miR-155 expression modulates microglia functions *in vitro* and in the APP/PS1 mouse model of Alzheimer's disease

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Abstract

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Alzheimer's Disease (AD) is characterized by the accumulation of extracellular Amyloid-β (Aβ) as well as both CNS and systemic inflammation. Microglia, myeloid cells resident to the CNS, use microRNAs to rapidly respond to inflammatory signals. MicroRNA (miRNA) profiles are altered in the tissue, circulating monocytes, and serum of AD patients. MiR-155 is a specific miRNA that modulates the phasic inflammatory responses of innate immune cells, however its precise role in AD pathogenesis remains unknown. We hypothesized that miR-155 participates in AD pathophysiology by regulating microglia responses to Aβ *in vitro* and *in vivo*. In cultured neonatal microglia, we observed that modulation of miR-155 expression impacts the internalization of fibrillar Aβ at the plasma membrane *and* to low-pH compartments. In mouse models of AD, microglia specific knock-out of miR-155 decreased accumulation of Aβ. In addition, we also observed that microglia specific deletion of miR-155 acutely increased seizures and seizure-related mortality in two mouse models of AD. Reduced Aβ plaques after miR-155 deletion in microglia suggests increased clearance and the hypothesis that in AD models, microglia facilitate epileptogenesis by increased internalization of synaptic

material along with removal of Aβ. Together, these findings identify miR-155 expression in microglia as a potential regulator of synaptic homeostasis and microglia responses to Aβ in mouse models of AD.

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Chapter 1
Introduction

Alzheimer's disease

In 1907, Alois Alzheimer reported the results of an autopsy on Auguste Deter, a 55-year-old woman who had died from a progressive behavioral and cognitive disorder (Alzheimer, 1906; Hippius & Neundorfer, 2003). Alzheimer keenly observed the presence of two distinctive pathological changes in Deter's brain post-mortem: 1) called neurofibrillary tangles, abnormal intracellular aggregates eventually shown to be composed of hyper-phosphorylated forms of the microtubule-associated protein tau, and 2) neuritic plaques, dystrophic neuronal processes surrounding extracellular plaques (Alzheimer, 1906). In the 1980s, it was shown that dystrophic plaques were primarily composed of a 4.2-kDa peptide, 40 or 42 amino acids in length that was cleaved from a larger precursor, later identified as Amyloid Precursor Protein (APP) (Glenner & Wong, 2012; O'Brien & Wong, 2011). The peptide isolated by Glenner & Wong has come to be known as the Aβ peptide, short for amyloid-β peptide. Alzheimer's disease (AD) is now recognized as the most common form of elderly dementia, characterized by these pathological hallmarks (Glenner & Wong, 1984, 2012). Mid-life onset genetic forms of AD are rare. However, they are an important subset to investigate since late onset sporadic cases present with similar pathology. AD currently afflicts 26 million people worldwide with projections of a fourfold increase in that number by 2050 (Y. Huang & Mucke, 2012). Clinically, AD is characterized by progressive memory loss and cognitive decline. The slow pace of disease progression can decrease the accuracy of the diagnosis Alzheimer's disease (Bonham, Desikan,

Yokoyama, & Alzheimer's Disease Neuroimaging, 2016; Busciglio, Lorenzo, Yeh, & Yankner, 1995). Therefore, the lack of biomarkers and knowledge about AD pathophysiology have contributed to the challenges in creating diagnostic and therapeutic approaches for AD.

Since its initial characterization, insights into the pathophysiology of AD are rooted in the analysis of post-mortem tissue of patients. The pathological hallmarks of AD include senile plaques and neurofibrillary tangles. Plaques are classified in several types primarily dependent on the plaque's morphology (compact vs diffuse) or location (spatial association with synapses vs blood vessels), which could suggest that degeneration of a specific population of cells might be connected to a specific morphological feature of plaque (Armstrong, 2006). Plaques, although heterogeneous in their composition, are primarily composed of aggregated amyloid-β-42 peptide APP (lacking C terminus). Other major components of plaques include Apolipoproteins, α1-antichymotrypsin, sulphated glycosaminoglycans, and complement factors (C1q, C3, C4) (Armstrong, 2006; Lian et al., 2016). At the pathological level, the association of immune system proteins with plaques was an initial clue suggesting involvement of the immune response in AD pathophysiology(Bonham et al., 2016; Rasmussen, Nordestgaard, Frikke-Schmidt, & Nielsen, 2018).

Neurofibrillary Tangles (NFTs) can also be observed post-mortem tissue. NFTs occur intracellularly and are made up primarily of aggregated Tau bearing abnormal posttranslational modifications, including hyperphosphorylation, glycosylation, ubiquitination, polyamination, nitration and proteolysis, which may represent mechanisms within neurons to remove damaged or aggregated proteins (Armstrong,

2006). Traditionally, it has been posited that Tau functions primarily to stabilize microtubules and that its aggregation in AD leads to deficits and cell death through a loss-of-function mechanism (Bonham et al., 2016; Busciglio et al., 1995). However, it has been recently shown that Tau may facilitate or enhance excitatory neurotransmission by regulating the distribution of synaptic activity-related signaling molecules (Rajmohan & Reddy, 2017). Upon abnormal post-translational modifications, Tau becomes enriched in the dendrites at synaptic spines where it can interfere with synaptic transmission. Aβ may promote this postsynaptic enrichment of Tau through a process that involves members of the microtubule affinity-regulating kinase (MARK) family (Busciglio et al., 1995; Rajmohan & Reddy, 2017). Therefore, several studies have made efforts to link plaques to NFT pathology.

Efforts to link plaque pathology to NFT pathology have led to the development of the amyloid cascade hypothesis (ACH) (Armstrong, 2006; Bondarev, Antonets, Kajava, Nizhnikov, & Zhouravleva, 2018). This hypothesis was first formulated when mutations in APP revealed a pathogenic role in AD. The ACH posits that an increase in the processing of APP to the highly amyloidogenic peptide $A\beta_{1\text{-}42}$ and the formation plaques is the primary pathological event that precedes subsequent pathological changes (including NFT formation) in AD progression (Bondarev et al., 2018; Hardy & Higgins, 1992). For example, mutations in *Presenilin 1* (*PSEN1),* and *Presenilin 2* (*PSEN2)* were identified to result in autosomal dominant familial AD, where greater numbers of plaques and NFTs were observed compared with cases of sporadic AD(Benitez et al., 2013; Cruchaga et al., 2012). Although no direct link between $A\beta_{1\text{-}42}$ deposition and NFT pathology has been established, there are clinical cases supporting the idea that

mutations in PS1, and aberrant $A\beta_{1-42}$ production, may contribute to increases in Tau deposition (Benitez et al., 2013; Cruchaga et al., 2012; Guerreiro, Gustafson, & Hardy, 2012). Although familial AD (FAD) accounts for less than 1% of cases, there is pathological and clinical overlap with sporadic forms of AD (Cruchaga et al., 2012; Oakley et al., 2006).

During the early phases of disease, AD leads to pyramidal neuron loss in two main brain regions, entorhinal cortex and CA1 region of the hippocampus, contributing to overall cortical atrophy that is observed. It has been demonstrated that much of the total brain atrophy is due to loss of neuronal processes, as opposed to neuron death (Busciglio et al., 1995; Y. Huang & Mucke, 2012). Current diagnostic MRI technologies have allowed for the observation of decreases in cortical thickness in multiple brain regions of AD patients. Brain imaging approaches have enabled correlations between cognitive decline to observed brain atrophy, and may be used to presage the conversion from mild cognitive impairment to full AD presentation (Y. Huang & Mucke, 2012). Furthermore, alterations in neuronal networks can be detected in patients with AD and people at risk of AD by functional MRI (Frisoni, Fox, Jack, Scheltens, & Thompson, 2010; Putcha et al., 2011). This correlates with decreased hippocampal volume and cortical thinning in AD susceptible regions in patients at risk of AD (Salloway et al., 2009; Salloway et al., 2011). Numerous studies in mouse models and patient data suggests that AD does not just “silence” neuronal networks, but it causes aberrant network activity that may interfere with learning, memory and cognitive functions (Hollnagel et al., 2019; Palop et al., 2003; Palop & Mucke, 2010). Overstimulation of specific neuronal populations due to aberrant connectivity may lead

to excitotoxicity and AD pathology (Y. Huang & Mucke, 2012; Palop et al., 2003; Palop & Mucke, 2010).

Genetics of Alzheimer's disease

AD likely results from complex interactions between multiple genetic, epigenetic and environmental factors. Mutations in three genes, amyloid precursor protein (*APP)*, *Presenilin 1* (*PSEN1),* and *Presenilin 2* (*PSEN2)*, lead to autosomal dominant familial AD. Familial AD (FAD) accounts for less than 1% of cases but overlaps pathologically and clinically with sporadic forms of AD (Glenner & Wong, 2012; Y. Huang & Mucke, 2012; O'Brien & Wong, 2011). APP is produced in large quantities in neurons and is metabolized rapidly. Multiple pathways exist for APP proteolysis, some of which lead to generation of the secreted Aβ peptide and some of which do not (Figure 1.1). After sorting in the endoplasmic reticulum (ER) and Golgi, APP is delivered to the axon where it is transported to synaptic terminals. However, the crucial steps to APP processing occur in the trans-Golgi network (TGN) and at the plasma membrane before transport to synaptic terminals via clathrin-coated vesicles. At the cell membrane APP is cleaved by α-secretase and then γ-secretase. This process alone will not generate Aβ peptides. At the plasma membrane, APP can be reinternalized in clathrin-coated pits into another endosomal compartment containing both the proteases BACE1 and γ-secretase. Here, cleavage of APP results in the production of Aβ, which is then dumped into the extracellular space (a process that occurs through vesicle recycling) or can be degraded in lysosomes (Glenner & Wong, 1984, 2012; Tam, Seah, & Pasternak, 2014).

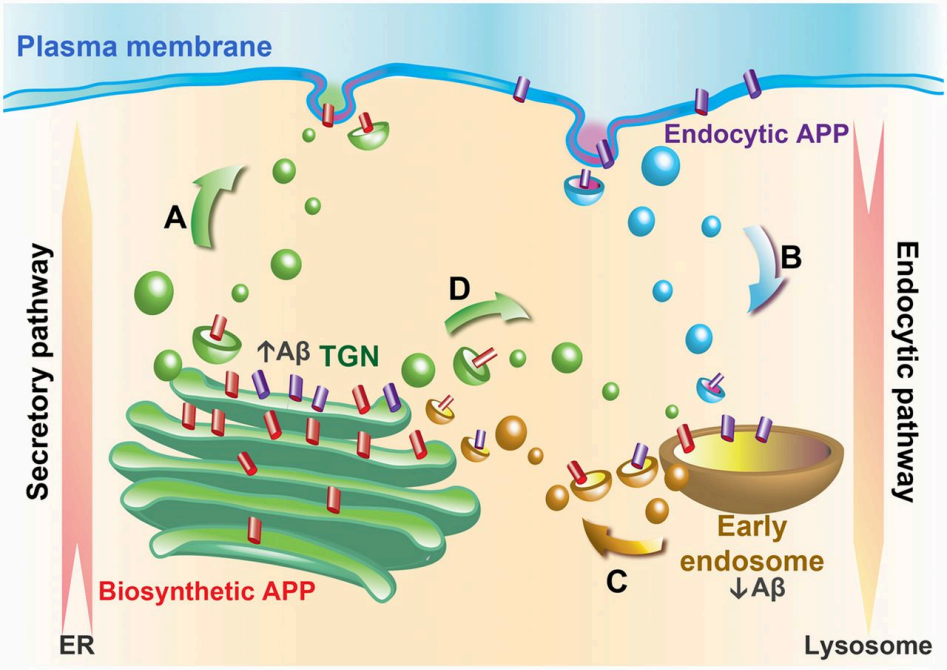

Figure 1.1: Model for APP trafficking in Aβ production. **A)** For Amyloid precursor protein (APP) to be cleaved into $A\beta_{40 \text{ or } 42}$ in the Trans Golgi Network (TGN), newly synthesized APP (red) at the Endoplasmic Reticulum (ER) first traffics along the secretory pathway via the TGN to the plasma membrane. **B)** APP is then internalized via endocytosis to reach early endosomes. **C)** The endocytic pool of APP (now purple) is recycled to the TGN in a retromer-dependent manner, where Aβ is produced. **D)** The biosynthetic pool of APP (red) in the TGN can also be trafficked directly to endosomes in a process mediated by the AP-4 complex **C)** and be retrieved back to the TGN in a retromer-dependent manner for Aβ production. **From**: Wai-Yan Choy, et. Al., 2012. Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid β (Aβ) production in the trans-Golgi network. PNAS.

There are 32 *APP*, 179 *PSEN1* and 14 *PSEN2* gene mutations that result in early-onset, autosomal dominant, fully penetrant FAD. *PSEN1* and *PSEN2* mutations primarily lead to coding modifications within one of the nine transmembrane domains (Guerreiro et al., 2012; Holtzman, Morris, & Goate, 2011; Thinakaran & Koo, 2008). However, the precise molecular mechanisms by which mutations in FAD genes lead to the pathological hallmarks of AD has not been determined. Mutations in *APP*, *PSEN1*

and *PSEN2* cause early onset (<60 years old) autosomal dominant AD. Down Syndrome patients (trisomy 21) are also at high risk for AD due to an additional copy of the APP gene which resides on chromosome 21. This lead to the assumption that over expression of APP is a cause of early onset AD. Increased APP gene expression can also be caused by mutations in the promoter region, and this may be a risk factor that leads to Late-Onset AD (LOAD; >60 years old). Rare variants in APP, PSEN1, PSEN2 and ADAM10 have also been identified in LOAD families (Benitez et al., 2013; Cruchaga et al., 2012; Del-Aguila et al., 2018).

LOAD is also strongly influenced by genetic factors. Polymorphisms in apolipoprotein ε4 (*APOE4*) have been genetically linked to sporadic AD. More specifically, *APOE4* variants have been linked to semi-dominant inheritance for late-onset AD (Karch & Goate, 2015). There is a gene dose effect on increasing the risk and the age of onset of the disease (Corder et al., 1993; Karch & Goate, 2015). A Genome Wide Association Study (GWAS) study identified *APOE4* as the only major gene associated with age-related cognitive decline in humans (Lambert et al., 2013). The gene-dose effect that the *APOE4* variant has on cognition may develop prior to the presentation of AD symptoms. The identification of additional novel loci that can predict LOAD risk is essential to our understanding of the underlying mechanisms of AD pathogenesis. Genome wide associated studies (GWAS) have identified polymorphisms in or near several genes that are associated with the risk of developing AD: *ABCA7*, *CLU*, *CR1*, *CD33*, *CD2AP*, *EPHA1*, *BIN1*, *PICALM*, *MS4A* (Harold et al., 2013; Karch & Goate, 2015; Lambert et al., 2013). Additional loci Ire identified in a meta-analysis of these large LOAD consortium datasets: *CASS4*, *CELF1*, *DSG2*, *FERMT2*, *HLA DRB5*

DBR1, *INPP5D*, *MEF2C*, *NME8*, *PTK2B*, *SLC24H4 RIN3*, *SORL1*, *ZCWPW1, QCK, ACE, ADAM10, ADAMTS1, and WWOX* (Kunkle et al., 2019; Lambert et al., 2013). Through GWAS studies, variants in genes involved in lipid metabolism, endocytosis, and the inflammatory response have been identified but not yet thoroughly characterized in animal models of the disease (Karch & Goate, 2015). The identification of common variants that have small effects on AD risk has begun to create a broader picture of the processes and pathways involved in AD pathogenesis.

