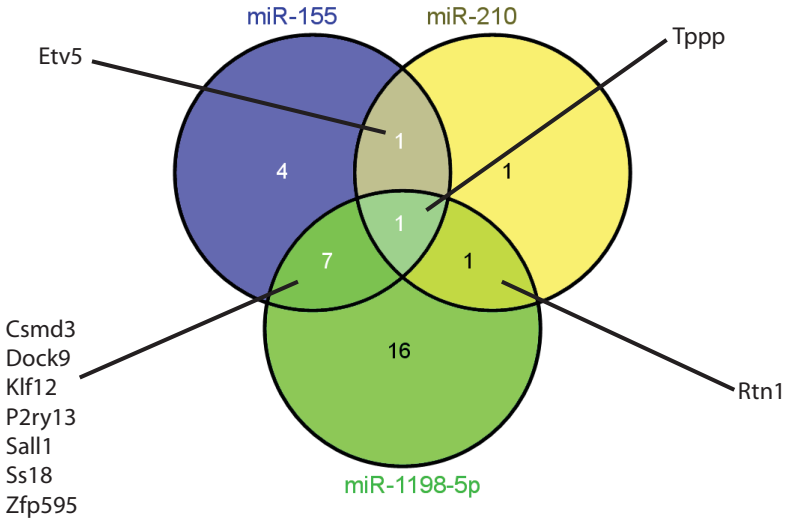
miR-503 expression is downregulated in response to LPS at 12 and 17 weeks. Mice overexpressing human wild type SOD1 (WT) were injected with 5mg/kg LPS 24 hours before microglia isolation (WT LPS). Microglia were isolated at 4, 6, 8, 10, 12, and 17 weeks. Total RNA was isolated from 5-8 spinal cords and small RNA sequencing was performed. Alignment was performed using SHRiMP and relative expression levels were determined using the DESeq tool. WT LPS was compared to WT. Values are relative expression for a single replicate.

Figure 2.11. Overlap of predicted targets of differentially expressed miRNAs.

A. Overlap of upregulated genes.



B. Overlap of downregulated genes.



Predicted targets of the downregulated (miR-182, miR-503, miR-668) and upregulated (miR-155, miR-210, miR-1198-5p) miRNAs were determined using Targetscan. Targets were examined in our microglia RNA-seq dataset, and those with expression changes that correlated with changes in miRNA expression (ie, if a miRNA was upregulated, targets were downregulated, and if a miRNA was downregulated, targets were upregulated) and with a p-value < 1e-4 and fold change > 2 were identified. The overlap of these significant target lists was determined using the Venny tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Figure 2.12. Hypoxia-induced genes are upregulated in G93A microglia.

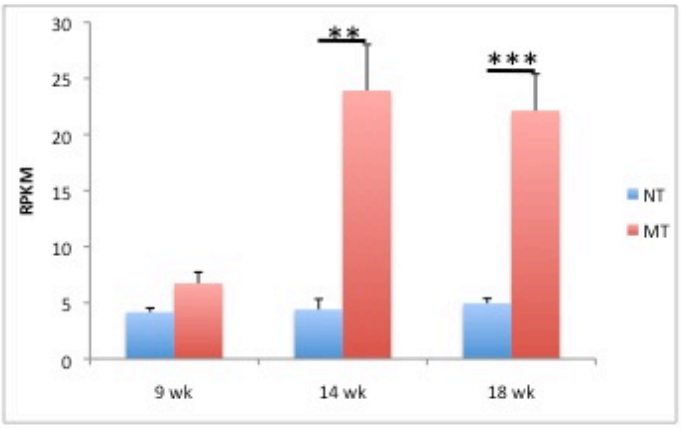
A. HIF-1α is upregulated in G93A microglia at 14 and 18 weeks. Microglia were acutely isolated from the spinal cords of non-transgenic (NT) and G93A (MT) SOD1 mice at 9, 14, and 18 weeks, and total RNA from microglia isolated from individual mice was examined by RNA-seq. **p<0.003, *** p<0.0008, Student's t test, NT 9, NT 14, NT 18 (n=3), MT 9 (n=5), MT 14 (n=7), MT 18 (n=9). Values represent mean +/- SEM.

B. miR-155 is upregulated in acutely isolated G93A microglia at 10, 12, and 17 weeks. Microglia were acutely isolated from the spinal cords of mice overexpressing human wild type (WT) and G93A (MT) SOD1 mice at 4, 6, 8, 10, 12, and 17 weeks. Total RNA was isolated from 5-8 spinal cords and pooled to yield a biological replicate. RNA quantity and quality were examined using a Nanodrop and Bioanalyzer, respectively. miR-155-specific reverse transcription and Taqman qPCR were performed. qPCR were normalized to snoRNA 135. * p<0.01, ** p<0.002, Student's t test, WT 4 (n=4), MT 4 (n=4), WT 6 (n=4), MT 6 (n=4), WT 8 (n=3), MT 8 (n=5), WT 10 (n=4), MT 10 (n=5), WT 12 (n=5), MT 12 (n=4), WT 17 (n=5), MT 17 (n=6). Values represent mean +/- SEM.

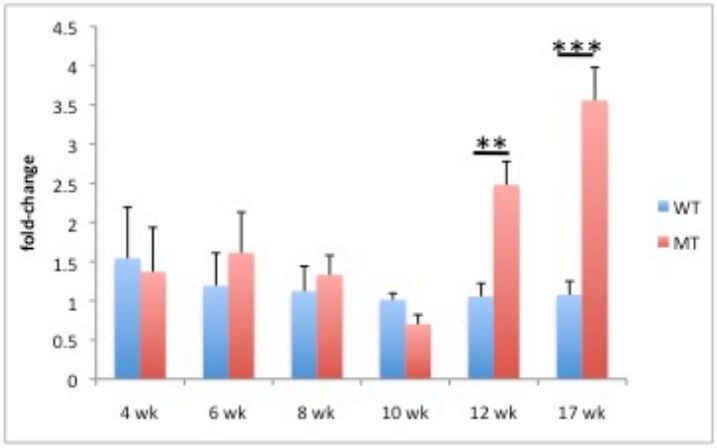
C. Model for hypoxia-induced changes. In G93A microglia, HIF-1α is upregulated. HIF-1α directly binds the promoter of miR-210 to induce its expression. miR-210 is then able to bind and downregulate its predicted mRNA targets, Etv5, Tppp, and Rtn1. miR-155 also targets Tppp and Etv5 for downregulation.

Figure 2.12, continued.

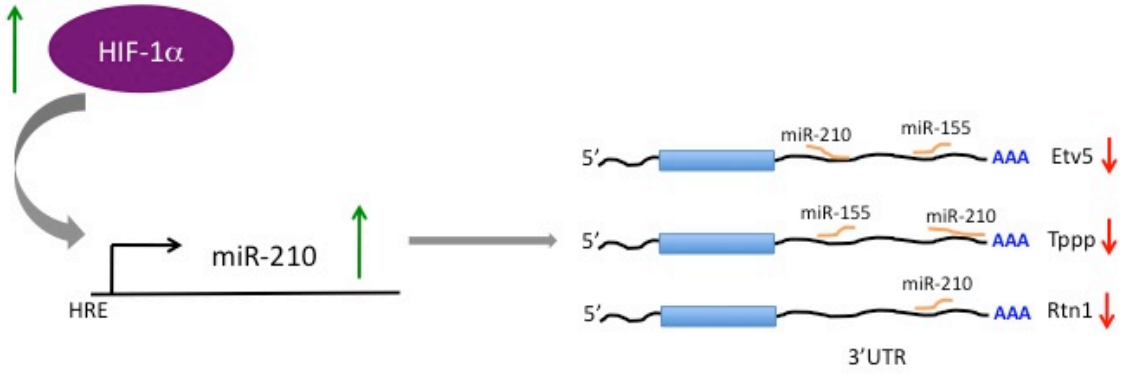
A. HIF-1α is significantly upregulated in G93A microglia at 14 and 18 weeks.