It remains uncertain whether the link between aging and neurodegenerative disorders is specifically caused by aging-related deterioration or if it reflects the time required for the genetic or epigenetic pathogenic processes to occur. Aβ aggregation stemming from mutations in APP may contribute to cognitive dysfunction by impairing specific populations of inhibitory interneurons that normally regulate the activity of excitatory principal cells (Verret et al., 2012). Aging, remains as the most important known non-genetic risk factor for the development of late-onset AD. A medical history of head injury, hyperlipidemia, hypertension, diabetes and obesity are key environmental factors that also influence AD risk (Karch & Goate, 2015). A combination of a genetic factor (i.e. *APOE4*) with one or more of these environmental factors may increase the risk of LOAD and age-related cognitive decline. Therefore, while LOAD etiology may be complex and multifactorial.

FAD mouse models recapitulate some key pathological features of both FAD and sporadic LOAD (eg. Plaque pathology). Although mouse models are a reasonable tool with which to interrogate very specific questions regarding AD pathology, (eg. Kinetics Aβ plaques) there are major caveats to consider while using mouse models. For

example, there will be limitations when directly applying findings from mouse models to sporadic cases of AD in humans, since most mouse models of AD closely model FAD(Onos et al., 2019). Further, some mouse models of AD show little to no change in synaptic degeneration in the context of Aβ aggregation (Garcia-Alloza et al., 2006). Still, the use of AD mouse models fills a unique niche in research of this neurodegenerative disease, where very specific questions regarding pathological changes can be elucidated (Neuner, Heuer, Huentelman, O'Connell, & Kaczorowski, 2019).

Epigenetic Changes in Alzheimer's disease

Mouse models and post-mortem tissue analysis of AD patients have shown that epigenetic dysregulation occurs in multiple levels in AD. It is not understood if epigenetic changes are a cause of AD or secondary to Aβ accumulation (Brouwers et al., 2006). Lack of complete concordance between monozygotic twins supports the notion that epigenetic mechanisms will modulate AD risk. Epigenetic changes associated with AD include abnormal DNA methylation, histone modifications and significant changes in microRNA (miRNA) expression (Chouliaras et al., 2010). Pharmacological inhibition of DNA methylation in the hippocampus impaired memory consolidation after learning a task, promoted histone deacetylation, and improved and promoted learning and memory in AD mouse models as measured by increased learning-related gene expression in aged wild-type mice (Peleg et al., 2010). Furthermore, studies have shown that histone acetylation improved learning and memory in a mouse model of AD and increased learning-related gene expression in aged wild-type mice, suggesting that learning and memory are epigenetically regulated

in health and in disease, such as AD (Fischer, Sananbenesi, Wang, Dobbin, & Tsai, 2007).

Significant changes in miRNA expression occurs during aging and AD progression. These changes contribute to epigenetic modulation of gene expression that occurs during disease. microRNAs (miRNAs) are epigenetic modulators of gene expression at the post-transcriptional level, acting as key modulators of physiological events, like the setting developmental timing, or initiating and maintaining an inflammatory response (Ryan M. O'Connell, Rao, & Baltimore, 2012; Ponomarev, Veremeyko, & Weiner, 2013). Interestingly, immunomodulatory miRNAs may be regulated in an age-dependent manner. However, it is not yet clear whether this regulation contributes to the heightened inflammatory environment observed in aging and aging-related disorders (Ryan M. O'Connell, Rao, Chaudhuri, & Baltimore, 2010). The use of mouse models has been advantageous for many groups seeking answers to this question. For example, we have shown that deficiency of *PSEN2* leads to an exaggerated pro-inflammatory response by microglia, which is associated with decreased miR-146a expression (Jayadev et al., 2013). In addition, alterations in other miRNAs related to normal aging and altered in aging-associated diseases. Therefore, modulation and dysregulation of microRNAs can contribute to disease pathogenesis and is a field that remains largely unexplored.

CNS and Peripheral innate immune inflammation in Alzheimer's disease

Numerous studies support the principle that a heightened circulating inflammatory response is observed in AD patients at multiple stages of disease (Amor et al., 2014). Animal models of systemic inflammation support the link between

peripheral inflammatory insults and CNS degeneration. Animal models of induced chronic neuroinflammation develop age-associated AD pathology, increased Aβ plaque burden and aggregated phosphorylated Tau (Nazem, Sankowski, Bacher, & Al-Abed, 2015; Orre et al., 2014).

Circulating monocytes are recruited to the brain during disease. Human monocytes phagocytose Aβ, a function that can be assessed as an AD relevant measurement of peripheral immune function during disease (Fiala et al., 2005) . In turn, clearance or lysosomal degradation of Aβ by peripheral and resident immune cells is potentially important to the equilibrium impacting Aβ deposition in the brain, as the localization of Aβ is dynamic, and equilibrium between Aβ plasma and CSF may alter Aβ deposition in brain (Mitrasinovic & Murphy, 2003). There may be a benefit of reducing Aβ accumulation via early intervention by immune cell mediated clearance. While these interventions are not proven to be successful at the therapeutic level, they support the hypothesis that inflammatory pathways influence Aβ clearance, and that Aβ clearance and accumulation may be a key component of AD pathogenesis. The identification of novel pathways or targets that modulate inflammatory cells directly will further the efficacy of immunomodulatory AD therapy by potentially obviating the need to design therapeutic antibodies required to cross the blood brain barrier.

Microglia-mediated inflammation in Alzheimer's disease

Microglia are tissue-resident macrophages that perform CNS-specific functions. "Resting" microglia contribute to CNS homeostasis by monitoring changes in their environment, influencing neuronal connectivity and plasticity in healthy adult tissue (Kierdorf & Prinz, 2017; Lynch, 2009). They derive from a unique lineage of

erythromyeloid precursors (EMPs) in the embryonic yolk sac. EMPs infiltrate the brain during early development, differentiate into microglia, and maintain their population by self-renewal, although this remains a topic of debate in the literature (Kierdorf et al., 2013). Microglia distribute themselves throughout the CNS and continuously scan their surroundings (Casano & Peri, 2015). Microglia share many traits with other subsets of tissue-resident macrophages, including dependence on the CSF1 receptor (CSF1R) for differentiation and survival, a requirement for PU.1 as an essential lineage-determining transcription factor (LDTF), the ability to efficiently internalize and degrade tissue debris, and quickly trigger an inflammatory response following detection of pathogens or tissue damage (Bohlen et al., 2017; Kierdorf et al., 2013). In addition to responding to injury and infection, microglia carry out functions that are specific to the CNS environment, including secretion of neurotrophic factors and participation in the developmental refinement of synaptic networks (Hong, Dissing-Olesen, & Stevens, 2016). Upon activation, microglia can acquire a range of phenotypes that can either contribute to disease or ameliorate disease progression. However, full depletion of microglia exacerbates inflammation and brain injury in mouse models (Jin et al., 2017). Although dysregulated microglia are implicated in the pathogenesis of several neurodegenerative and psychiatric conditions, the mechanisms controlling developmental, homeostatic, and pathogenic phenotypes and gene expression profiles of microglia remain poorly understood.

The microglia-mediated inflammatory response is essential for immune defense against pathogens and managing non-infectious tissue damage (X. Hu et al., 2012; Kierdorf & Prinz, 2017). Microglia are capable of a variety of inflammatory responses to

altered environmental stimuli. At the time of brain injury, microglia rapidly transform their morphology, proliferate, release pro-inflammatory cytokines and increase expression of immunomodulatory surface antigens (Xu, He, & Bai, 2016). Therefore, signals that initiate the host defense response lead microglia to substantially alter gene expression, behavior and morphology. Pro-inflammatory and anti-inflammatory tissue repair patterns of microglia activation develop in consecutive phases. These phases mirror some aspects of inflammatory regulation by other tissue macrophage populations (W. Su, Aloi, & Garden, 2016; Xu et al., 2016). Still, microglia have unique ontogeny, evolutionary roles and epigenetic exposures that distinguish them from other tissue macrophages (Durafourt et al., 2012). Faulty regulation of these phasic responses by microglia carries an enormous potential for dysfunction and destructive cascades that lead to neurotoxicity (Xu et al., 2016). Microglia within or derived from the adult CNS have not been extensively studied to date at the cellular-behavioral level, making the molecular mechanisms that regulate microglial behaviors incompletely understood.

Microglia inflammatory activity is acquiesced by inhibitory contacts with healthy neurons through the CX3CR1 or TREM2 receptors as well as by astrocyte secretion of anti-inflammatory TGF-β (Butovsky et al., 2014; Colonna & Butovsky, 2017; Faustino et al., 2019). In the diseased brain, however, microglia exhibit a sustained pro-inflammatory response, which can evolve to a chronic pro-inflammatory state. Moreover, microglia exhibit an age-related decrease in phagocytic capabilities for endogenous proteins and become less sensitive to regulatory signals (Fiala et al., 2005). Studies in young and aged retina and brain suggest that aged microglia maintain a pro-inflammatory state that ultimately impedes inflammatory resolution

(Okunuki et al., 2018). Optimal regulation of immune behaviors by microglia is crucial to adult and aged CNS health.

Several mechanisms may compromise the cessation of the inflammatory response in AD. These may include the ongoing formation of Aβ plaques and positive feedback loops between inflammation and chronic amyloidogenic -APP processing (Caldeira et al., 2017). Furthermore, accumulation of Aβ fibrils and neuronal debris that further activate receptors, establishes a non-resolving, or chronic inflammatory state (Mizuno, 2012). For example, Beclin1, an intracellular regulator of microglial function is reduced in brains of AD patients. Beclin1 has a role in retromer-mediated sorting of cellular components, like: TREM2, APP, BACE1 and CD36 (in the endolysosomal pathway) (Lucin et al., 2013). The factors that contribute to neurotoxicity and degeneration lead to chronic microglia activation. The non-cell autonomous processes in AD are rooted in microglia regulation from beneficial modulators of pathology to chronic activation of inflammatory pathways. However, a major caveat is that to be targeted for therapy, a pathway should be exclusive to microglia and not have a crucial function in other cell types. Therefore, investigating the molecular mechanisms that mediate the chronicity of the microglia mediated inflammatory response is crucial to understanding inflammation in AD.

MiRNAs in innate immune cell activation

MicroRNAs (miRNAs) are small non-coding RNAs (~22 nucleotides) involved in many physiological and pathological processes. miRNAs can reduce or enhance the response to inflammatory stimuli by altering expression of proteins involved in immune signaling pathways (Ryan M. O'Connell et al., 2012; Ryan M. O'Connell et al., 2010).

miRNA genes are most commonly transcribed by RNA polymerase II, or in some instances by RNA polymerase III (Bartel & Chen, 2004). miRNAs can be found in the introns of protein-coding genes or as independent genes. A primary transcript can contain a single miRNA or multiple miRNAs that are processed out of the same transcript. Pre-miRNAs are initially processed by Drosha and DGCR8 in the nucleus, then exported to the cytoplasm where the miRNA hairpin is cleaved by Dicer resulting in a miRNA duplex. One of the miRNA strands is loaded into the RNA-induced silencing complex (RISC) and guides this complex to the 3′ untranslated regions (UTRs) of target messenger RNAs (mRNAs) suppressing target protein expression or promoting degradation of the mRNA, depending on the completion of complementarity between the miRNA and the target sequence (Bartel & Chen, 2004; Ryan M. O'Connell et al., 2010).

Dynamic transcriptional changes occur after immune cells are initially exposed to inflammatory cues, leading to the up or down regulation of immune-response genes that coordinate the timing and amplitude of inflammatory responses. Along with changes in expression of protein-coding genes during these activation state, miRNA transcripts also change expression. Several miRNAs have been shown to change their expression levels during inflammation and do so in a variety of different immune cell types (Ryan M. O'Connell et al., 2010). Several different microRNAs are quickly upregulated during the inflammatory response in a cell-specific manner. Amongst these, miR-155 and miR-146a were originally identified as miRNAs that are upregulated by NF-κB transcriptional activation (Ryan M. O'Connell, Chaudhuri, Rao, & Baltimore, 2009; Zhao et al., 2011). Even though miRNA expression during inflammation is controlled at the transcriptional

level, there is evidence that protein regulators of the miRNA biogenesis pathways can also be affected during inflammatory responses.

Mature miRNAs expressed in immune cells and loaded into RISC preferentially target signaling proteins, transcription factors, and regulators of cell death, leading to reduced expression of these factors. These classes of proteins play influential roles in how extracellular signals are received and processed by immune cells. Therefore, reductions in their levels affect both the development and functional responses of inflammatory cells during inflammation.

As microRNAs serve to fine tune protein expression, investigators have interrogated thoroughly if small differences in protein concentrations in immune cells have a meaningful effect on physiological processes. One example of the significant impact of reductions in protein effectors is the transcription factor PU.1, a target of miR-155 (Dahl et al., 2003), although this has yet to be thoroughly investigated in microglia. Mice haploinsufficient for PU.1 show excess production of granulocytes at the expense of monocyte development. The impact of PU.1 haploinsuficiency in tissue resident macrophages, like microglia, remains a topic of study. Another example is seen in mice heterozygous for the negative signaling regulator SOCS1, also a target of miR-155, where they display a heightened sensitivity to endotoxemia (Dahl et al., 2003; Kinjyo et al., 2002). These examples indicate that the miRNA binding sites found in the 3′ UTRs of genes that serve as inflammatory regulators are functionally important for an appropriately tuned inflammatory response. The functional relevance of miRNAs expressed by immune cells during various types of inflammatory responses has

become apparent. Further work to elucidate the impact of miRNAs in inflammation and disease pathology is needed.

miR-155 and miR-146a are inflammatory microRNAs

The pro-inflammatory microRNA, miR-155 is coded by the B cell integration cluster gene (*Bic*) that was found to play a role in B cell leucosis (Faraoni, Antonetti, Cardone, & Bonmassar, 2009). MiR-155 is upregulated in myeloid cells in response to several pro-inflammatory stimuli, such as LPS, IFNγ, and TNFα (Rodriguez et al., 2007). This suggests a role for miRNAs as modulators of inflammatory activation in both microglia and macrophages. MiR-155 was shown to promote tissue inflammation by enhancing the production of Th17 cells, and it is highly induced during macrophage inflammatory responses and in multiple sclerosis lesions (Yao et al., 2012). Furthermore, miR-155 has been implicated in upregulating pro-inflammatory cytokine secretion by targeting SOCS1 and SHIP1 message for degradation and downregulating IL-10 production (Cao, Liu, Song, & Ma, 2005; Kinjyo et al., 2002; Ryan M. O'Connell et al., 2009). As a microRNA of inflammatory function, expression of miR-155 is highly regulated in immune cells.