B. miR-210 is significantly upregulated at 12 and 17 weeks.



C. Model for hypoxia-induced changes.

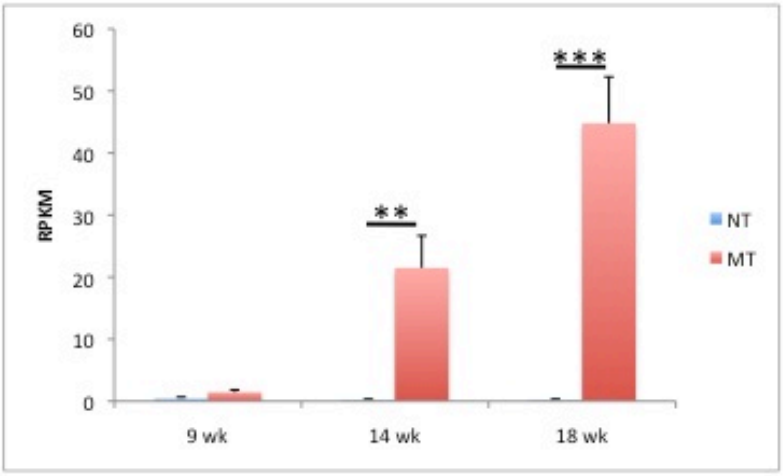


IGF1 and Nox 2 are upregulated in G93A microglia

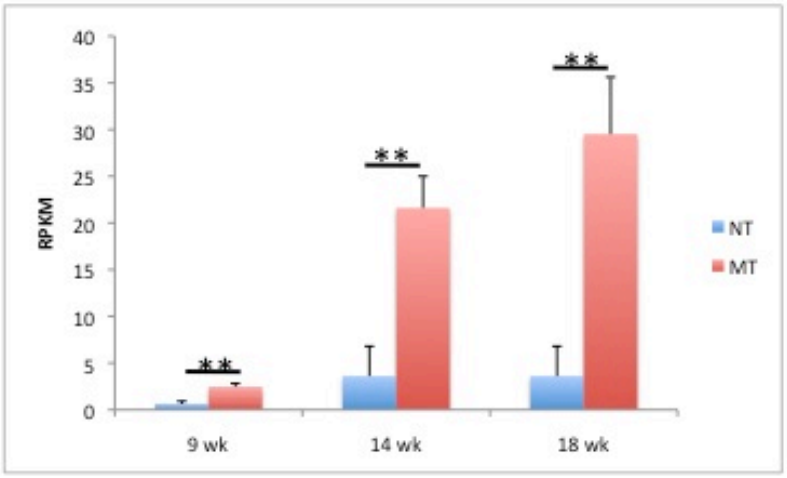
miR-182 and miR-668, two of the miRNAs downregulated at end-stage, are predicted to target insulin-like growth factor 1 (IGF1) and Nox2 (miR-182 is predicted to target both and miR-668 is predicted to target just IGF1). IGF1 is a neuroprotective factor that has been observed to be upregulated in G93A microglia during disease progression (*42*). IGF1 activates AKT pro-survival signaling in motor neurons (*43*) and has been shown to significantly extend lifespan when administered to ALS mice (*44, 45*). Nox2, an NADPH oxidase, is increased in classically activated microglia (*46*) and has been shown to be neurotoxic to motor neurons by small molecule inhibition and knockout studies in ALS transgenic mice (*47*). I examined both of these mRNAs in the microglia RNA-seq dataset. Both mRNAs are significantly upregulated at 14 and 18 weeks, which correspond to disease onset and end-stage, respectively (Figure 2.13). The upregulation of both protective and toxic factors is interesting considering the complicated biology of microglia. That two miRNAs are predicted to target IGF1 is particularly interesting since there is very high induction of IGF1 from symptom onset, and the mechanism of this upregulation is not understood.

Figure 2.13. Predicted targets of miR-182 and miR-668 are upregulated.

A. IGF1 is upregulated in G93A microglia.



B. Nox2 is upregulated in G93A microglia.



A. IGF1 is upregulated in G93A microglia. miR-182 and miR-668 are both predicted by Targetscan to target IGF1. As both these miRNAs are downregulated, their targets would be upregulated. IGF1 levels were examined in the microglia RNA-seq dataset. **p<0.007, *** p<0.0004, Student's t test, NT 9, NT 14, NT 18 (n=3), MT 9 (n=5), MT 14 (n=7), MT 18 (n=9). Values represent mean +/- SEM.

B. Nox2 is upregulated in G93A microglia. miR-182 is predicted by Targetscan to target Nox2. As miR-182 is downregulated, its targets would be upregulated. Nox2 levels were examined in the microglia RNA-seq dataset. **p<0.008, Student's t test, NT 9, NT 14, NT 18 (n=3), MT 9 (n=5), MT 14 (n=7), MT 18 (n=9). Values represent mean +/- SEM.

Discussion

Microglia are an important non-neuronal cell type involved in ALS disease progression. These cells are activated and proliferate in the spinal cord, in parallel with motor neuron degeneration (*7, 48*). miRNAs, which are small, non-coding molecules that are important regulators of gene expression have not been extensively examined in ALS. To date, skeletal muscle-specific miR-206 is the only miRNA shown to be involved in disease pathogenesis (*21*). The role of miRNAs in microglia during ALS disease progression has not been previously examined.

By examining the miRNA sequencing from primary microglia over an extended time course, I was able to identify miRNAs that are highly and moderately expressed in primary spinal cord microglia (Figure 2.2). To my knowledge, this study is the first identification of miRNAs dysregulated in microglia in ALS. miR-155 is highly and significantly upregulated with disease progression, starting at 10 weeks, which corresponds to early symptom onset, and increasing to end-stage (Figure 2.5A). miR-155 has been well established as a pro-inflammatory miRNA that is involved in many aspects of the immune response including the regulation of helper T cell differentiation (*49*), induction during the macrophage inflammatory response (*50*), and involvement in normal immune function of B, T, and dendritic cells (*22*). I also observe that miR-155 is upregulated in response to LPS (*34, 51*). As LPS is known to activate microglia, causing increased phagocytosis, secretion of cytokines, and induction of nitric oxide synthase (*36*), this suggests that the upregulation of miR-155 might be due to general microglial activation that occurs with ALS disease progression. However, the upregulation of miR-155 as early as 10 weeks is earlier than has been previously reported for gliosis and microglia activation in the SOD1 G93A transgenic mouse (http://jaxmice.jax.org/strain/002726.html) (*52*).

miR-210 is also significantly upregulated in G93A microglia with disease progression, starting at 12 weeks (Figure 2.6A). miR-210 has not previously been shown to be involved in neurodegenerative diseases or expressed in microglia. This miRNA is considered a hypoxamir, as it is highly induced by hypoxia due to direct binding by HIF-1α to its promoter in hypoxic conditions (*40, 53*). miR-210 has been found to target nucleotide excision repair enzymes (*54*), promote osteoblastic differentiation (*55*), and control mitochondrial metabolism (*56*). I observe that HIF-1α is also upregulated in the G93A microglia (Figure 2.12A). The upregulation of HIF-1α was recently reported in total spinal cord from G93A SOD1 transgenic mice, but not in a specific cell type (*57*). It has been suggested that HIF-1α might be responding to the oxidative stress conditions in ALS due to the increased levels of reactive oxygen species, and its upregulation might be a neuroprotective response (*58*). Thus, it is possible that in G93A microglia, oxidative stress and hypoxia cause the induction of HIF-1α which directly upregulates miR-210. miR-210 then downregulates its predicted targets Etv5, Tppp, and Rtn1 (Figure 2.12C). As noted above, miR-155 also targets Etv5 and Tppp, and remarkably is also hypoxia-inducible and is itself able to target HIF-1α (*59*). Thus, miR-210 and miR-155 appear to be coordinately regulated and cooperate to downregulate a specific set of miRNAs.

I also found that miR-1198-5p is upregulated at end-stage, and miR-182, miR-503, and miR-668 are downregulated at end-stage (Figure 2.9A, 2.9C). miR-503 is also downregulated in response to LPS activation, suggesting this miRNA might respond to numerous stimuli (Figure 2.10**).** I used Targetscan to examine the predicted targets of these miRNAs in the microglia RNA-seq dataset. The Venny tool was used to determine the overlap of predicted targets of the downregulated miRNAs and the upregulated miRNAs (Figure 2.11, Table 2.1). With regards to the upregulated miRNAs, it was interesting that all three miRNAs are predicted to target Tppp

Table 2.1. Description of up and downregulated mRNAs predicted to be targeted by more than one miRNA.