MicroRNAs are rapidly induced in response to an environmental signal, and expression of microRNA genes and clusters can be correlated to different immune cell activation states. The Garden lab recently demonstrated that miR-155 participates in pro-inflammatory activation of microglia in a middle cerebral artery occlusion/reperfusion (MCAO/R) model of neuroinflammation (Wei Su et al., 2014). Mice deficient in miR-155 have a diminished immune response, while mice overexpressing miR-155 develop chronic-inflammatory states and hematopoietic malignancies (R. Hu et al., 2014;

Johansson, Malmhall, Ramos-Ramirez, & Radinger, 2017). We demonstrated that in microglia miR-155 targets the anti-inflammatory transcription factor c-Maf for degradation (Wei Su et al., 2014). In general, microRNAs fine tune expression of effector genes, and mature miR-155 targets mRNAs of signaling proteins, transcription factors and regulators of cell death. Although the role of miR-155 has been extensively studied in peripheral immune cells, less is known regarding the role of miR-155 in neuroinflammation or the microglia response to CNS disease.

Gaps in Knowledge

A growing number of studies have begun to highlight the significance of microglia and miRNAs in AD (Mizuno, 2012). These studies also suggest that dysfunctional miRNA regulation is associated with neurodegeneration, senescence and cellular aging. The identification of altered miRNA levels in the post-mortem AD brain compared to age matched controls raises the possibility that these epigenetic regulators influence AD pathogenesis (Pogue & Lukiw, 2018). For example, amyloid-β (Aβ) generation and degradation is regulated by miRNAs through regulation of APP mRNA, splicing and proteolytic cleavage in neurons (Tam et al., 2014). Aβ, in turn, has also been shown to impact miRNA expression levels. MiRNAs additionally regulate the degradation of Aβ in monocytes and can both directly and indirectly lead to increased Aβ brain deposition (Ponomarev et al., 2013). Mouse models of AD can additionally highlight the significance of disrupted miRNA expression *in vivo*. For example, early upregulation of miR-155 is observed in the 3XTG model of AD is observed by *in situ* hybridization, prior to development of Aβ plaques (Guedes et al., 2014). Thus, miRNAs are regulated in AD and can be dysregulated, contributing to AD pathology and alter an AD phenotype *in*

vivo. In this thesis, we have examined the role of miR-155 in modulating the microglia-mediated responses to disease associated molecular patterns (DAMPS), like fibrillar Aβ *in vitro,* and *in vivo* using mouse models of AD.

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Chapter 2

miR-155 influences expression of miR-146a, intracellular handling and internalization of fibrillar $A\beta_{1-42}$ by microglia *in vitro*

Introduction

Neuroinflammation is a central hallmark of CNS injury including traumatic brain injury and neurodegenerative diseases like Alzheimer's disease (AD; (Barrientos, Kitt, Watkins, & Maier, 2015). Microglia are the specialized population of innate immune cells within the CNS parenchyma. The microglia-mediated inflammatory response is essential for immune defense against pathogens and managing non-infectious tissue damage (Lynch, 2009; Zrzavy et al., 2018). In response to injury, microglia rapidly transform their morphology, phagocytic abilities, release pro-inflammatory cytokines and chemokines, and increase expression of immunomodulatory surface antigens (Lourbopoulos, Erturk, & Hellal, 2015). Therefore, signals that initiate the host defense response to pathogen or damage associated molecular patterns (PAMPS or DAMPS respectively) lead to microglial activation that initiates an increased neuroinflammatory environment in the CNS that may propagate injury or disease states.

Microglia initiate consecutive phases of pro-inflammatory and anti-inflammatory activation during response to injury. Microglia, like macrophages, are capable of a variety of inflammatory responses to changes in environmental homeostasis (Yamasaki et al., 2014). The macrophage pro-inflammatory response develops in response to pro-inflammatory cytokines such as interferon-γ (IFN-γ) and toll like receptor (TLR) ligands (Xu et al., 2016). Activated macrophages perform functions important for pathogen suppression such as generation of reactive oxygen species (ROS) and secretion of pro-inflammatory factors that may cause injury to neighboring cells. In contrast, anti-

inflammatory signals such as interleukins (IL)-4, -10 or -13, and transforming grown factor (TGF)-β downregulate pro-inflammatory activation, initiating an anti-inflammatory response that promotes tissue remodeling and repair (Henry, Huang, Wynne, & Godbout, 2009). Microglia expression of activation markers differs quantitatively but not qualitatively from that observed in peripheral macrophages (Zrzavy et al., 2018). Microglia respond to similar IFN-γ and TLR activation with pro-inflammatory behaviors and downregulate pro-inflammatory activation in response to IL-4, IL-10 and TGF-β. Microglia behavior during inflammation can lead to: i) loss of normal function in other glial cells that can subsequently trigger downstream damage to neurons; ii) direct neuronal toxicity; iii) participation in excessive synaptic stripping (Butovsky & Weiner, 2018). Faulty regulation of microglia phasic responses to inflammatory signals has enormous potential to cause dysfunction and destructive cascades that lead to neurotoxicity in disease. In chronic CNS diseases, microglia exhibit a sustained pro-inflammatory response, which can evolve to a long term pro-inflammatory state (Butovsky & Weiner, 2018). Moreover, in normal aging, microglia lose phagocytic capabilities and become less sensitive to regulatory signals (Lucin et al., 2013; Mitrasinovic & Murphy, 2003). Therefore, investigative efforts in the field should focus on elucidating the factors that modulate the ability of microglia to clear extracellular forms of Aβ.

A central goal of the field is to understand the key regulators of microglia behaviors and magnitude of the inflammatory response to DAMPs. These DAMPs can include exposure to amyloid-β (A*β*) fibrils or plaques that occur in AD and upregulate microglia pro-inflammatory responses. Microglia utilize microRNAs to rapidly respond to

inflammatory signals. MicroRNAs (miRNAs) are small non-coding RNAs (~22 nucleotides) involved in many physiological and pathological processes (Ryan M. O'Connell et al., 2012). A single miRNA can regulate hundreds of genes and up to 90% of human genes are potentially regulated by miRNAs. miRNAs downregulate the expression of mRNA targets by binding to complementary sequences in the 3-prime untranslated region (UTR) of the message. Several reports suggest that miRNAs play an important role in regulating the responses of innate immune cells, including microglia (Ryan M. O'Connell et al., 2012; Ryan M. O'Connell et al., 2010). Since a single miRNA can modulate expression of many genes during inflammation, miRNAs may serve as regulators of microglia inflammatory behaviors during disease.

Recently, the Garden lab demonstrated a role for miR-155, in pro-inflammatory activation of microglia in a middle-cerebral artery occlusion and reperfusion (MCAO/R) model of neuroinflammation. In this model, miR-155 targets the anti-inflammatory transcription factor c-Maf for degradation (Wei Su et al., 2014). MiR-155 is upregulated in macrophages, monocytes, and microglia in response to several pro-inflammatory stimuli, including LPS, IFNγ, and TNFα (Durafourt et al., 2012; X. Hu et al., 2012; Lynch, 2009). Furthermore, miR-155 was highly upregulated during macrophage inflammatory responses and in multiple sclerosis lesions (O'Loughlin, Madore, Lassmann, & Butovsky, 2018). miR-155 has been implicated in upregulating pro-inflammatory cytokine secretion by targeting the Suppressor of Cytokine Signaling 1 (SOCS1) message for degradation and downregulating cytokines that act via the JAK/STAT pathway (Kinjyo et al., 2002; Yao et al., 2012). Mice deficient in miR-155 have a diminished immune response, while mice overexpressing miR-155 develop chronic-

inflammatory states and hematopoietic malignancies (R. Hu et al., 2014). Therefore, miR-155 expression is a key initial driver of the microglia mediated pro-inflammatory response.

miRNAs can also suppress expression of pro-inflammatory functions to attenuate pro-inflammatory cytokine expression. A well-studied anti-inflammatory miRNA in innate immune cells is miR-146a. miR-146a acts as a negative feedback regulator of inflammatory activation to dampen NF-κB signaling by downregulating expression of the effectors TRAF6 and IRAK1 (Boldin et al., 2011). Therefore, miR-146a negatively regulates expression of pro-inflammatory cytokines, like IL-6 and TNFα. In microglia, miR-146a expression is regulated by Presenilin 2, a protein mutated in familial AD patients (Jayadev et al., 2013). Expression of both miR-155 and miR-146a is altered in the post-mortem AD brain and in mouse models of AD (Guedes et al., 2014; R. Hu et al., 2014; Zhao et al., 2011). However, the role of these miRNAs in microglia (eg. Clearance of insoluble Aβ) in AD remains largely unexplored.

Studies have shown that while miR-146a promotes anti-inflammatory functions, knock-out of miR-146a accelerates the aging phenotype, which can be abrogated by conditional deletion of miR-155 from T cells (R. Hu et al., 2014). This phenotype is not observed in miR-155 -/- and miR-146a -/- mice. Therefore, we asked if miR-155 and miR-146a expression influenced microglia responses. We hypothesized that microglia inflammatory responses to pro-inflammatory stimuli (eg, LPS, fibrillar Aβ) involve miR-155 and miR-146a induction in a manner mimicking that of peripheral cells (Figure 2.1). Here, we aimed to understand the role of miR-155, a known modulator of inflammatory responses in microglia, its connection to a second anti-inflammatory microRNA, miR-

146a, and how these two microRNAs modulate microglia phenotypic activation *in vitro* in response to Aβ. We identified a novel role of miR-155 expression in microglia. We observed that miR-155 is not only a driver of miR-146a expression but additionally is a key modulator of the kinetics of fibrillar amyloid-β (Aβ) processing pathways that lead to effective lysosomal degradation.

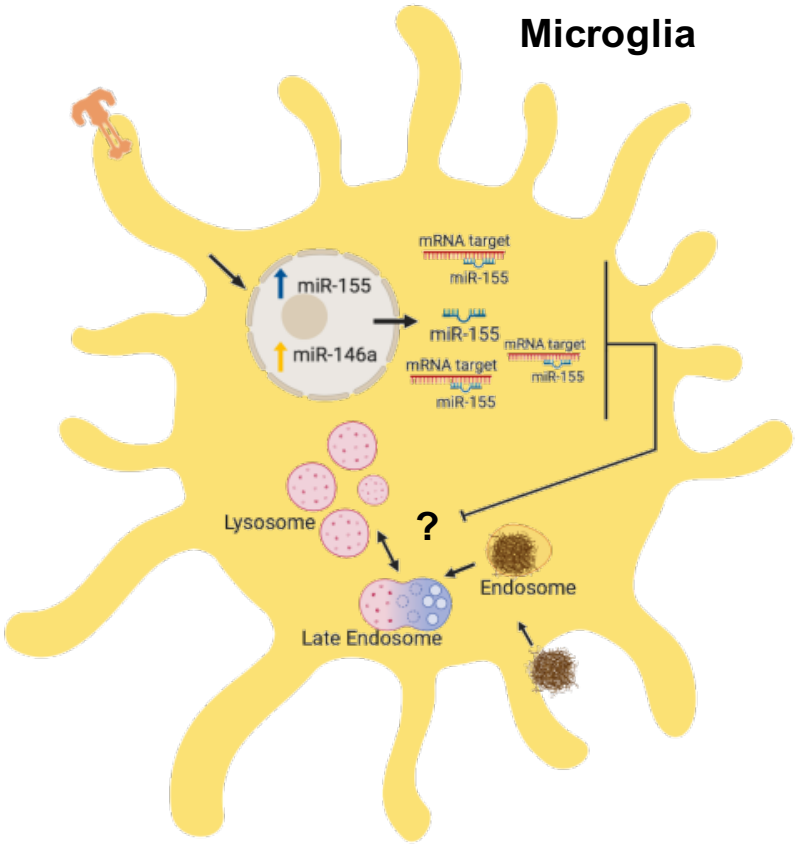

Figure 2.1 Hypothesized model *in vitro*: microglia processing of Aβ will involve modulation of the lysosomal processing pathway which is influenced by miR-155 expression.

Methods

Animals

Wild-type (WT) C57/BL6 mice were used in this study. The floxed miR-155 mice, referred to as "miR-155 flx", possess loxP sites flanking exon 2 of the mBIC gene that codes for miR-155 on both alleles. The miR-155 overexpressing animals, referred as "mBIC-FSF", contain a transgene inserted into the Rosa 26 Locus that contains a floxed stop sequence upstream of the mBIC gene. The miR-146a floxed animals, referred to as "miR-146a flx", were obtained from Li Fang at the University of California, San Diego and have been published previously (Park et al., 2012). All mice were maintained in a specific pathogen-free facility and all procedures were performed in accordance with a University of Washington IACUC-approved protocol. All mice were group-housed with *ad libitem* access to food and water on a 14/10 light dark cycle (lights on at 0600 hours).

Mixed Glia Culture Generation and Microglia Isolation

Cortices Ire dissected from P3-P4 cortex of neonates from miR-155 flx, mBIC-FSF, miR-146a flx or WT to prepare a mixed glia culture system. After isolation from the skull and removal of all meninges and deep brain structures, cortices were mechanically dissociated and enzymatically digested with trypsin (0.25%) at 37°C for 25 minutes. To generate a single cell suspension, tissue was mechanically dissociated in D10C [DMEM; Dulbecco's Modified Eagle Medium high glucose (Gibco, Life Technologies, Grand Island, NY), 10% heat inactivated horse serum (Gibco, Life Technologies), 10% nutrient mixture F-12 ham (Sigma-Aldrich, St. Louis, MO), 2mM L-glutamine (Sigma-Aldrich), 10mM HEPES (Sigma-Aldrich)], 4-6 times in a cycle of resuspension and pelleting. The collected cell suspension was plated in tissue culture grade flasks coated

with 1X poly-D-Lysine (Sigma) and allowed to seed the flask for 1-3 days. The media was then changed to fresh D10C then supplemented with 20% L929 conditioned D10C (Möller et al., 2000). Microglia were harvested 7-10 days after initial culture preparation by agitating flasks and recovering cells floating in media. Isolated microglia were then plated in poly-D-Lysine (Sigma) coated tissue culture grade plates at a density specific for experiments.

rAAV production, purification and titration

Initial studies were conducted with recombinant AAV vector serotype 2 (rAAV2) expressing Cre recombinase or Luciferase were generated according to our previously published work(W. Su, Kang, et al., 2016). In short, rAAV2-Cre or rAAV2-Luciferase were prepared by calcium phosphate transfection (CaPO4 co-precipitation) into HEK293D cells with the corresponding shuttle plasmid and pDG2 packaging capsid (for rAAV2). Co-transfection of HEK293D cells with the calcium phosphate method was carried out using pDG(*)-encoding the appropriate capsid (CAP) gene for packaging viral genome and AAV vector plasmids (two plasmid system) to produce high-titer infectious AAV . Six hours post-transfection, medium was changed to serum-free media and cultured for 48-72 hours. Cells and virus-containing medium were harvested by scraping cells off the plate and intracellular vectors were released by performing 3 freeze-thaw cycles (dry ice/ethanol then 37 ºC water bath). rAAV2 was purified by affinity chromatography using a heparin column and concentrated using 100K Amicon Ultra-4 centrifugal filters (Millipore, Billerica, MA) and tittered by qRT-PCR with primers targeting Cre or Luciferase sequence. The remaining studies were conducted with

rAAV2-Cre driven by the IE1a promoter, rather than a CMV promoter. This rAAV2-Cre was purchased form the Duke University viral generation core facility.

LPS treatment of cultured microglia, in vitro miR-155 and miR-146a manipulation and Real-time Quantitative Reverse Transcriptase PCR

For microRNA expression studies in WT microglia, cells were harvested from mixed glia cultures and plated in D10C with MCSF (D10C + MCSF; R&D) at a density of 1.0 x 10^5 cells per Ill (15mm) in 4 or 24 well plates previously coated with poly-D-Lysine. Prior to lipopolysaccharide (LPS) treatment of WT cells, existing D10C +MCSF media was replaced with Macrophage Specific Serum Free Media (M-SFM) for 18 hours. Media was then replaced with D10C + M-CSF containing 100ng/mL of LPS (Sigma Chemical). For microRNA manipulations, microglia were harvested from mixed glia cultures prepared from miR-155 flx, miR-146a flx or mBIC-FSF mice and plated in D10C with MCSF (D10C + M-CSF; R&D) at a density of 1.0 x 10^5 cells per well (15mm) in 4 or 24 Ill plates previously coated with poly-D-Lysine. One day after initial plating, media was replaced with virus-containing D10C + M-CSF at 1.0 x 10^9 vg and the infection continued for 7 days.

Cells were harvested for RNA analysis using Qiazol lysis reagent and RNA was extracted using the miRNeasy kit (Qiagen, Valencia, CA). We used the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Life Technologies) with 5' stem loop specific primers for miR-155, miR-146a and a control small nucleolar (Sno) RNA, Sno202. cDNA was generated using 15ng of total RNA from each sample. The Taqman Master mix No UNG (ABI) was used for qPCR analysis, as were the complementary primer probes for miR-155, miR-146a and Sno202 amplification. We used a standard

curve for each microRNA to quantify microRNA levels in each sample. The standard curve was generated using equivalent amounts of cDNA from mimics for miR-155 and miR-146a and total levels of transcript were calculated and quantified using the standard curve.

pHrodo-Abeta$_{1-42}$ synthesis and titration for phagocytosis assays

Lyophilized Amyloid-β_{1-42} ($A\beta_{1-42}$) Protein (Bachem) was resuspended in sterile, endotoxin-free 1X PBS in a sterile, tissue-culture environment to achieve a concentration of 100 μM of peptide. The resuspended peptide left to aggregate and fibrilize for 48 hours at 37°C. The pH-sensitive dye, pHrodo (Invitrogen) was used to label the fibrillar Aβ (fAβ-pHrodo). fAβ was labeled following the manufacture's guidelines for use of the dye. Alexa 488, a non-pH sensitive dye, was used to label fAβ (fAβ-488). fAβ-488 was generated using an aliquot of 100uM fibrilized Aβ and following the Alexa 488 protein labeling kit instructions (Thermo) for labeling large proteins. The labeled forms of Aβ were aliquoted and stored at -80°C.

Cells were plated at a density of 1.0 x 10^5 cells per 15mm well. Cells were exposed to a range of fAβ-pHrodo and fAβ-488 (5 μM, 1μM, 500nM, 250nM) to determine the quantity of fAβ to be used for each phagocytosis assay experiment. Data were analyzed using FlowJo v10 and the average geometric mean of fAβ-pHrodo (PE) and fAβ-488 (FITC) intensity reflect the extent of phagocytosis or internalization, respectively. We chose to use 1uM fAβ-pHrodo and fAβ-488 for all assays since we observed a minimum of 70% phagocytosis or fluorescence intensity of each peptide preparation.

Phagocytosis assay as a measure of microglia behavior post LPS stimulation or microRNA manipulation

Microglia were harvested from mixed glia cultures and plated at a density of 1.0 x 10^5 cells/15mm lll or 2.0 x 10^4 cells per 96 well plate. After 7 days of AAV2-Cre infection in D10C + M-CSF, media was aspirated and replaced with D10C + M-CSF with 1 μM of fAβ-pHrodo or fAβ-488 for 6, 12, 18, or 24 hours. Cells treated with LPS (100 ng/mL) were treated throughout the duration of the time course (maximum of 48 hours). After the end of the time course and in both experimental paradigms, media was aspirated away from cells, and cells were trypsinized with phenol red-free 0.25% trypsin (Gibco) for 5 min at 37°C. After neutralizing the trypsin with equal volumes of FACS (HBSS -/-, 1mM Hepes and 10% heat-inactivated FBS) media, cells were stained with DAPI (1ng). Levels of fAβ-pHrodo or fAβ-488 were detected in each sample using the LSR II (BD Bioscience) or the Amnis Imaging Flow Cytometer (Millipore). Data were analyzed using FlowJo v10 and the average geometric mean of fAβ-pHrodo (PE) and fAβ-488 (FITC) intensity reflect the extent of phagocytosis or internalization, respectively.

Results

Expression of miR-155 is quickly upregulated in microglia exposed to LPS.

MiR-155 expression in blood derived macrophages is tightly regulated and is an outcome of TLR/NF-kB signaling, ultimately promoting the pro-inflammatory phase of inflammation. We tested if miR-155 expression is upregulated in response to LPS stimulation and what the time course of this expression is in mouse primary cultured microglia. We took mouse primary neonatal microglia isolated from mixed glia cultures, then stimulated them with 100 ng/mL of LPS over 5 days (Figure 2.2 A). qPCR was performed from RNA extracted from cells at each day to detect miR-155 and miR-146a induction. We observed a fast and significant upregulation of miR-155 after one and two days of LPS treatment that slowly decreased over a five-day period (Figure 2.2 B, Ordinary One-way ANOVA with Bonferroni's multiple comparisons. For miR-155: Day 1: $p<0.0001$, Day 2: $p<0.0001$, Day 3: $p<0.01$, Day 4 and 5: not significant (n.s.). For miR-146a: Day 1: n.s, Day 2: n.s., Day 3: n.s., Day 4: $p<0.005$, Day 5: $p<0.005$). In contrast, miR-146a expression slowly increased during the first three days post LPS treatment. miR-146a upregulation peaked and remained constant at 4 and 5 days of LPS exposure, during a period of miR-155 downregulation. The relative ratios of miR-155 to miR-146a show the degree of change of each microRNA (Figure 2.1 C). These findings demonstrate that LPS induction of miR-155 and miR-146a expression in microglia recapitulates the expected timing of induction in other immune cells (blood derived macrophages) and supports the hypothesis that in microglia, miR-155 participates in a TLR signaling mediated induction of pro-inflammatory responses while miR-146a is activated later when its anti-inflammatory function should begin.

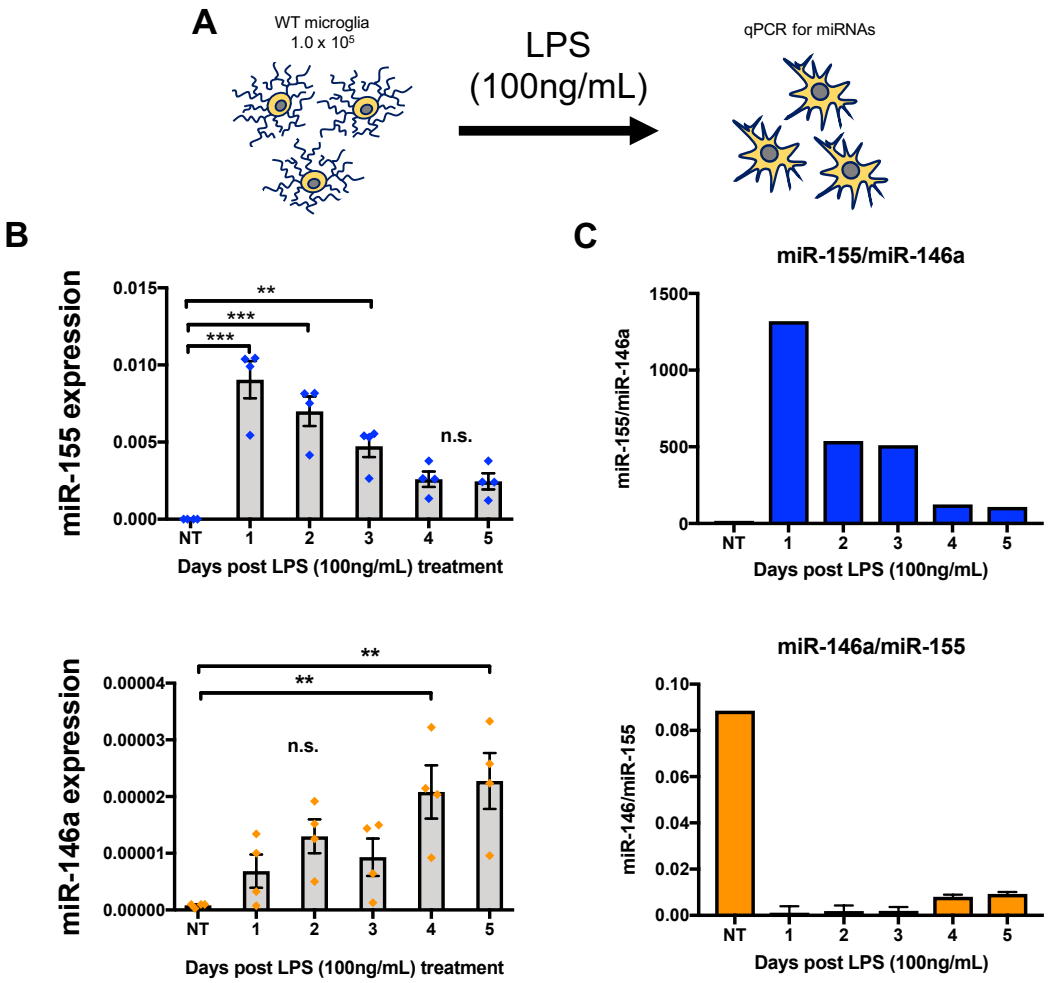

Figure 2.2: Microglia respond to LPS with phased induction of miR-155 and miR-146a similar to macrophages. A) Primary mouse neonatal microglia were plated and exposed to 100ng/mL of LPS, then total RNA was extracted and analyzed for changes in microRNA expression **B)** Quantification of miR-155 and miR-146a expression over time post LPS exposure (Ordinary One-way ANOVA with Bonferroni's multiple comparisons. For miR-155: Day 1: $p<0.0001$, Day 2: $p<0.0001$, Day 3: $p<0.01$, Day 4 and 5: not significant (n.s.). For miR-146a: Day 1: n.s, Day 2: n.s., Day 3: n.s., Day 4: $p<0.005$, Day 5: $p<0.005$). C) Relative changes of miR-155 to miR-146a plotted as a ratio.

In the absence of a strong pro-inflammatory stimulus, miiR-155 overexpression is sufficient for miR-146a induction in mouse primary cultured microglia.

We next asked if miR-155 up-regulation is sufficient to induce expression of miR-146a in the absence of a strong inflammatory stimulus (LPS). We infected neonatal cultured microglia harvested from mice harboring a flox-stop-flox mBIC transgene with rAAV2-Cre for five days. When these cells are express Cre-recombinase, the stop codon is excised, resulting in overexpression of miR-155 (Figure 2.3 A). We employed real time (RT)-qPCR to quantify changes in expression of both miR-155 and miR-146a. We observed that miR-155 overexpression was sufficient to significantly induce miR-146a expression in primary cultured microglia (Ordinary One-way ANOVA with Tukey's multiple comparisons, $p<0.0001$, Figure 2.3 B and C). These findings further support the hypothesis that miR-155 is a determining factor of the coordinated miRNA component of the response to TLR activation in microglia, since exogenous induction of miR-155 alone is sufficient to induce miR-146a.

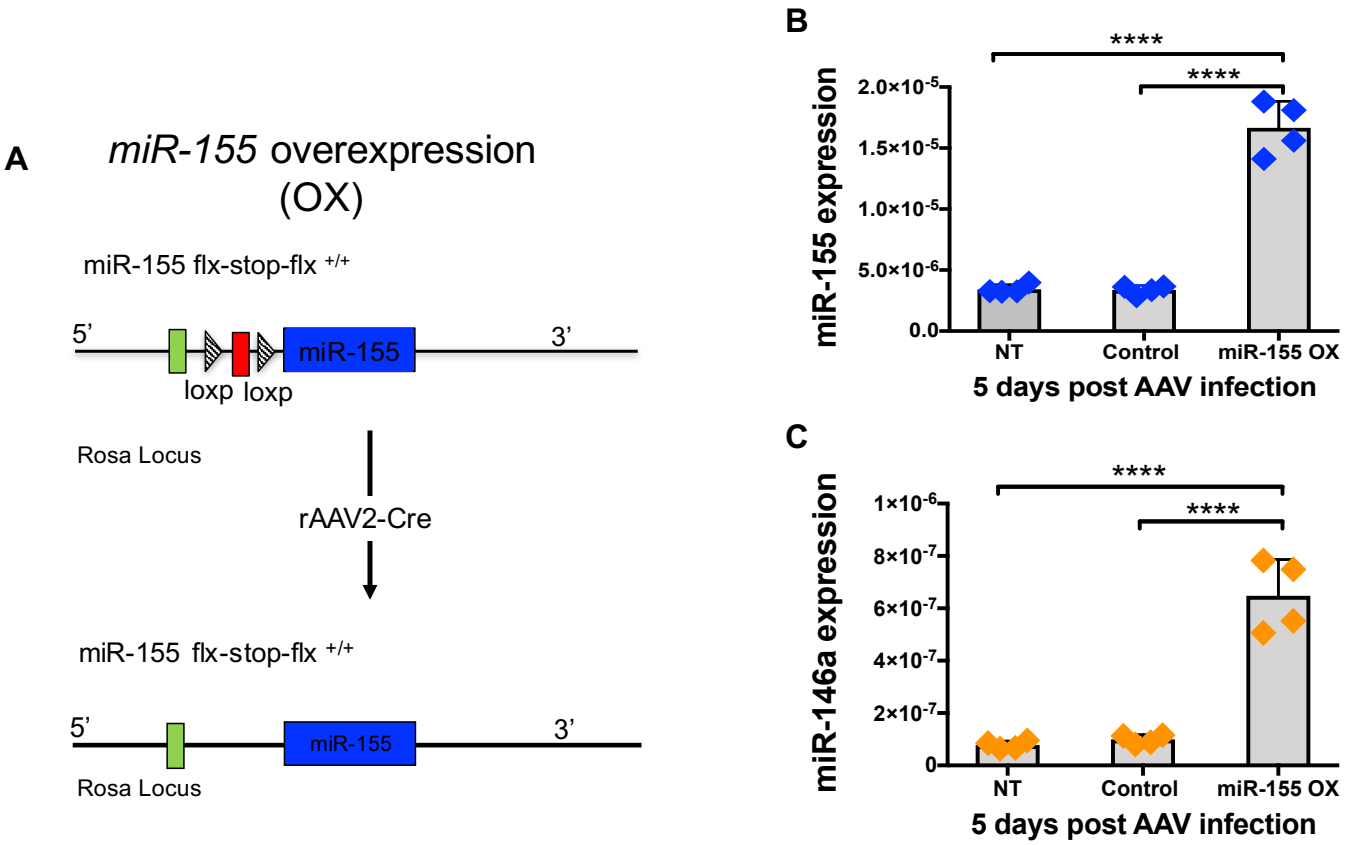

Figure 2.3: **In the absence of a strong inflammatory stimulus, overexpression of miR-155 is sufficient to induce expression of miR-146a**. A) mBIC-FSF neonatal microglia were plated and treated with rAAV2-Cre for 5 days. Then total RNA was extracted and analyzed for changes in microRNA expression (Ordinary One-way ANOVA with Tukey's multiple comparisons, $p<0.0001$ for Figure 2 B) and C) Quantification of miR-155 overexpression and miR-146a levels as a result of miR-155 overexpression.