A. Upregulated genes.

Gene	Description	miRNA
Abcg1	involved in macrophage cholesterol and phospholipid transport	miR-182, 503
B2m	component of MHC Class I molecules	miR-182, 668
Bcl2	regulator of apoptosis	miR-182, 503
Chst11	carbohydrate sulfotransferase	miR-182, 668
Coro1c	WD repeat family protein	miR-182, 503, 668
Eif4b	eukaryotic initiation factor	miR-503, 668
Ell2	RNA polymerase elongation factor	miR-182, 503
Fmn1	participates in adherens junctions and actin polymerization	miR-182, 503
Galc	galactosylceramidase	miR-182, 503, 668
Igf1	neuroprotective hormone, upregulation observed in ALS	miR-182, 668
Kcnj2	potassium channel	miR-182, 503
Ptchd1	candidate for X-linked intellectual disability	miR-182, 503, 668
Rap2b	Ras-related protein	miR-182, 668
Sdc3	Syndecan 3	miR-182, 503
Slc11a2	metal ion transporter, associated with disease duration in ALS	miR-503, 668
Slc7a1	high affinity cationic amino acid transporter	miR-503, 668
Slc7a2	low affinity cationic amino acid transporter	miR-182, 503, 668
St8sia6	sialyltransferase	miR-182, 503

(*42*, *60-65*)

Table 2.1, continued.

B. Downregulated genes.

Gene	Description	miRNA
Csmd3	CUB and sushi multiple domains 3	miR-155, 1198-5p
Dock9	GEF, activates Cdc42	miR-155, 1198-5p
Etv5	ETS family transcription factor, regulates COX2	miR-155 210
Klf12	developmentally-regulated transcription factor	miR-155, 1198-5p
P2ry13	purinoceptor	miR-155, 1198-5p
Rtn1	associated with ER, involved in neuroendocrine secretion	miR-210, 1198-5p
Sall1	zinc finger transcriptional repressor	miR-155, 1198-5p
Ss18	actin-associated, functions in matrix-specific adhesion	miR-155, 1198-5p
Tppp	linked to multiple sclerosis myelin lesions	miR-155, 210, 1198-5p
Zfp595	zinc finger protein	miR-155, 1198-5p

(*39*, *66-71*)

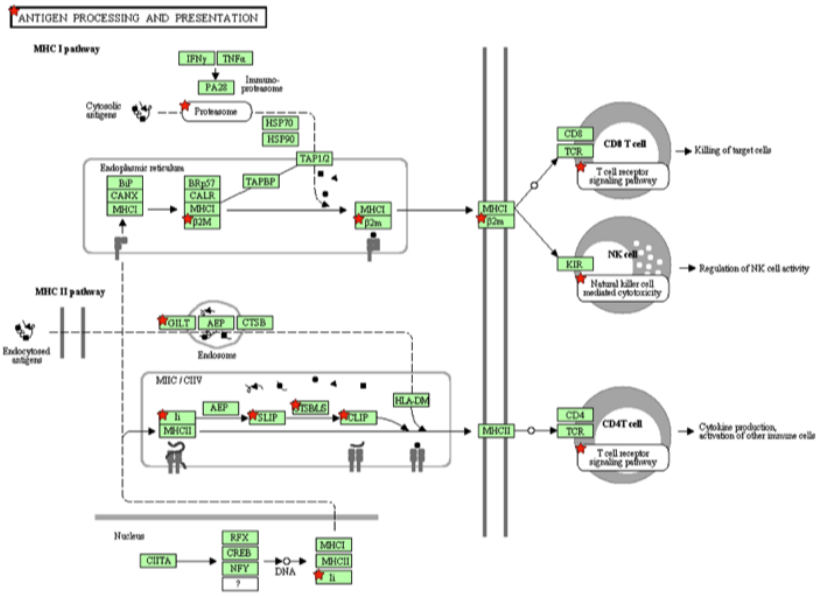
(Figure 2.7B, 2.8B), a brain-specific protein normally expressed in oligodendrocytes. It has been shown that changes in its expression are characteristic of some neurodegenerative diseases where neuronal expression occurs with accumulation in Lewy body inclusions, which mark degenerating neurons (*72*). Thus, it is possible that the downregulation of Tppp could be a neuroprotective effect. Csmd3 and P2ry13, a purinergic receptor, are also predicted to be targeted by both miR-155 and miR-1198-5p (Figure 2.7B), and both Csmd3 and P2ry13 mRNAs are constitutively expressed microglia mRNAs (unpublished data, communicated by Isaac Chiu). In addition, the upregulation of the divalent metal ion transporter Slc11a2 is interesting. This transporter mediates iron transport in cerebral endosomal compartments, and a specific polymorphism in this gene was found to be associated with shorter duration of ALS in a cohort of French sporadic patients (*60*).

The mRNAs predicted to be targeted by the downregulated miRNAs also show overlap. There are more genes in this group because there are more genes upregulated in G93A microglia than downregulated, as a general trend (unpublished data, Maniatis lab). Of particular interest, miR-182 and miR-668, two of the miRNAs downregulated in G93A microglia at end-stage, are predicted to target Nox 2 and IGF1 (miR-182 is predicted to target both and miR-668 is predicted to target just IGF1) (Figure 2.13). IGF1 is a neuroprotective molecule that is upregulated in microglia during ALS progression (*42*) and supports neuronal survival (*73*). However, Nox2, the phagocytic NAPDH oxidase, is increased in classically activated microglia and exacerbates traumatic brain injury (*46*). It has also been shown that NADPH oxidase is activated in the spinal cord of sporadic ALS patients and SOD1 G93A transgenic mice and that inactivation of the enzyme delays neurodegeneration and extends survival (*74*). The upregulation of both protective and toxic factors is interesting, as microglia typically respond to

different stimuli to act in *either* a neuroprotective or neurotoxic manner. Neurotoxic activation is typically associated with increased production of molecules including cytokines, reactive oxygen species, and Nox2. Neuroprotective activation likely leads to an increase in phagocytosis and neuronal survival molecules, like IGF1. In a model of brain ischemia, IGF1 was shown to be upregulated in resident microglia (*75*). These observations suggest the interesting possibility that microglia respond in both protective and toxic ways during disease progression, possibly based on their proximity to damaged neurons. One hypothesis of ALS disease progression in humans, "focality", posits that neurodegeneration initiates stochastically in the spinal cord and spreads in the upper and lower motor neuron levels and the periphery (*76-78*). Degeneration and other deficits spread contiguously, and it is possible that that microglia and astrocytes are required for this spreading.

Using the DAVID bioinformatic tool (*31, 32*), I examined functional categories of predicted targets of the differentially expressed miRNAs. I pooled the upregulated targets of the downregulated miRNAs and the downregulated targets of the upregulated miRNAs. Due to the numbers of genes I examined, I only had sufficient statistical power to obtain valuable information for the upregulated genes. Of interest, there is an enrichment for genes involved in glycosaminoglycan degradation (KEGG pathway mmu00531), suggesting mutant microglia upregulate genes to degrade a specific component of the extracellular matrix. In addition, there is enrichment for genes involved in antigen processing and presentation (KEGG pathway mmu04612) (Figure 2.14). When activated, microglia have the ability to act as antigen-presenting cells to present antigenic compounds to T cells in order to mount an adaptive immune response (*73, 79*). It has also been shown that both CD4+ and CD8+ T cells infiltrate the spinal cord with disease progression in SOD1 ALS transgenic mice (*42*). Thus, it is particularly

Figure 2.14. Gene ontology analysis of upregulated genes shows an enrichment in antigen processing and presentation.



The DAVID tool was used to analyze the functional categories of the predicted upregulated targets. The KEGG pathway antigen processing and presentation (mmu04612) was enriched. * indicates genes in this pathway that are upregulated.

interesting that upregulated genes in microglia are involved in both the MHC I and MHC II pathway, and go on to activate both CD4+ and CD8+ T cells. Although the levels of these genes cannot be directly attributed to the downregulation of specific miRNAs, that G93A microglia have increased expression of these genes in concert with the downregulation of miRNAs that target them is compelling.

In summary the data presented demonstrate that miRNAs are differentially expressed in SOD1 G93A microglia compared to WT microglia. Specifically, miR-155 and miR-210 are significantly upregulated during ALS disease progression. These data also add to our general understanding of miRNAs in ALS, as well as complement the other studies being done in the Maniatis lab to understand the role of non-neuronal cells is ALS disease progression.

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Chapter 3.

Discussion

My thesis is the first study directed towards the identification of miRNAs that are dysregulated in cells of the nervous system in a transgenic mouse model of ALS. Previously, only skeletal muscle-specific miR-206 had been found to be upregulated during ALS disease progression. The authors proposed that this upregulation delays ALS progression by promoting the regeneration of neuromuscular synapses in mice (*1*). Thus my identification of 6 miRNAs that are up or downregulated in SOD1 G93A microglia is novel and adds to our understanding of the specific contributions of microglia in ALS disease progression. In particular, the significant upregulation of miR-155 and miR-210 in the SOD1 G93A transgenic mouse model is particularly interesting. Although miR-155 is involved in inflammatory and immune processes, it has not previously been observed to be dysregulated in microglia in ALS. miR-210, however, has not previously been implicated in microglia or neurodegenerative diseases.