MiR-155 and miR-146a are induced by fAβ exposure

We observed that LPS stimulation leads to induction of miR-155 expression in microglia. Since $fA\beta_{1-42}$, like LPS, binds to the TLR4 receptor, we were interested in determining how $fA\beta_{1-42}$ exposure would influence expression of miR-155 or miR-146a. We asked if expression of miR-155 and miR-146a was modified by exposing cells in culture to varying concentrations of $fA\beta_{1-42}$ (1μM, 5 μM or 10 μM). We observed that

changes in expression of miR-155 occurred after cells were exposed to 10 μM concentrations of $fA\beta_{1-42}$, but remained unchanged with lower concentrations of $fA\beta_{1-42}$ (Appendix Figure 1). This observation, although not statistically significant, suggests a trend that high concentrations of $fA\beta_{1-42}$ may also modulate expression of miR-155 and miR-146a in microglia.

Pro-inflammatory activation alters levels of fibrillar $A\beta_{1-42}$ in low-pH compartments in microglia

One measurable phenotype of activated microglia is the extent of phagocytic ability after exposure to different disease or damage associated molecular patterns (DAMPS). In AD, microglia cluster around plaques and can internalize and degrade fibrillar forms of $A\beta_{1-42}$ ($fA\beta_{1-42}$) as part of a tissue reparative activation state (He, Luo, Shen, et al., 2016; He, Luo, Yang, et al., 2016; Koenigsknecht-Talboo & Landreth, 2005; Ma et al., 2013; Weldon et al., 1998). In addition, several microRNAs have been shown to indirectly modulate levels of Aβ in monocytes from patients with AD (Tiribuzi et al., 2014). Since miR-155 drives microglia pro-inflammatory activation states, we hypothesized that pro-inflammatory activated cells would phagocytose less $fA\beta_{1-42}$. To test the hypothesis that pro-inflammatory activated microglia are less likely to contain internalized protein in low-pH compartments for degradation (lysosomal pathway) we designed an assay (Figure 2.4 A) where stimulated cells are exposed to $fA\beta_{1-42}$ tagged with a pH sensitive rhodamine ($fA\beta_{1-42}$-pHrodo), which fluoresces once it is localized to low-pH compartments. After stimulating cells with LPS for 24 hours, when we had observed the highest level of miR-155 induction, we exposed cells to 1uM $fA\beta_{1-42}$-pHrodo. After 24 hours of LPS (100ng/mL) exposure, microglia showed a significant

reduction in fAβ_{1-42}-pHrodo localized in lysosomal compartments 6 hours after initial exposure to fAβ_{1-42}-pHrodo. This was compared unstimulated cells also exposed to fAβ_{1-42} (Welche's t-test, $p<0.001$, Figure 2.4 B).

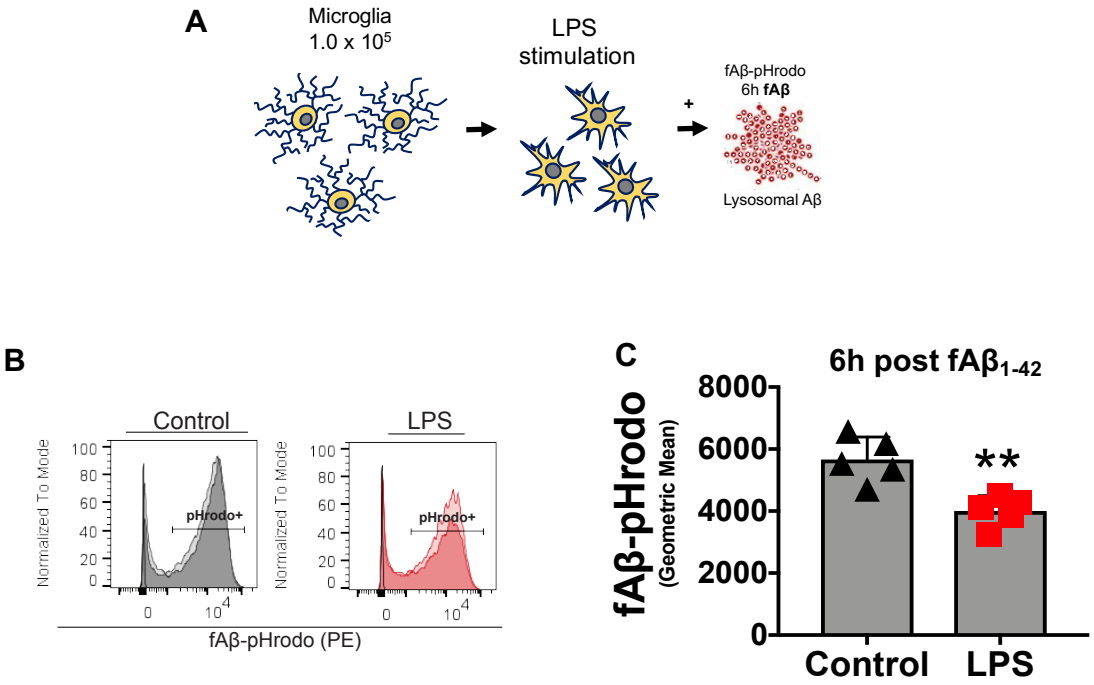

Figure 2.4: miR-155 induction by LPS influences microglia lysosomal transit of fibrillar Aβ_{1-42}. A) Wild-type (WT) microglia were plated and treated with 100ng/mL of LPS for 24 hours. **B)** Then cells were treated with 1uM for 6 hours of fAβ_{1-42}. Lysosomal fAβ levels were detected using flow cytometry (LSRII) and data were analyzed using FlowJo and Prism7 (Welche's t-test, $p<0.001$).

Finally, we wanted to preliminarily assess if the observed differences in fAβ_{1-42}-pHrodo levels in low-pH compartments stemmed from a reduction in total low-pH compartments. After treating cells with LPS for 24 hours, cells were stained with Lysotracker-Deep Red (647) to allow for the visualization of low-pH compartments by flow cytometry. There was no significant difference in the total signal of Lysotracker Deep Red after LPS treatment in the same time frame as the fAb$_{1-42}$ internalization

(Appendix Figure 2). Therefore, this change in intracellular handling of fAβ_{1-42}-pHrodo signal is unlikely to be secondary to an effect of LPS treatment on the total volume of low-pH compartments.

miR-155 expression alters levels of fibrillar Aβ_{1-42} in low-pH compartments in microglia

Upregulation of miR-155 in response to a pro-inflammatory stimulus leads to a pro-inflammatory activation state resulting in cytokine production and reduction in the phagocytic, tissue-reparative phenotypes (R. M. O'Connell, Taganov, Boldin, Cheng, and Baltimore (2007). However, the effect of miR-155 expression on the tissue-reparative phenotypes of microglia in response to fAβ_{1-42} remains largely unexplored. We asked whether conditional deletion of miR-155 would increase intracellular handling of fAβ_{1-42} to low-pH compartments in microglia. We hypothesized that deletion of a pro-inflammatory driver of microglia activation would upregulate microglia anti-inflammatory phenotypes and intracellular processing of fAβ_{1-42} from the extracellular space. To test this hypothesis, we infected miR-155 flx or mBIC-FSF primary microglia with rAAV2-Cre for 7 days to conditionally knock-out or conditionally overexpress miR-155, respectively. On day 7, We exposed cells to 1uM fAβ_{1-42}-pHrodo for 6 hours (Figure 2.5 A). Using flow cytometry, we observed that conditional deletion of miR-155 in microglia lead to a significant increase of fAβ_{1-42}-pHrodo signal in low-pH compartments. In turn, we observed that miR-155 overexpression lead to decreased fAβ_{1-42}-pHrodo in low-pH compartments (Figure 2.5 B-C, One-way ANOVA with Sidak's multiple comparisons, $p<0.0001$). This result suggests a new role for miR-155 in modulation of intracellular processing of fAβ_{1-42} by microglia.

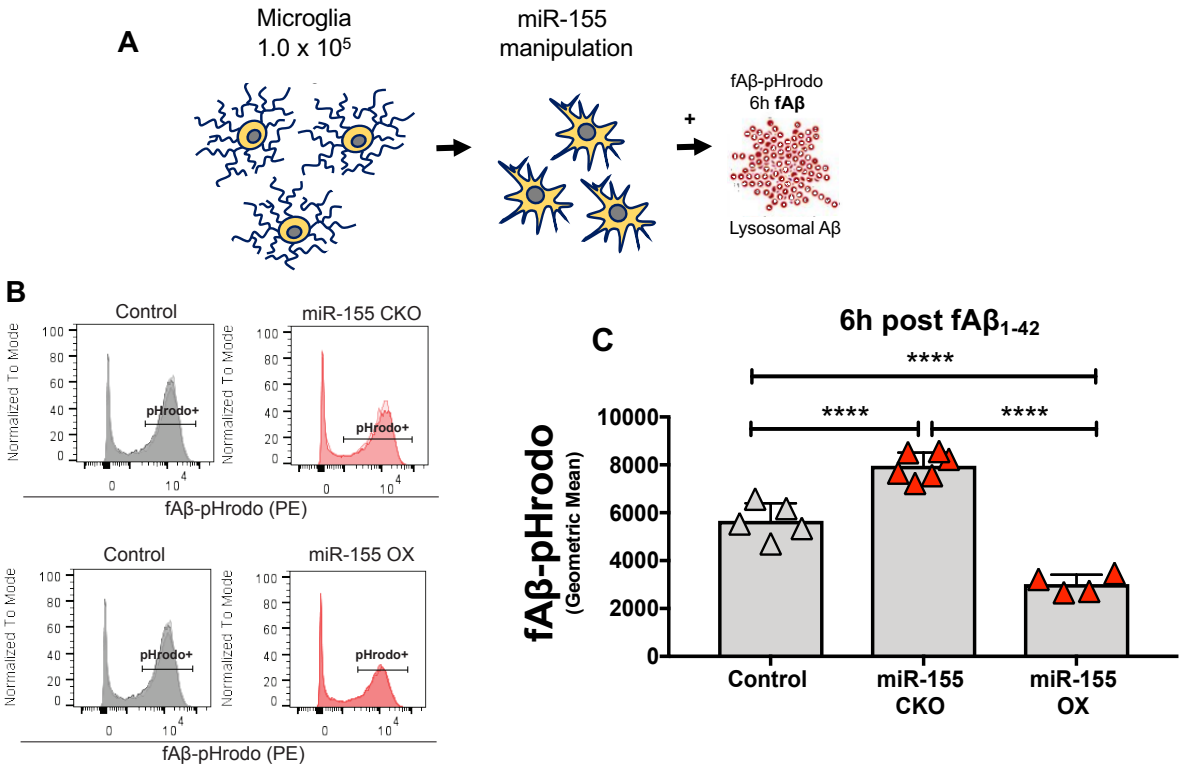

Figure 2.5: miR-155 influences microglia lysosomal transit of fA$\beta_{1\text{-}42}$. A) miR-155 $^{flx/flx}$ or mBIC-FSF microglia were plated and infected with rAAV2-Cre for 7 days. **B)** Then cells were treated with 1uM for 6 hours of fA$\beta_{1\text{-}42}$. Lysosomal fAβ levels were detected using flow cytometry (LSRII) and data were analyzed using FlowJo and Prism7 (Ordinary One-way ANOVA with Sidak's multiple comparisons, $p<0.0001$).

LPS stimulation in primary microglia, or miR-155 manipulation alone, modifies intracellular handling of A$\beta_{1\text{-}42}$ to at the plasma membrane and low-pH compartments

We observed that miR-155 influences intracellular handling of fA$\beta_{1\text{-}42}$ to low-pH compartments. We then asked if expression of miR-155 modified the intracellular handling of fA$\beta_{1\text{-}42}$ to low pH compartments or entry via the plasma membrane. We hypothesized that total internalization from the extracellular space and shuttling of protein to low-pH compartments would be decreased compared to untreated cells. We

designed a separate assay where microglia were exposed to either $fA\beta_{1-42}$-pHrodo or $fA\beta_{1-42}$ tagged with a non-pH sensitive fluorophore ($fA\beta_{1-42}$-488, Figure 2.6 A). To initially test this hypothesis, we stimulated WT cells with LPS (100 ng/mL) for 24 hours, at the point of highest induction of miR-155 we had observed. Next, we exposed cells to $fA\beta_{1-42}$-pHrodo or $fA\beta_{1-42}$-488 for 6, 12,18, and 24 hours (Figure 2.6 B and C). Cells were then analyzed by flow cytometry. The geometric mean of each fluorophore was calculated from the population of cells and represents the signal from total internalized $fA\beta_{1-42}$ or $fA\beta_{1-42}$ in low-pH compartments. Then, the average geometric mean of each signal was normalized to levels of $fA\beta_{1-42}$-pHrodo or $fA\beta_{1-42}$-488 of untreated (WT) microglia. We observed that LPS-stimulated microglia show a reduction $fA\beta_{1-42}$ in low-pH compartments over time (Figure 2.6 B, filled black triangles. 2-way ANOVA, significant differences of main effect in time, genotype and interaction $p<0.0001$). In turn, $fA\beta_{1-42}$-488 signal was increased in LPS stimulated microglia only at 24 hours (Bonferroni multiple comparisons, $p<0.05$, Figure 2.6 C, filled black triangles). These data suggest that in microglia, LPS treatment leads to downregulation of protein processing via lysosomal pathways even though total cellular internalization of $fA\beta_{1-42}$ increases at later times.

Similarly, since we observed that miR-155 influences levels of $fA\beta_{1-42}$ in low-pH compartments, we asked 1) if miR-155 only contributed to $fA\beta_{1-42}$ internalization by microglia into low-pH compartments or 2) was there an impairment in $fA\beta_{1-42}$ handling after miR-155 manipulation at plasma membrane. We hypothesized that miR-155 expression influences microglia internalization and intracellular handling of $fA\beta_{1-42}$ over time, where conditional miR-155 overexpression contributed to impairments of this

process, and conditional miR-155 knock-out would upregulate pathways involved in intracellular handling. To test this hypothesis, we infected miR-155 flx or mBIC-FSF cells with rAVV2-Cre for 7 days to conditionally delete or overexpress miR-155, respectively. On day 7 of infection, we exposed microglia to $fA\beta_{1-42}$-pHrodo or $fA\beta_{1-42}$-488 for 6, 12, 18, or 24 hours, then (as before) measured the levels of $fA\beta_{1-42}$-pHrodo or $fA\beta_{1-42}$-488 using flow cytometry. Cells were then analyzed by flow cytometry. The geometric mean of each independent fluorophore was calculated to represent the total then normalized to levels of $fA\beta_{1-42}$-pHrodo or $fA\beta_{1-42}$-488 of untreated (WT) microglia.

We observed that conditional knock-out of miR-155 in microglia lead to fast intracellular handling of $fA\beta_{1-42}$-pHrodo over time, with a significant increase of $fA\beta_{1-42}$-pHrodo signal in low-pH compartments at 6 hours, compared to untreated cells, miR-155 overexpressing cells, as well as cells treated with LPS (red-filled triangles, 2-way ANOVA, multiple comparisons (Bonferroni) $p<0.0001$, Figure 2.6 B). Interestingly, with conditional knock-out of miR-155, we did not observe deficits of internalization at the plasma membrane, since miR-155 knock-out resulted in significantly increased $fA\beta_{1-42}$-488 internalization at 6 and 12 hours (green-filled triangles, 2-way ANOVA, Bonferroni multiple comparisons $p<0.0001$, Figure 2.6 C) compared to untreated cells, miR-155 overexpressing cells, and cells treated with LPS. At 18 hours, there was a significant decrease in $fA\beta_{1-42}$-pHrodo signal in mR-155 overexpressing cells and cells stimulated with LPS, compared to miR-155 conditional knock-out microglia and untreated WT microglia. Conditional overexpression of miR-155 lead to a significant increase in $fA\beta_{1-42}$-488 internalization at 6 hours compared to untreated WT cells, similarly to the phenotype observed with LPS stimulation. In microglia, miR-155 overexpression did not

result in a statistically difference in total internalization as compared to LPS treated cells. Interestingly, conditional knock-out of miR-155 in microglia resulted in a statistically increased internalization phenotype compared to untreated cells, miR-155 overexpressing cells, and LPS treatment. These data suggest a new role of miR-155 in $fA\beta_{1\text{-}42}$ intracellular processing, where conditional knock-out of miR-155 leads to increases in intracellular handling of $fA\beta_{1\text{-}42}$ and the impairments resulting from miR-155 induction or overexpression do not result in deficits in internalization of $fA\beta_{1\text{-}42,}$ but of intracellular handling.

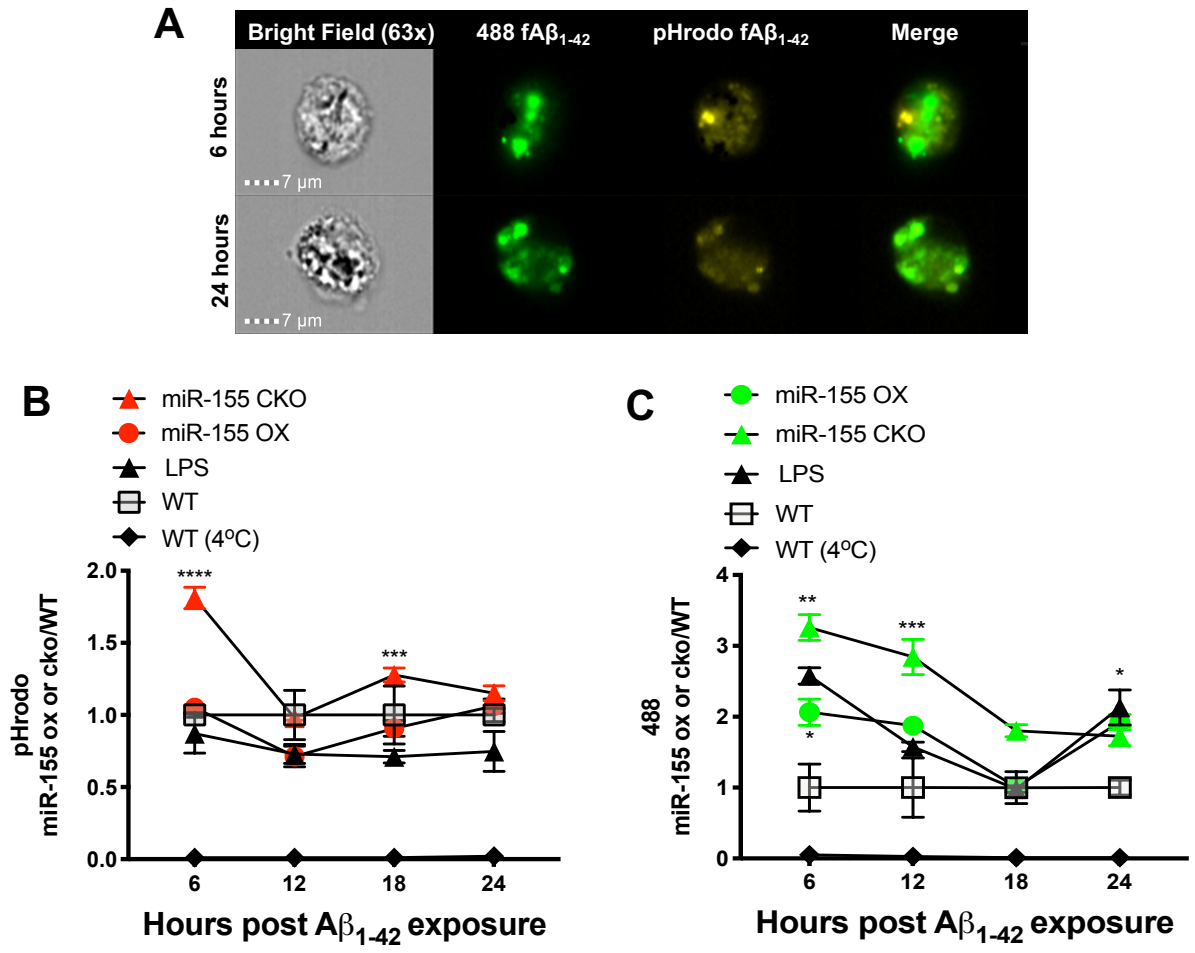

Figure 2.6: LPS stimulation in primary microglia, or miR-155 manipulation alone, modifies extracellular processing of $A\beta_{1-42}$ to low-pH compartments and at the plasma membrane. A) Representative images of WT microlgia exposed to $fA\beta_{1-42}$-pHrodo or $fA\beta_{1-42}$-488 at 6 hours (where fluorophores do not colocalize) and 24 hours (where fluorophores colocalize). B) Microglia (WT, miR-155 flx, or mBIC-FSF) were plated infected with rAAV2-Cre for 7 days or treated with 100ng/mL of LPS (WT only) for 24 hours. Then, cells were treated with 1uM of $fA\beta_{1-42}$ tagged with a pH sensitive rhodamine or C) a non-pH sensitive fluorophore for 12, 18, and 24h. Total internalization of $fA\beta_{1-42}$ or $fA\beta_{1-42}$ in low-pH compartments was detected using flow cytometry (LSRII) and data were analyzed using FlowJo and Prism7 (Two-way ANOVA with Bonferroni multiple comparisons *** = p<0.001; ** = p<0.01, * = p<0.05).

Discussion

Neuroinflammation is a hallmark of progressive neurodegenerative disease. Microglia play a central role in orchestrating the inflammatory environment in the brain in response to injured and apoptotic neurons and other DAMPs. During AD pathogenesis, different species of Aβ are produced, aggregate, and are a principal component of the plaques that are hallmark of disease pathology. Adult microglia, like peripheral immune cells, can quickly respond to infection, cell damage, and inflammatory signals such as soluble and aggregated forms of Aβ. We identified a novel role for miR-155, a microRNA driver of pro-inflammatory responses, in internalizing and handling fibrillar forms of $A\beta_{1-42}$ via promotion of the endolysosomal degradation pathways. We hypothesized that miR-155 not only modified expression of an anti-inflammatory microRNA, miR-146a, but that expression of pro-inflammatory miR-155 downregulated microglia intracellular handling of $A\beta_{1-42}$ *in vitro* (Figure 2.7). We found that conditional knock-out of miR-155 altered the ability of microglia to internalize fibrillar forms of $A\beta_{1-42}$ into lysosomal compartments. In turn, overexpression of miR-155 decreased this process, suggesting a role for miR-155 in modulating gene expression networks influencing lysosomal function in addition to inflammatory gene expression. These data increase the understanding of the complex patterns of expression and interactions between pro- and anti-inflammatory miRNA-regulated pathways and their quick upregulation or downregulation in response to LPS or Aβ.

Microglia interactions with $A\beta_{1-42}$ in AD pathogenesis

Microglia are uniformly distributed in the brain and continuously survey their immediate environment for pathogens, foreign materials and apoptotic cells. Microglia inflammatory activity is silenced by inhibitory contacts with healthy neurons through the

CX3CR1 or TREM2 receptors as well as by astrocyte secretion of anti-inflammatory TGF-β (Butovsky et al., 2014; Colonna & Butovsky, 2017; Faustino et al., 2019). Upon injury, microglia rapidly extend processes to the site of injury, then migrate to the lesion sites, recognize the pathogen, and release pro-inflammatory cytokines and increase expression of immunomodulatory surface antigens that lead to pro-inflammatory states (Xu et al., 2016). Therefore, signals that initiate the host defense response lead microglia to substantially alter gene expression and behavior. Pro-inflammatory and anti-inflammatory tissue repair patterns of microglia activation develop inconsecutive phases. These phases mirror some aspects of inflammatory regulation by other tissue macrophage populations (W. Su, Aloi, et al., 2016; Xu et al., 2016). Faulty regulation of these phasic responses by microglia carries an enormous potential for dysfunction and destructive cascades that lead to neurotoxicity (Xu et al., 2016). In this work we identified a novel role for a known driver of inflammation in modulating microglia response phenotypes.

Several mechanisms are involved in the inflammatory response in AD. These may include the ongoing formation of $A\beta_{1\text{-}42}$ aggregates that contribute to the positive feedback loops between inflammation and APP processing. In the brains of AD patients, and in AD mouse models, microglia are found closely associated with the amyloid plaques and exhibit an 'activated' pro-inflammatory phenotype. Several studies using radiolabeled or fluorescent labeled A*β* (Paresce et al. 1996) and direct injection of fibrillar A*β* into rat brains further demonstrated the capability of microglia to internalize A*β*. Here we identified a novel role for miR-155 expression in microglia regulation of internalization and lysosomal transport of $A\beta_{1\text{-}42}$. We found that miR-155 was

upregulated quickly in response to pro-inflammatory stimuli (LPS) and to A$\beta_{1\text{-}42.}$ In turn, conditional deletion of miR-155 leads to microglia upregulation of A$\beta_{1\text{-}42}$ transit into the low pH endosome/lysosomal compartment. Continued accumulation of Aβ fibrils and neuronal debris that further activate inflammatory cell signaling pathways, establishes a non-resolving chronic inflammatory state (Mizuno, 2012). An intracellular regulator of microglial function, Beclin1, is reduced in brains of AD patients. Beclin1 has a role in retromer-mediated sorting of cellular components, like: TREM2, APP, BACE1 and CD36 (in the endolysosomal pathway) (Lucin et al., 2013). These receptors and co-receptors are modulated or predicted to be modulated by miR-155 expression (R. S. Huang, Hu, Lin, Lin, & Sun, 2010; Jay et al., 2015; Lucin et al., 2013). Further work is required to understand the mechanism by which miR-155 regulates expression of receptors known to interact with Aβ fibrils, contributing to AD pathology and microglia activation phenotypes.

Microglia pro-inflammatory and anti-inflammatory activation

The role of miRNAs in inflammation and neurological disease has been extensively studied in cell types other than microglia, like T-follicular helper cells and peripheral macrophages. In these studies, it was shown that miR-146a acts as a negative regulator of inflammation by suppressing NF-kB transcriptional activity, miR-155 acts to potentiate the pro-inflammatory response. Monocytes that overexpress miR-146a showed a dampened inflammatory response, and TRAF-6 and IRAK-1 are the major miR-146a targets that mediate this effect (Boldin et al., 2011). In addition, miR-146a was also shown to negatively regulate expression of pro-inflammatory cytokines, like IL-6 and TNF-a. In contrast, miR-155 enhances pro-inflammatory responses by

suppressing expression of anti-inflammatory proteins and may act as a negative feedback regulator of miR-146a. MiR-146a expression was downregulated in primary Presenilin 2 knock-out microglia while NF-kB signaling activity was increased, as detected using a luciferase reporter assay (Jayadev et al., 2013). Although these studies have shown a clear role of miR-155 and miR-146a in inflammatory regulation, the role of microRNA regulation in modifying cellular functions in response to AD related DAMPS remained unexplored in microglia.

Altered expression of miRNAs leads to changes in gene expression that contribute to disease states in cancer, metabolic disorders, and neurological abnormalities (Kiko et al., 2014; Mendell & Olson, 2012; Pogue & Lukiw, 2018; Ponomarev et al., 2013). While up-regulated miRNAs have the potential to reduce the transcript levels of their target mRNAs on a genome-wide scale, in disease processes only certain mRNAs are preferentially affected in their expression patterns. Therefore, the targeting and modulation of microRNAs is a potential approach for therapeutic development. In the AD brain, expression of microRNAs in specific functional groups is significantly upregulated, including inflammatory microRNAs miR-155 and mIR-146a. However, the specific cell types contributing altered miRNA expression in bulk CNS samples and the physiological implications this upregulation in the disease processes remain largely unexplored. We show that in primary microglia, miR-155 and miR-146a are interconnected regulators, acting together in modulation of different stages of the innate immune response to PAMPS (LPS) or DAMP (Aβ), like blood-derived macrophages (Elton et al., 2013; O'Connell et al., 2010). In addition, we identified a

novel role for miR-155, demonstrating that miR-155 expression is involved in catabolism of fibrillar A$\beta_{1\text{-}42}$.