By examining the miRNA sequencing from primary microglia over an extended time course, I was able to identify the miRNAs that are highly and moderately expressed in resting primary spinal cord microglia (Table 2.1). This is the first genome-wide examination of miRNAs expressed in microglia, so this alone is a valuable set of data for our lab as well as the general field. The comparison of LPS-induced WT SOD1 microglia to non-induced WT SOD1 microglia also provides information about the miRNAs that are up or downregulated in the presence of LPS. This made it possible to determine whether changes in miRNA levels were specific to disease, or an aspect of broad microglial activation, for example miR-155 and miR-503. miR-155 and miR-503 are both dysregulated by LPS in the same manner as during disease progression, suggesting that these changes might occur in response to more than one type of trauma, for example chronic disease and acute LPS treatment.

Hypoxia, miR-210, and ALS

Hypoxia-inducible factor 1α (HIF-1α) is a master regulator of cellular oxygen homeostasis and is activated by hypoxia. In the brain, HIF-1α expression is induced by hypoxia in neurons, astrocytes, and ependymal and endothelial cells (*2, 3*). There is accumulating evidence that activation of HIF-1α could potentially exert neuroprotective effects in both neuronal and non-neuronal cells (*4*). In ALS, there is substantial evidence that mitochondrial dysfunction is an early pathogenic process in the disease and contributes to disease progression (*5*). There is a significant decrease in the capacity for mitochondria in the brain and spinal cord of SOD1 G93A transgenic mice to load Ca^{2+}, which leads to elevated Ca^{2+} levels (*6*). This increase in Ca^{2+} has been found to induce reactive oxygen species and oxidative stress in primary motor neurons from G93A transgenic mice (*7*). In addition, there is evidence that mitochondrial reactive oxygen species can act as signaling molecules and trigger protective responses through HIF-1α activation (*4*). Taken together, this suggests that induction of HIF-1α could be neuroprotective in neurodegenerative diseases. For example, using primary cultured astrocytes to study Alzheimer's disease, it was shown that induction of HIF-1α reduces astrocyte activation by Aβ, thus supporting neuronal survival (*8*).

The disease progression of ALS is closely related to hypoxia. Motor neurons are particularly susceptible to hypoxic conditions due to their high oxygen consumption and relatively low capability for antioxidant mechanisms (*9*). An early symptom of disease is diaphragm muscle weakness, which is involved in episodes of intermittent hypoxia and reoxygenation (*10*). It was also recently found that HIF-1α is dysregulated in monocytes, which are circulating microglia precursors, from sporadic ALS patients (*11*).

miR-210 is considered the master hypoxamir, and is highly activated in hypoxia in

response to upregulation of HIF-1α (*12*). HIF-1α directly binds the hypoxia responsive element on the miR-210 promoter (*13*). miR-210 has been found to be involved in numerous processes including the induction of angiogenesis (*14*), repression of mitochondrial metabolism (*12*), and inhibition of cell proliferation (*15*). However, it has not previously been shown to be involved in ALS. I observed that miR-210 is upregulated in G93A microglia at 12 and 17 weeks, which corresponds to symptom onset and end-stage, respectively (Figure 2.10B), and this is likely due to the upregulation of HIF-1α in G93A microglia (Figure 2.10A). Several predicted targets of miR-210 are significantly downregulated in our microglia RNA-seq data. Rtn1 expression is correlated with neuronal differentiation, and its downregulation might be a toxic effect (*16*). However, Tppp is a brain-specific protein normally expressed in oligodendrocytes and changes in its expression are characteristic of some neurodegenerative diseases where neuronal expression occurs with accumulation in Lewy body inclusions which mark degenerating neurons (*17*). Thus, it is possible that the downregulation of Tppp could be a protective effect.

Future directions

Current/ongoing plans

In the immediate future, I plan to carry out *in situ* hybridization experiments with spinal cord sections using locked nucleic acid (LNA) probes (Exiqon) to examine the localization of miR-155 and miR-210. miR-146a will be used as a positive control since I found that this miRNA is constitutively and highly expressed in microglia (Figure 2.2). It is interesting to consider the possibility that miR-155 and miR-210 are expressed in distinct populations of microglia within the spinal cord, corresponding to neurotoxic and neuroprotective phenotypes, respectively.

Further look into targets/relevance

Even though we have the RNA-seq data from acutely isolated microglia, thus localizing the observed mRNA changes to microglia, it will be beneficial to also do immunohistochemistry on spinal cord sections to examine protein levels of the target mRNAs. In concert with the *in situ* hybridization described above, this would visually place the miRNA and its mRNA targets in the same cell. For particularly compelling targets, for example IGF1 and Nox2, it would also be interesting to perform luciferase assays with the 3'UTR of the target mRNA downstream of a luciferase reporter. Transfection of these reporters into the N9 microglia cell line, along with exogenous miRNA would allow us to determine if the specific UTR is regulated by the miRNA. If deletion of the target site in the same reporter abolishes the ability of the miRNA to downregulated luciferase, it would be strong indication that the miRNA is in fact targeting that mRNA. To further understand the effects of the specific miRNAs in vitro, the N9 microglia cell line can be transduced with lentiviruses to express SOD1 G93A or WT SOD1 (*18*). Knockdown and overexpression of specific miRNAs can then be carried out in these transduced cells.

Examine how alternative 3'UTR usage affects miRNA targeting

The lab has RNA-seq data from acutely isolated microglia from non-transgenic and SOD1 G93A mice at 9, 14, and 18 weeks, corresponding to pre-symptomatic, symptom onset, and end-stage, respectively. Alternative splicing analysis of this dataset has revealed substantial splicing changes in disease, particularly alternative last exon usage, which would cause an alternative 3'UTR. As discussed previously, alternative 3'UTRs could remove miRNA target sites, thus affecting the ability of a miRNA to target its cognate mRNA. For example, Pax3, a myogenic regulator, that is transiently expressed during activation of adult muscle stem cells and

quiescent stem cells is regulated by miR-206 (*19*). In most quiescent and activated adult stem cells miR-206 suppresses Pax3 expression. However, quiescent stem cells that express high levels of Pax3 also express high levels of miR-206. In these cells, Pax3 transcripts undergo alternative polyadenylation, resulting in a shorter 3'UTR, and thus removal of miR-206 target sites. An intriguing candidate for the removal of target sites due to an alternative terminal exon in G93A microglia is macrophage scavenger receptor 1 (Msr1). Msr1 is a predicted target of miR-155, however it is upregulated in the G93A microglia (Figure 3.1A) suggesting it is not targeted by miR-155. Interestingly, analysis of the Msr1 3'UTR using Targetscan shows that it has two 3'UTRs; a short 3'UTR with no predicted miRNA target sites and a long 3'UTR with a highly conserved miR-155 target site. Specific examination of the genomic location of Msr1 using the microglia RNA-seq shows that at 18 weeks the G93A microglia specifically express the short UTR, whereas the control microglia do not express this short UTR (Figure 3.1B). This suggests that with disease progression, G93A microglia selectively express the alternative short Msr1 3'UTR, which is not sensitive to the increased levels of miR-155.

By a mechanism we do not fully understand, the predominant splicing change observed in the microglia RNA-seq data alternative last exon usage, which would lead to an alternative 3'UTR (Table 3.1)**.** At the mRNA level in this dataset, there are many changes in splicing factors and spliceosome components, so this could be the cause of these changes (unpublished data, communicated by Isaac Chiu). The mRNAs affected by these changes will have to be examined using bioinformatics to determine whether the alternative 3'UTRs cause the addition or removal of miRNA target sites. Then the microglia RNA-seq data can be used to examine how levels of these mRNAs correlate with miRNA levels.

Table 3.1. Alternative splicing events in microglia RNA-seq dataset.

9 weeks

Event	Count
Alternative first exon	27
Alternative last exon	329

14 weeks

Event	Count
Alternative first exon	34
Tandem UTR	3
Retained intron	2
Alternative last exon	496

18 weeks

Event	Count
Alternative first exon	70
Alternative 5' ss	1
Cassette exon	1
Tandem UTR	2
Alternative 3' ss	2
Retained intron	8
Skipped exon	5
Alternative last exon	390

MISO was used to identify alternative splicing events in the RNA-seq dataset from acutely isolated SOD1 G93A microglia compared to non-transgenic microglia during disease progression (*20*).