Targeting the behavior of microglia has been suggested as a potential novel therapeutic strategy for modulating the progression of neurodegenerative disorders. Microglia function could be directed toward clearance accumulated Aβ, suppressing autoimmune attacks on the CNS, fighting CNS viral infection, or promoting tissue repair functions rather than neurotoxic inflammation in response to acute CNS injury or neurodegenerative diseases. Given the important roles for miRNAs in regulating gene expression during inflammation, we hypothesize that the power of miRNAs could be harnessed to influence microglia activity and modulate the neuroinflammatory response in CNS disease. Here, we showed that conditional expression of miR-155 in microglia *in vitro* is sufficient to alter expression of an anti-inflammatory miR-146a and to skew the protein-processing functions of microglia. Deletion of miR-155 leads to an increased proportion of A$\beta_{1\text{-}42}$ into lysosomal compartments, suggesting that miR-155 expression can modulate targets associated with degradation of Aβ.

Using appropriate gene therapy tools, miRNA modulation could be a strategy to fine-tune the immune response, skewing microglia behavior according to the specific requirements for each disease setting. We further suggest that miR-155 and miR-146a are two specific miRNA molecules with demonstrated roles in microglia that are likely to serve as important future biomarkers of disease and potential therapeutic targets in several CNS disorders. Further pre-clinical work should focus on elucidating the role of these microRNAs *in vivo* in models of disease.

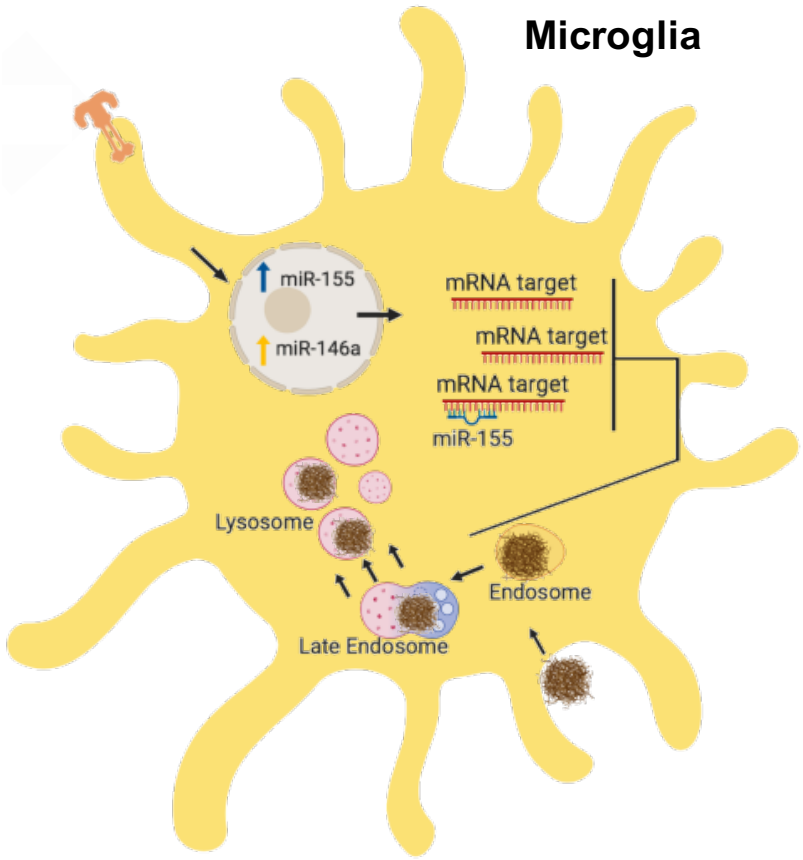

Figure 2.7: Model. Summary of findings: 1) microglia response to pro-inflammatory stimuli includes modulation of mIR-155 and mIR-146a, like in peripheral macrophages. 2) Microglia clearance of $fA\beta_{1\text{-}42}$ involves modulation of intracellular processing pathways are influenced by miR-155 expression. Conditional knock-out of miR-155 leads to increases in intracellular handling of $fA\beta_{1\text{-}42}$ and the impairments resulting from miR-155 induction or overexpression do not result in deficits in internalization of $fA\beta_{1\text{-}42,}$. This is not a deficit at the plasma membrane, but a change in intracellular handling of $fA\beta_{1\text{-}42}$.

Chapter Conclusions and Future Directions

Further understanding of the signals that lead to microglia activation, and regulation from beneficial to detrimental, lies in understanding the modulation of inflammatory pathways. We identified a role for miR-155, an inflammatory microRNA, in modulating expression of an anti-inflammatory miR-146a in microglia. In addition, We found that conditional deletion or overexpression of miR-155 altered the ability of microglia to transfer internalized Aβ to lysosomal compartments after internalization, an important pathway that leads to clearance of pathogenic forms of Aβ in AD. Therefore, investigating the molecular and epigenetic regulators that mediate the chronicity of the microglia mediated inflammatory response is crucial to understanding in AD pathology. Future studies should focus on investigating the functions of unexplored targets of miR-155, modifiers of lysosome biogenesis and composition (like TFEB/C family of transcription factors), the chronicity of miR-155 regulating effectors involved in the transfer of Aβ to lysosomal compartments, and receptors for Aβ (like CD36, TREM2). Overall, improved understanding of the role of miR-155 in microglia and its impact in Aβ processing can enrich our understanding of the pathways that contribute to Alzheimer's disease pathology.

Chapter 3

miR-155 expression in microglia modulates inflammation and pathology *in vivo* in mouse models of AD

Introduction

Alzheimer's disease (AD) is the most common form of age-related dementia and is characterized by progressive memory loss and cognitive decline. AD disease progression begins decades before diagnosis which results in a valuable window for therapeutic intervention before irreversible neurodegenerative changes and consequent cognitive loss occur. However, since AD etiology involves multiple genetic, epigenetic and environmental factors, our current understanding of AD pathogenesis does not allow for the design of preventative or disease modifying therapeutics.

Pathologically, AD is characterized by the accumulation of extracellular Amyloid-β (Aβ) plaques and CNS and systemic inflammation. Mutations in the three genes – the Amyloid Precursor Protein (*APP*), Presenilin 1 (*Psen1*) and Presenilin 2 (*Psen2*) – lead to autosomal dominant familial forms of AD (FAD; <60 years of age), which overlaps clinically and pathologically with sporadic presentations of late onset AD (LOAD; >60 years of age). The protein products from FAD genes contribute to APP processing into secreted Aβ peptides that accumulate into fibrils and extracellular plaques. A common goal of AD research efforts is to create and use animal models that provide insight into the molecular pathogenesis of AD, providing key correlations to clinical outcomes. Therefore, manipulating the genes associated with FAD in animal models serves as a logical approach to investigate the molecular mechanisms of familial and sporadic AD pathogenesis. There are many well-studied mouse models of AD, and differences in these models allow for the study of distinct pathological hallmarks that occur during

disease time course that mimic aspects of the disease in humans. The APPswe/PS1dE9 (APP/PS1) mouse model expresses human mutant-APP (APPswe: KM594/5NL) and a mutant form of PSEN1 (dE9: deletion of exon 9) which leads to Aβ plaque deposition by 4 months with a progressive increase in plaque number up to 12 months, exhibiting changes in pathology and a decline in working memory at 6 months of age in various behavioral tasks (eg, Morriz Water maze) (Garcia-Alloza et al., 2006; Jankowsky et al., 2001). The 5xFAD mouse model of AD also develops Aβ plaque pathology, though in a significantly shorter timeframe than the APP/PS1 model of AD (Oakley et al., 2006). Mouse models of AD have been readily utilized to investigate mechanisms of AD progression. However, questions involving the pathophysiology of non-cell autonomous mechanisms of disease remain unexplored.

Neuroinflammation is a significant contributor to AD progression but the precise role of the cellular modulators of inflammation is unclear. Microglia are the specialized resident myeloid cell population in the CNS that mediate innate immune responses. During a CNS inflammatory response, microglia maintain tissue homeostasis via debris containment, phagocytizing debris including misfolded or aggregated proteins that lead to the upregulation of tissue repair signaling cascades.

Several mechanisms may compromise the cessation of the microglia-mediated inflammatory response in AD. These may include the ongoing formation of Aβ and positive feedback loops between inflammation and APP processing. Furthermore, accumulation of Aβ fibrils and neuronal debris that further activate receptors, establishes a non-resolving inflammatory, or chronic state (Koenigsknecht-Talboo et al., 2008; Mitrasinovic & Murphy, 2003).

A central mechanism mediating microglia inflammatory activation involves post-transcriptional epigenetic modulation of inflammatory effector genes. MicroRNAs (miRNAs) regulate the phasic responses of both developmental and physiological events (Ryan M. O'Connell et al., 2010). As epigenetic modulators of gene expression, miRNAs influence the timing and amplitude of the innate immune response. Several studies demonstrate that miRNA profiles are altered in tissue, circulating monocytes and serum of AD patients (Kiko et al., 2014; Pogue & Lukiw, 2018). More specifically, certain miRNAs with inflammatory functions are up- or down-regulated in the AD brain, including miR-155 (Li et al., 2011; Lukiw, Zhao, & Cui, 2008; Pogue & Lukiw, 2018). MiRNAs can reduce or enhance immune signals by altering expression of proteins involved in immune signaling pathways. A single miRNA has the potential to modulate expression of hundreds of genes (Ryan M. O'Connell et al., 2012; Ryan M. O'Connell et al., 2010). Upon sensing an inflammatory stimulus, microglia upregulate expression of a set of miRNAs that drive a pro-inflammatory response and/or serve as negative feedback regulators of the pro-inflammatory functions. MiR-155 can set the magnitude and timing of the pro-inflammatory response by targeting the 3’UTR of mRNAs that encode specific anti-inflammatory mediators (Ryan M. O'Connell et al., 2009; Ye et al., 2016). Mice deficient in miR-155 have a diminished ability to respond to inflammatory stimuli, like LPS, while mice overexpressing miR-155 develop chronic-inflammatory states and hematopoietic malignancies (Bala et al., 2011; Du et al., 2014).

Previously, the Garden lab has reported that p53 activation resulting in miR-155 expression is required for microglia induction in response to pro-inflammatory signals. In microglia, miR-155 targets the anti-inflammatory transcription factor c-Maf for degradation (Wei Su et al., 2014). Furthermore, miR-155 targets mRNAs coding for additional molecules involved in suppressing the inflammatory response including, SHIP1 and SOCS1, leading to increased pro-inflammatory gene expression. In this study, we hypothesized that conditional knock-out of miR-155 in the APP/PS1 mouse model of AD would lead to upregulation of anti-inflammatory targets of miR-155, decrease insoluble levels of Aβ, and decrease plaque pathology (Figure 3.1).

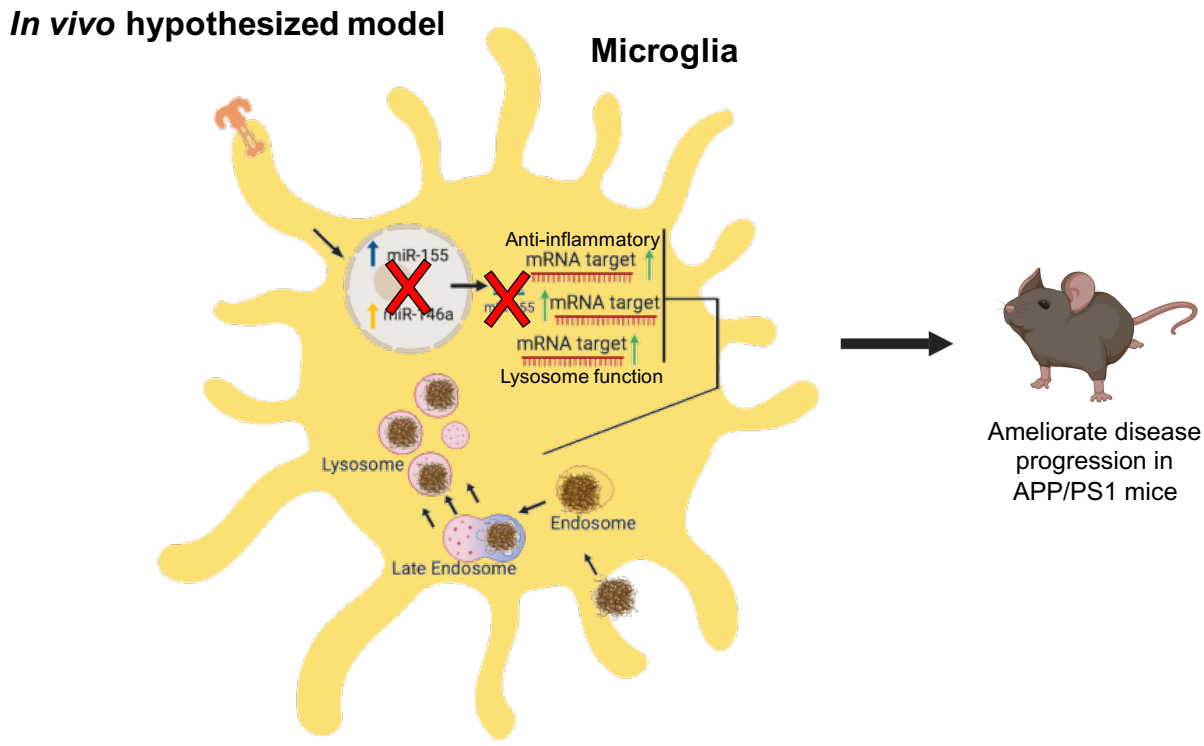

Figure 3.1: Hypothesized model *in vivo*. Microglia processing of Aβ will involves modulation of the intracellular handling pathway which are influenced by miR-155 expression, leading to upregulation of anti-inflammatory targets of miR-155, decrease insoluble levels of Aβ, and decrease plaque pathology in the APP/PS1 mouse model of AD.

Methods

Mouse model breeding and tamoxifen treatment scheme

APPswe/PS1dE9 (APP/PS1) or 5xFAD heterozygous mice were crossed twice with homozygous floxed miR-155 mice (miR-155$^{flx/flx}$) to obtain homozygous miR-155$^{flx/flx}$ alleles in the APP/PS1$^{+/-}$ or 5xFAD$^{+/-}$ background (miR-155$^{flx/flx}$ x APP/PS1$^{+/-}$ or 5xFAD$^{+/-}$). Mice with miR-155$^{flx/flx}$ APP/PS1$^{+/-}$ or 5xFAD$^{+/-}$ were crossed with miR-155$^{flx/flx}$ CX3CR1$^{CreER/CreER}$ mice to obtain APP/PS1$^{+/-}$ miR-155$^{flx/flx}$ CX3CR1$^{CreER/+}$ or 5xFAD$^{+/-}$ miR-155$^{flx/flx}$ CX3CR1$^{CreER/+}$ mice miR-155$^{flx/flx}$ CX3CR1$^{CreER/+}$ littermates. At 8 weeks of age, mice were treated with a one-time 20mg dose of tamoxifen or corn oil vehicle by oral gavage. End points were harvested at 6, 9, and 12 months of age. All animals were maintained in a C57/BL6 background and were handled described in our approved UW IACUC protocol (IACUC protocol number: 3254-04: Molecular and Cellular Basis of Neurodegeneration).