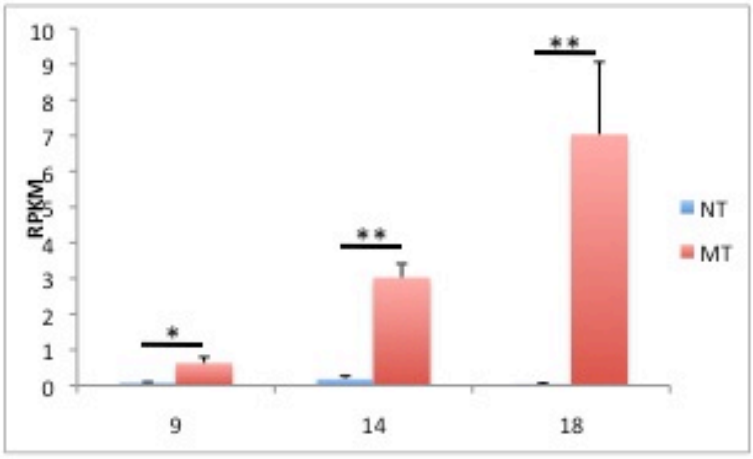
Figure 3.1. Msr1 and miR-155.

A. Msr1 is significantly upregulated in G93A microglia at 9, 14, 18 weeks. Msr1 levels were examined in the microglia RNA-seq dataset. *p<0.04, **p<0.009, Student's t test, NT 9, NT 14, NT 18 (n=3), MT 9 (n=5), MT 14 (n=7), MT 18 (n=9). Values represent mean +/- SEM.

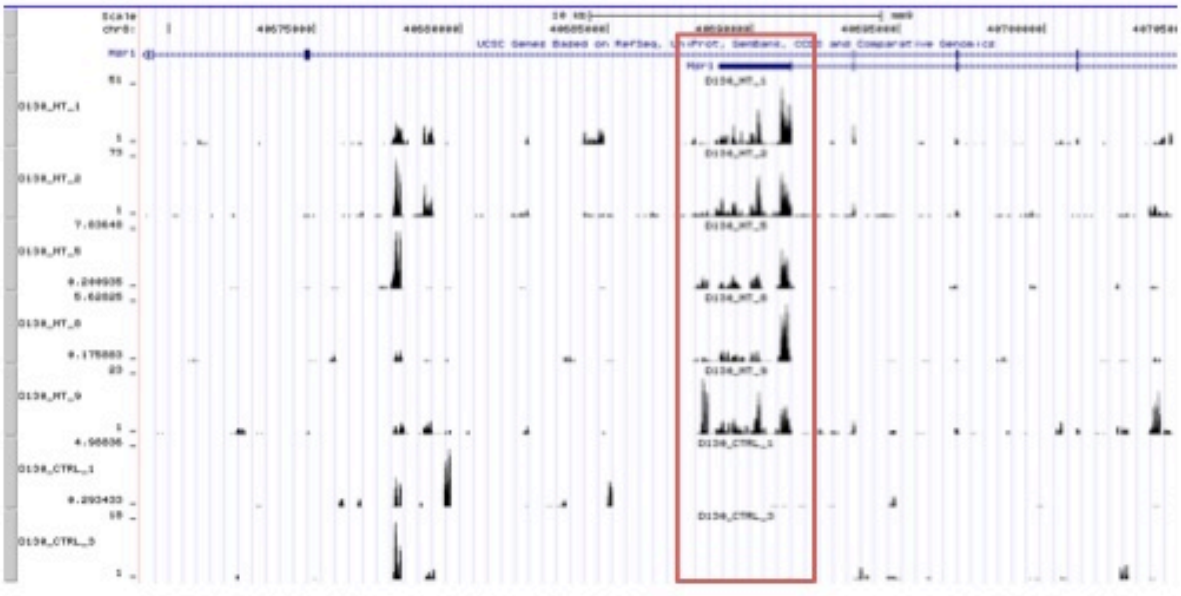
B. At 18 weeks, G93A microglia selectively express the short Msr1 3'UTR which lacks any miRNA target sites. Targetscan shows that Msr1 has two 3'UTRs: a short 3'UTR with no miRNA target sites and a long 3'UTR with a highly conserved miR-155 target site. The genomic region surrounding the Msr1 3'UTRs is shown for the microglia RNA-seq dataset. The top five rows correspond to G93A 18 week microglia and the bottom two are non-transgenic 18 week microglia. The short alternative Msr1 3'UTR is boxed.

Figure 3.1, continued.

A. Msr1 is upregulated in G93A microglia at 9, 14, and 18 weeks.



B. At 18 weeks, G93A microglia selectively express the short Msr1 3'UTR which lacks any miRNA target sites.



Determine if miRNA changes also occur in sporadic ALS patients

Although it is obviously challenging to obtain primary microglia from sporadic ALS patients, it is possible to obtain monocytes from the blood of patients. Monocytes are white blood cells that are part of the immune system. During hematopoeisis, some hematopoeitic stem cells differentiate into monocytes before settling in the central nervous system and further differentiating into microglia (*21*). It would be very interesting to see if the miRNA changes I observe in acutely isolated microglia from G93A transgenic mice also occur in sporadic ALS cases. It was recently shown that the HIF-1α pathway is dysregulated in monocytes from sporadic ALS patients (*11*). Thus, I hypothesize that miR-210 would also be dysregulated in monocytes from sporadic ALS patients.

miR-155 knockout mice

In the past year there have been numerous studies linking the cytokine interferon-γ (IFNγ) to ALS disease progression, particularly the effects of mutant astrocyte-supplied IFN. Aebischer *et al*. observed that mutant SOD1 astrocytes release IFNγ, which activates the LIGHT-lymphotoxin-β receptor death pathway (*22*). Similarly it has also been shown that IFN signaling is activated in G93A transgenic mice, with an increase in IFN-stimulated genes in the spinal cords of presymptomatic G93A mice (*23*). Specifically, the upregulated IFN-stimulated genes were found in the astrocytes. Recently, it was shown that miR-155 is a positive regulator of IFNγ production in natural killer cells (*24*). These observations are in concert with my data showing significant miR-155 upregulation in G93A microglia at 10, 12, and 17 weeks (Figure 2.4A) and provide further evidence for the involvement of IFN and the inflammatory response in ALS disease progression. Thus, I am currently crossing miR-155 null mice (B6.Cg-

Mir155tm1.1Rsky/J, Jackson Laboratory) with SOD1 G93A mice (B6.Cg-Tg(SOD1*G93A)1Gur/J, Jackson Laboratory) to generate miR-155 null mice that are also overexpressing human G93A SOD1. If miR-155 is removed, we hypothesize that there could be an extension in lifespan due to the removal of this pro-inflammatory factor that also positively regulates IFNγ. Although we are currently breeding these mice, it is likely that I will no longer be in the lab to determine the full effects of miR-155 deletion on disease progression. A post-doc in the lab will continue breeding these mice and characterize the resulting phenotype. As miR-155 is a pro-inflammatory miRNA expressed in numerous immune cell types in addition to microglia, including T cells, B cells, dendritic cells, and natural killer cells (*24-26*), there could be significant improvement in disease progression and extension in lifespan. In addition to the generation of these mice, we could also inject miR-155 shRNA viruses into the spinal cords of SOD1 G93A mice to determine if there is any effect on the transgenic mice. This would be a less time consuming experiment and would provide some indication as to the possible benefits of decreased levels of miR-155 expression.

Although similar studies with miR-210 would be very interesting, there is not currently a miR-210 knockout mouse available. Due to the many roles of miR-210, it is possible that an inducible miR-210 deletion mouse might be necessary to study its involvement in disease. This would then allow the specific deletion of miR-210 in microglia. An alternative to a mouse model would be the injection of miR-210 shRNA viruses into the spinal cords of SOD1 G93A mice to determine if there is an effect on lifespan and disease progression.

Implications

Our lab is deeply interested in how the various cell types of the nervous system contribute to ALS disease progression. We have numerous types of detailed analyses, including RNA-seq datasets from mouse ES cell-derived motor neurons overexpressing G93A SOD1, primary astrocytes from SOD1 G93A transgenic mice, acutely isolated microglia from SOD1 G93A transgenic mice, total spinal cord from SOD1 G93A transgenic mice, and induced pluripotent stem cell-derived motor neurons from patients with various TDP43, FUS, and SOD1 mutations. My thesis work provides a paradigm for similar analyses in other cell types, such as astrocytes. The use of the miRNA sequencing dataset in conjunction with our acutely isolated microglia RNA-seq dataset allowed me to simultaneously examine the levels of miRNAs and their predicted mRNA targets. The identification of dysregulated miRNAs allows us to make hypotheses regarding the mRNAs they are potentially targeting. An interesting example is IGF1, a neuroprotective factor, which has been shown to be upregulated in G93A microglia (Figure 2.11A) (*18*). To date, it is not know how IGF1 is upregulated, so the identification of two miRNAs that are downregulated with ALS disease progression that target IGF1, suggests these miRNAs might be involved in the significant upregulation of IGF1 observed in ALS. A thorough understanding of how miRNA expression is changing in microglia during disease allows us to identify some of the mechanisms leading to the mRNA level changes that are occurring in microglia, and thus more deeply understand the contributions of this specific cell type to disease progression. In addition, this has helped develop a framework for bioinformatic analyses for future miRNA sequencing and the different ways to probe available RNA-seq datasets.

More specifically, my work suggests new directions for research on microglia, miRNAs, and ALS. Only two studies thus far have examined hypoxia and HIF-1α in ALS (*11, 27*), and no previous studies have observed that hypoxia-inducible miR-210 is be upregulated in ALS. As it has been hypothesized that upregulation of HIF-1α could be protective, this would be very interesting to investigate further. In general, the identification of miRNAs dysregulated in microglia in ALS provide many potential avenues of research both specifically in microglia and also to understand the role of miRNAs in cells of the nervous system during disease progression.

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Appendix 1.

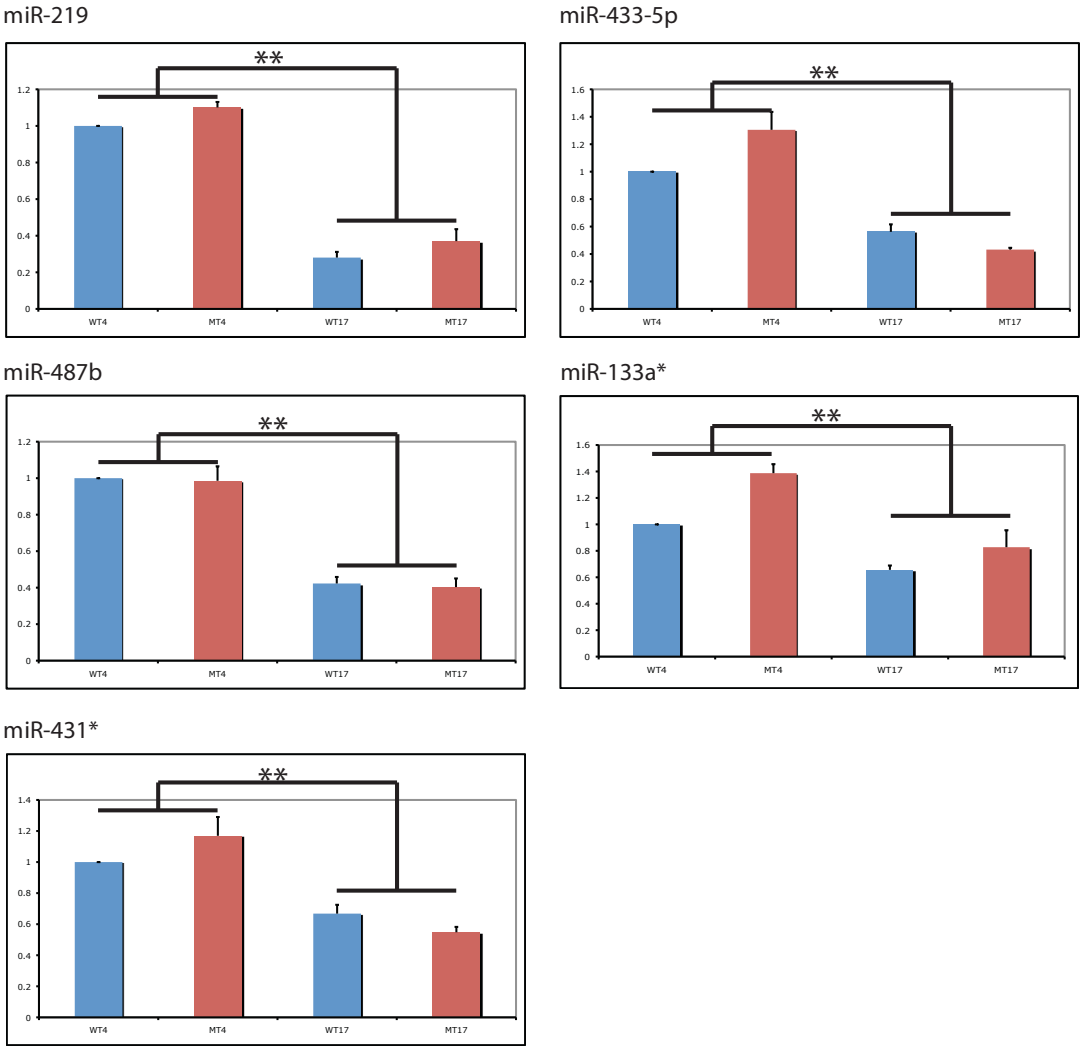
miRNAs are dysregulated in total spinal cord from SOD1 G93A transgenic mice.

Previous studies have shown that there are significant changes in gene expression that correlate with disease progression in the SOD1 mouse model of ALS, however the majority of studies carried out thus far have examined mRNA transcripts (*1*). Only two papers have addressed ALS and miRNAs in more detail. Williams *et al*. showed that miR-206, a skeletal muscle-specific miRNA, delays ALS progression and promotes the regeneration of neuromuscular synapses (*2*). Haramati *et al*. observed that knockout of Dicer in motor neurons causes progressive motor dysfunction and made a correlative case for the involvement of miR-9. They found that miR-9 targets neurofilament subunits, the stoichiometry of which has been previously suggested to be dysregulated in ALS (*3*).

To identify miRNA expression changes that occur during ALS disease progression in total spinal cord, I examined miRNAs expressed in total spinal cords from mice overexpressing human SOD1 G93A (MT) compared to wild type SOD1 (WT). Spinal cords were removed from MT and WT mice at 4, 8, 12, and 17 weeks, and total RNA was isolated. Total RNA from the 4 and 17 week time points was examined by ABI Taqman low density array qPCR (a method to examine miRNAs on a global scale) (Biopolymer Facility, Harvard Medical School) and RNA from all four time points was examined by high throughput SOLiD sequencing (Biopolymer Facility, Harvard Medical School and Levy lab, HudsonAlpha Institue for Biotechnology). Based on the results of both of these analyses, I examined a number of miRNAs by individual Taqman qPCR assay.

Based on the TLDA qPCR results, I first focused on the 4 and 17 week time points. I performed Taqman qPCR assays and observed two main trends. There were a number of miRNAs that were differentially regulated with age (Figure A1.1). These miRNAs were all

Figure A1.1. miRNAs are differentially expressed with age in total spinal cord.



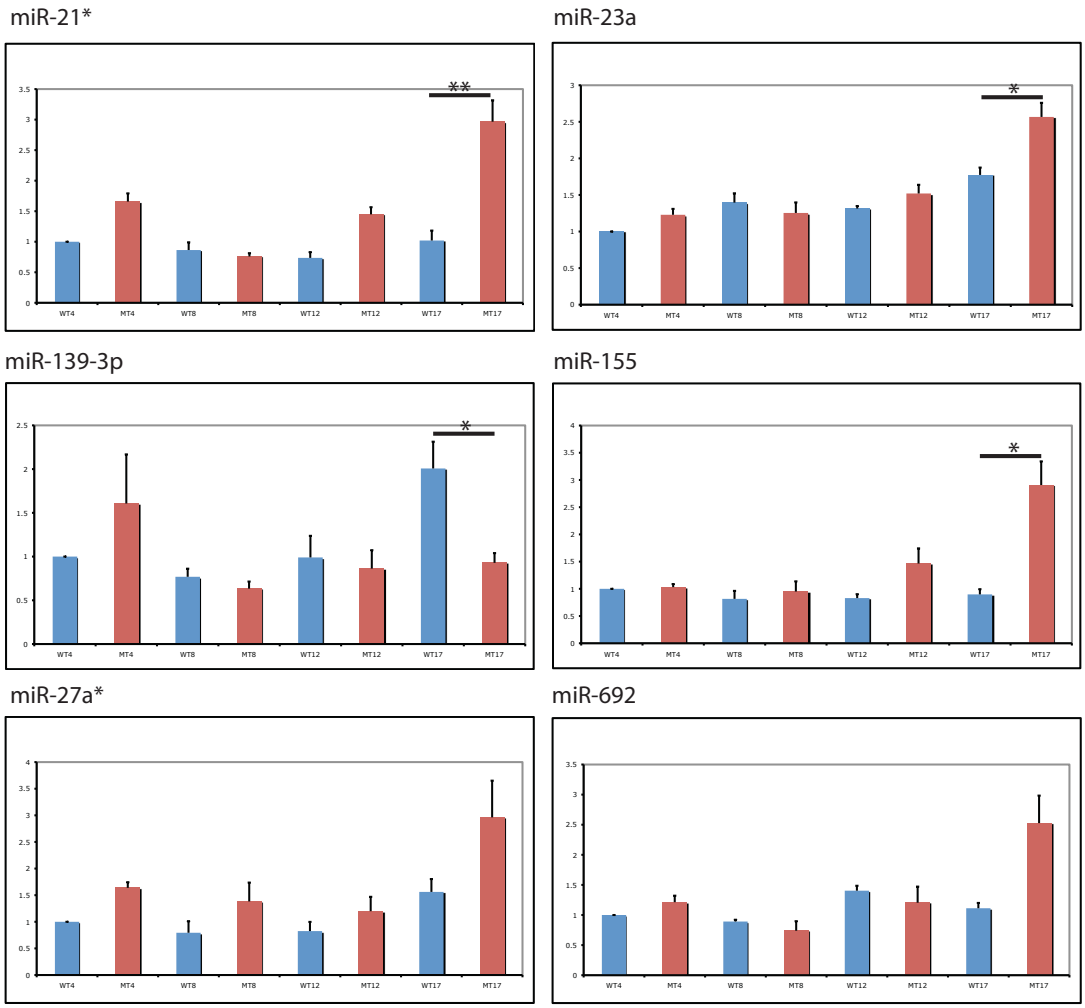
Mice overexpressing human wild type (WT) or G93A SOD1 were sacrificed at 4 and 17 weeks, corresponding to pre-symtptomatic and end-stage, respectively. Spinal cords were removed and total RNA was isolated. miRNA-specific reverse transcription and Taqman qPCR assays were used to examine miR-219, miR-433-5p, miR-487b, miR-133a*, and miR-431*. Results were normalized to snoRNA 135 and snoRNA 202. Each replicate is the spinal cord from an individual mouse. **$p<0.003$, Student's t-test, miR-219 [WT 4 (n=4), MT 4 (n=3), WT 17 (n=3), MT 17 (n=4)], miR-433-5p [WT 4 (n=4), MT 4 (n=4), WT 17 (n=4), MT 17 (n=4)], miR-487b [WT 4 (n=4), MT 4 (n=3), WT 17 (n=4), MT 17 (n=3)], miR-133a* [WT 4 (n=4), MT 4 (n=3), WT 17 (n=3), MT 17 (n=3)], miR-431* [WT 4 (n=5), MT 4 (n=5), WT 17 (n=5), MT 17 (n=5)]. Values represent mean +/- SEM.

expressed at similar levels in WT and MT spinal cords at 4 weeks, and then were downregulated at 17 weeks in both genotypes.

I also observed several miRNAs that were differentially expressed just at 17 weeks in the G93A spinals cords. As this was a disease-specific effect, I further examined the expression of these miRNAs in the complete time course (4, 8, 12, and 17 weeks). I observed that several miRNAs were dysregulated during disease progression but only at end-stage (Figure A1.2). miR-21*, miR-23a, miR-155, miR-27a*, and miR-431* are upregulated at end-stage and miR-139-3p is downregulated. Based on the observation of changes that occur late in disease, I hypothesized that these miRNAs might be due to the activation and proliferation of microglia that occurs during disease progression (*4, 5*). The upregulation of miR-155 was particularly compelling as it had been found to be important to the inflammatory response (*6, 7*). To investigate the changes in the microglia population during ALS disease progression, I performed qPCR on total RNA from the complete time course to examine levels of Cd11b and CD68 (Figure A1.3). CD11b is a general microglia and macrophage marker and CD68 is a marker for activated microglia (*8*). From these qPCR, I observed that microglia increase from 12 weeks (Cd11b) and are also activated starting at 12 weeks (CD68). This time point corresponds to symptom onset in this transgenic model. Thus, it is reasonable to hypothesize that some of the changes I observe at end-stage are due to microglia.

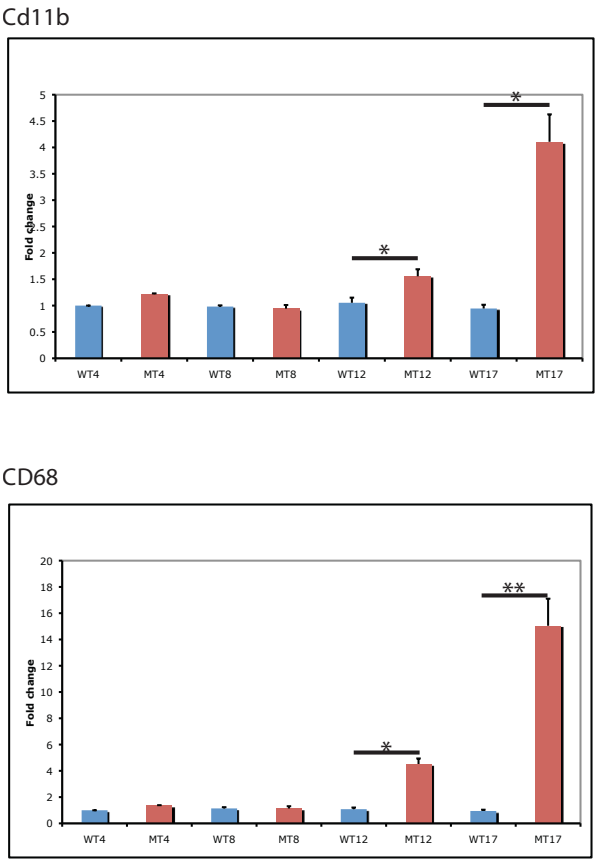
Although spinal cords are a physiologically relevant system, they contain a heterogeneous population of cells including motor neurons, astrocytes, microglia, and oligodendrocytes. Thus, it was very challenging to deconvolute the total spinal cord miRNA expression results. Based on these observations, at that point that I transitioned to focus solely on microglia.

Figure A1.2. miRNAs are dysregulated at end-stage in total spinal cord.



Mice overexpressing human wild type (WT) or G93A SOD1 were sacrificed at 4, 8, 12, and 17 weeks, corresponding to pre-symtptomatic (4 and 8), symptom onset (12), and end-stage (17). Spinal cords were removed and total RNA was isolated. miRNA-specific reverse transcription and Taqman qPCR assays were used to examine miR-21*, miR-23a, miR-139-3p, miR-155, miR-27a*, and miR-692. Results were normalized to snoRNA 135 and snoRNA 202. Each replicate is the spinal cord from an individual mouse. *$p<0.03$, **$p<0.006$, Student's t-test, miR-21* [WT 4, WT 8, MT 8, WT 12 (n=4), MT 4, MT 12, WT 17, MT 17 (n=3)], miR-23a [WT 4, WT 17 (n=4), MT 4, WT 8, MT 8, WT 12, MT 12, MT 17 (n=3)], miR-139-3p [WT 4, MT 4, WT 8, WT 12, MT 12, WT 17, MT 17 (n=4), MT 8 (n=3)], miR-155 [WT 4, MT 4, WT 8, MT 8, WT 12, MT 12, WT 17, MT 17 (n=4)], miR-27a* [WT 4, WT 8, WT 12, MT 12, WT 17, MT 17 (n=4), MT 4, MT 8 (n=3)], miR-692 [WT 4, MT 4, WT 8, MT 12, WT 17, MT 17 (n=4), MT 8, WT 12 (n=3)]. Values represent mean +/- SEM.

Figure A1.3. Microglia genes are upregulated in spinal cord with disease progression.



Mice overexpressing human wild type (WT) or G93A SOD1 were sacrificed at 4, 8, 12, and 17 weeks, corresponding to pre-symtptomatic (4 and 8), symptom onset (12), and end-stage (17). Spinal cords were removed and total RNA was isolated. Reverse transcription was performed and qPCR were carried out to examine Cd11b and CD68. Results were normaled to Gapdh and Rps17. Each replicate is the spinal cord from an individual mouse. *p<0.02, **p<0.006, Student's t-test, Cd11b [WT 4, WT 8, MT 8, WT 12, MT 12, WT 17, MT 17 (n=4), MT 4 (n=3)], CD68 [(WT 4, WT 8, MT 8, WT 12, WT 17, MT 17 (n=4), MT 4, MT 17 (n=3)]. Values represent mean +/- SEM.

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Appendix 2.

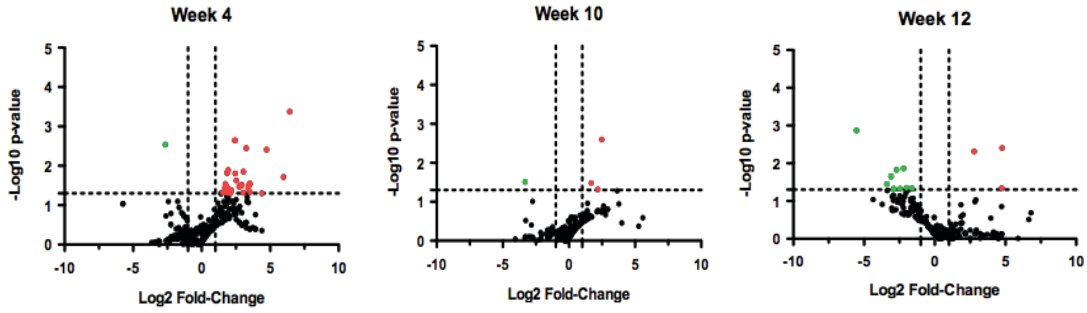
Microglial activation in response to LPS leads to differential expression of miRNAs.

Microglia activation occurs throughout the spinal cord in ALS models and increases with disease progression (*1, 2*). To distinguish between miRNA changes that were caused by acute activation versus chronic ALS disease mechanisms, I used lipopolysaccharide (LPS) to activate microglia. LPS, a bacterial-derived sugar, activates a toll-like receptor pathway and is known to activate microglia. This causes increased phagocytosis, secretion of cytokines, and induction of nitric oxide synthase (*3*). I performed intraperitoneal injection of 5mg/kg LPS 24 hours before microglia isolation (*4*). I injected mice overexpressing human wild type SOD1 (WT) at 4, 6, 8, 10, 12, and 17 weeks. Following total RNA extraction, the miRNA expression profiles were determine by small RNA sequencing. Using the DESeq tool to obtain relative expression levels, the results of the LPS-induced microglia (WT LPS) were compared to WT at each time point. I observed that miRNAs are differentially expressed in response to LPS activation (Figure A2.1).

A preliminary analysis of these data revealed that there are more LPS-induced changes at 4 weeks than at later time points. This could be due to the developing maturity of the immunocompetence of the mouse through 4 weeks of age (*5*). For example, natural killer cells do not begin to appear until ~3 weeks (*6*) and T cell-dependent antibody responses begin after 2 weeks and do not reach mature levels until 6-8 weeks (*7*). If the immune system of the mouse is not mature, it might be hypersensitive to external stimuli, such as LPS.

Comparison of WT LPS microglia to WT microglia also provide information about the miRNAs that are up or downregulated by the presence of LPS (Figure A2.2). miR-155 is the only miRNA observed to respond at almost all time points (Figure A2.2A), suggesting this is a signature of classical activation. I also observed miRNAs that only responded to LPS at later time points. miR-382 is only induced at 12 and 17 weeks (Figure A2.2B), miR-300 and miR-381 are induced only at 17 weeks (Figure A2.2C), and miR-32*, miR-331-3p, and miR-503

Figure A2.1. miRNAs are differentially expressed in response to activation by LPS.



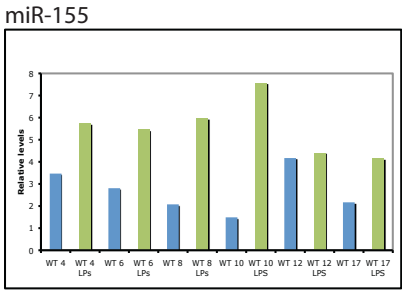
Mice overexpressing human wild type SOD1 (WT) were injected with 5mg/kg LPS 24 hours before microglia isolation (WT LPS). Microglia were isolated at 4, 6, 8, 10, 12, and 17 weeks. Total RNA was isolated from 5-8 spinal cords and small RNA sequencing was performed. Alignment was performed using SHRiMP and relative expression levels were determined using the DESeq tool. These data were compared to microglia from mice overexpressing human WT SOD1 that were not injected with LPS. Graphpad Prism was used to plot log2(fold-change) vs -log10(p-value) for the miRNA sequencing data, comparing WT LPS to WT. miRNAs that correspond to $p<0.01$ and log(fold-change) >1 or <-1 are colored with red or green. The data for microglia isolated at 4, 10, and 12 weeks is shown.

Figure A2.2. Differentially expressed miRNAs display different patterns of behavior.

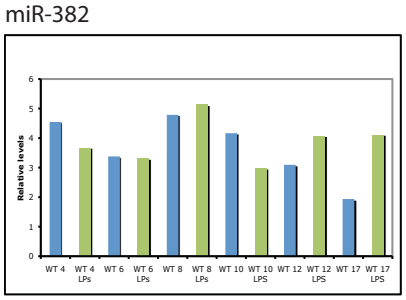
miRNA sequencing was performed on total RNA from microglia isolated from mice overexpressing human wild type SOD1 (WT) and WT mice injected with 5 mg/kg LPS 24 hours before microglia isolation (WT LPS) at 4, 6, 8, 10, 12, and 17 weeks. Alignment was performed using SHRiMP and relative expression levels were determined using the DESeq tool. The WT LPS microglia data was compared to the WT microglia data to identify miRNAs that are differentially expressed in response to LPS.

Figure A2.2, continued.

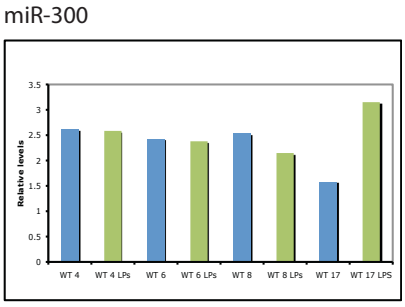
A. miR-155 is upregulated at all time points.



B. miRNAs are induced by LPS at later time points.



C. miRNAs are induced by LPS at 17 weeks only.



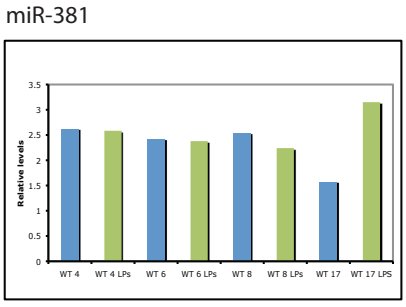
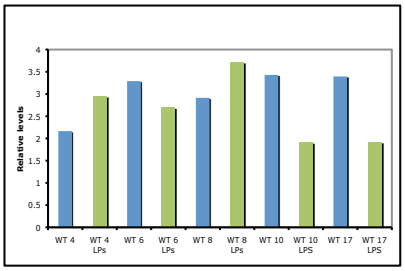


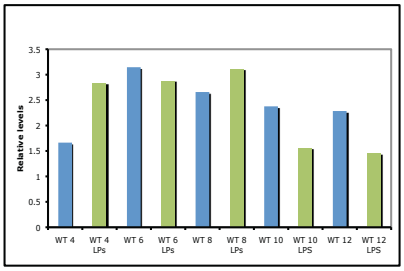
Figure A2.2, continued.

D. miRNAs are downregulated in response to LPS at later time points.

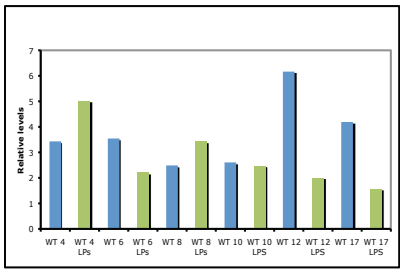
miR-32*



miR-331-3p



miR-503



are downregulated at 12 and 17 weeks (Figure A2.2D). It is interesting to observe that there are such different responses to LPS with age. Perhaps this is due to the maturation of the murine immune system.

These data provide the first global examination of how the expression of miRNAs change in response to acute LPS activation in the spinal cord. This adds to our understanding of microglia activation and allows us to differentiate between microglial miRNA changes that might be due to general activation versus specific diseases or pathological processes.

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