Ex vivo FACS isolation of adult microglia and total RNA extraction

Mice were anesthetized with Avertin (2.5%) until unresponsive to stimuli and then perfused with Hank's Balanced Salt Solution (HBSS -/-) with 1mM Hepes. Brain was then extracted, hemisected, and forebrain was placed in Accutase (Millipore, SRC005) to be mechanically processed and enzymatically dissociated for 30 min at 4^{o}C while shaking. Tissue was mechanically dissociated further with a serological pipette, then a micropipette, and strained through a 250u filter. The single cell suspension was resuspended in 100% Fetal Bovine Serum then in a 30% percoll solution overlayed with FACS Media (10% FBS, 1mM Hepes in HBSS -/-). Slurry was centrifuged at 800 x g for 15 min with slow acceleration and break. Percoll layer was then aspirated away from

the pellet and cells were washed once in FACS Media. Cells were stained with for CD45 expression (PE-Cy7-Rat anti-mouse CD45 antibody; BD Pharmigen). CX3CR1-YFP+ and Pe-Cy7-CD45^{+} (Microglia) cells were isolated and collected by FACS. Microglia were then pelleted at 2,500 rpm at 4 ^{o}C for 5 min, then lysed in buffer from the DNA/RNA Mini-prep kit (Zymo;11-385).

Detecting miR-155 deletion and changes inflammatory genes targeted by miR-155 by RT-qPCR

Total DNA and RNA were extracted from FACS isolated microglia using the DNA/RNA Mini-prep kit (Zymo;11-385). DNA was used for end-point PCR to detect presence or absence of miR-155 alleles using gel electrophoresis. Total RNA was quantified using nano-drop, and samples that did not meet criteria were excluded. Expression levels of miR-155 were detected using primer specific sequences for cDNA conversion using the TaqMan® MicroRNA Assay for mmu-miR-155-5p (Assay ID 002571). cDNA from MISSION® microRNA Mimics to hsa-miR-155 was used to generate a standard curve that would allow us to quantify total copy number of miR-155 per sample.

To detect changes in gene expression of targets for miR-155, total RNA was used for cDNA conversion using random primers. cDNA was then used for qPCR to detect changes in MAF (Forward 5'-CAACGGCTTCCGAGAAAAC-3', Reverse 5'-TCGCGTGTCACACTCACAT-3'), SOCS1 (Forward 5'-TCTGTCTCCCCCATCAGC-3', Reverse 5'-GCGTGCTACCATCCTACTCG-3'), SHIP1 (Forward 5'-GGCTGAGGAGGACACTGTAGAA-3', Reverse, 5'-CGGCAGACATAGGAATGTT-3') , CSF1R (primer sequence), and TFEB (Forward 5'-CAACGATGAGATGCTCAGCTA-3',

Reverse 5'-CTGTACACATCAAGTAGATTTCCAGAC-3'). Samples were normalized to a house keeping gene (GAPDH; Forward 5'-TGTGGAAGGGCTCATGACCA-3', Reverse 5'-CACCAGTGGATGCAGGGATG-3') and Ct values were used in a delta-delta-Ct analysis to determine fold change in RNA levels (Quant Studio 6). Prism 7 was used to graph changes in gene expression and run statistical analyses.

Soluble and insoluble protein extraction from fresh-frozen mouse brain

The protocol as in (Keene et al., 2019) was followed for total protein extraction from frozen mouse cortex. Briefly, 150 µl of chilled RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 150 mM sodium chloride, 50 mM Tris hydrochloride, 0.5 mM mag- nesium sulfate; all from Sigma-Aldrich; St. Louis, MO) with Complete Mini protease inhibitor (Millipore Sigma-Aldrich; St. Louis, MO) was immediately added to the tube. Samples were then sonicated on ice (3x pulses), and centrifuged for 30 min at 21,000 × g and 4 °C. Supernatants containing RIPA-soluble proteins were pipetted off into new 2 ml tubes. The remaining pellet was washed with an additional 50 µl RIPA buffer with Complete Mini, and centrifuged a second time for 30 min at 21,000 × g and 4 °C. The RIPA- buffer containing supernatant was then combined with the first RIPA-buffer containing supernatant. The combined RIPA-buffer supernatant contains soluble proteins, but not $A\beta_{1\text{-}42}$. For $A\beta_{1\text{-}42}$ extraction, 150 µl of chilled 5 M guanidine–hydrochloride (Gu–HCl) buffer containing Com- plete Mini was then added to the remaining pellet in tube, vortexed, then sonicated (on ice for 3 pulses). Then, suspension was centrifuged at 13,000 × g for 30 min at 4 °C to produce a Gu–HCl soluble supernatant containing the $A\beta_{1\text{-}42}$ fraction and other RIPA insoluble proteins. All extracts were aliquoted stored at −80 °C. Total protein content was determined in all

samples using a BCA kit (Pierce; Rockford, IL) together with colorimetric detection (absorbance at 562 nm) in a plate reader.

Electrocorticographic Recordings in miR-155 MG CKO AD mice

Mice were anesthetized with isoflurane and placed into a stereotaxic device where isoflurane anesthesia continued throughout surgery. Each mouse was implanted with ECoG electrodes, consisting of dental screws (Pinnacle Technology, Lawrence, KS; No. 8209: 0.10-in.). A midline incision was made above the skull. Recording electrodes were screwed through cranial holes as follows: over the left frontal cortex (1.5 mm lateral and 2 mm anterior to bregma) and over the right parietal cortex (1.5 mm lateral and 2 mm posterior to bregma), a ground electrode was placed over the visual cortex (1.5 mm lateral and 4.0 mm posterior to bregma), and a reference electrode was placed over the cerebellum (1.5 mm lateral and 6.5 mm posterior to bregma). Electromyogram (EMG) signals were obtained by placing a pair of silver wires into the neck muscles. The screws were connected, through silver wires, to a common 6-pin connector compatible with the Pinnacle recording device. The screws and connector were fixed to the skull with dental cement. APP/PS1$^{+/-}$ miR-155$^{flx/flx}$ CX3CR1$^{CreER/+}$ and 5xFAD$^{+/-}$miR-155$^{flx/flx}$ CX3CR1$^{CreER/+}$ mice were implanted at 7 weeks of age. Continuous recordings were made for 7 days (Pinnacle Technologies) to record a baseline reading. Mice were administered 20mg of tamoxifen via oral gavage at 8 weeks. Continuous recording persisted in single recording cages under a 12:12 LD cycle for 2-5 weeks or until recording was stopped or spontaneous death was recorded.

Once the cap was fully dried and set, mice were fitted with a preamplifier and tether, and connected to the Pinnacle Technology recording system, where they were

allowed 1 day to acclimate before recording started. The ECoG and EMG signals were sampled at 400 Hz with low-pass filters of 80 Hz and 100 Hz, respectively.

Interictal Spike Quantification

Epileptiform interictal spikes were identified semi-automatically over a 24-hour recording session with the Sirenia Seizure software (Pinnacle Technology, Lawrence, KS) using a line length threshold (Dubey & Ray, 2019) established for both ECoG channels, then confirmed visually by a manual scorer blind to experimental conditions. Spikes were then classified as occurring during either Wake, NREM or REM based on scores obtained through automated sleep classification.

Sleep Scoring and Processing

Raw ECoG/EMG data were automatically classified in 10-second bouts as either wake, NREM sleep or REM sleep using a previously described custom algorithm implemented in Python v2.7. Briefly, the power spectrums of the ECoG and EMG signals are calculated using Welch's method with an overlap window of 50%. Then, the following features for each 10-second epoch of sleep are calculated: delta power, the sum of ECoG power at frequencies ranging between 0.5 and 4 Hz; theta power, the sum of ECoG power at frequencies ranging between 6 and 12 Hz; and EMG RMS, the mean square root of the raw EMG signal. If EMG RMS was higher than a visually determined threshold for each 24-h recording session, typically set at 0.4 standard deviations above the mean, the epoch was classified as wake, otherwise it was classified as sleep. Next, sleep stage was classified as REM whenever the theta to delta power ratio during the epoch was higher than the average theta to delta ratio—over the whole 24-h recording session—plus 1 standard deviation. The remaining

epochs were classified as NREM sleep. This scoring method was based on heuristics for manually scoring sleep in previous studies, and validated by 2 independent experimenters that visually scored 10-second bins as previously described (Lee et al., 2016; McKinney, Dang-Vu, Buxton, Solet, & Ellenbogen, 2011), with a minimum of 90% score agreement in both cases.

Identifying changes in plaque pathology in MG CKO AD mice

Three sections of dorsal hippocampus, each 200um apart, were selected for each animal. Free floating sections were washed three times in 1X TBS then fixed for 20 minute at room temperature in 4% PFA. After two 1X TBS washes, sections underwent a 20 minute antigen retrieval in a sodium citrate buffer (pH=6.0) at 65 degrees and were cooled at room temperature for 20 min. After another two 1X TBS washes, sections were digested in 0.05% Proteinase K buffer for better plaque core visualization. After three 1X TBS washes, sections were incubated in blocking solution (1X TBS with 0.4% Triton X-100, 10% Donkey serum, 2% Bovine Serum Albumin, and 1% Glycine) at room temperature for 2 hours. Sections were incubated in half dilute blocking solution with primary antibodies (1:200 6E10, 1:500 Iba-1) for 18 hours at 4 degrees on shaker. Sections were washed three times in 1X TBST-T before secondary antibody (1:1000 Alexa 488 donkey anti mouse and 1:500 Alexa 594 donkey anti goat) incubation at room temperature for 3 hours. DAPI (1:1000) was added during last 30 min of secondary antibody incubation. After 2 final 1X TBS washes, sections were mounted with Vectashield containing DAPI and stored at 4 degrees in the dark until imaging.

Observer was blinded to all animal conditions. Fluorescent images were taken with an inverted microscope. Z-stacks (40um thickness, 1.5um step size) of three consecutive images in CA1 and CA3 were taken at 20X magnification and 1X1 binning with no overlapping regions. Channels across all images had identical exposure settings (100ms DAPI channel, 800ms FITC channel, and 1000ms CY3 channels). With Slidebook5, maximum projections along the z-axis of Z-stacks were created after nearest neighbor deconvolution. Quantification method was adapted after Harwell et al. 2016. Fiji was used to analyze: %area plaque area, plaque count, plaque diameter, %area of total MG, and %area of MG associated with plaque. All %area calculations and plaque diameter calculations are expressed in pixels.

Results

miR-155 deletion in APP/PS1 mice leads to upregulation of anti-inflammatory effector genes in microglia *in vivo*

To investigate the impact of miR-155 expression in microglia during pathological progression of the APP/PS1 mouse model of AD, we examined expression levels of miR-155 in microglia in APP/PS1 mice and in non-APP littermates. We hypothesized that miR-155 expression would be upregulated in aged APP/PS1 mice. We measured expression of miR-155 in sorted microglia from aged APP/PS1 mice and observed increased expression with age compared to wild-type littermates (not shown). Mice APP/PS1$^{+/-}$ miR-155$^{flx/flx}$ CX3CR1$^{CreER/+}$ and littermate controls (miR-155$^{flx/flx}$ CX3CR1$^{CreER/+}$) were given tamoxifen or corn oil at 2 months of age (Figure 3.2 A) by oral gavage. CX3CR1-YFP+/CD45+ cells were isolated using FACS and total DNA and

RNA extracted. Floxed alleles for miR-155 were confirmed as excised from the genomic DNA in FACS isolated microglia (Figure 3.2 B). Using qPCR we observed that miR-155 was upregulated in microglia in aged APP/PS1, while Cre-mediated excision of floxed miR-155 alleles reduced expression of miR-155 in tamoxifen treated animals compared to controls (Figure 3.2 C). Animals that did not express the APP/PS1 transgene, and received either tamoxifen or corn oil, did not show increased levels of miR-155 expression in isolated microglia. This data demonstrates that, miR-155 expression is induced in microglia from APP/PS1 mice but not in the absence of Aβ pathology while Cre mediated deletion of miR-155 prevents microglia miR-155 expression in both WT and APP/PS1 mice.

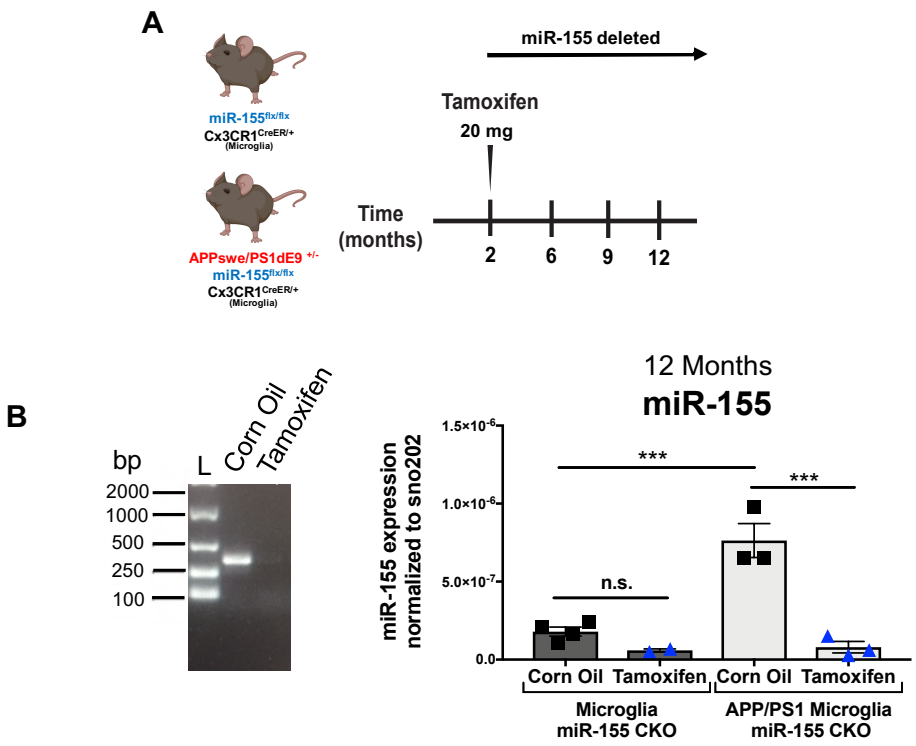

Figure 3.2: miR-155 is knocked-out of microglia *in vivo* the APP/PS1 mouse model of AD. A) APPswe/PS1dE9 mice were crossed with miR-155$^{flx/flx}$ x CX3CR1$^{CreER/+}$ mice to generate APPswe/PS1dE9 x miR-155$^{flx/flx}$ x CX3CR1$^{CreER/+}$ (with tamoxifen: APP/PS1 MG miR-155 CKO mice; with corn oil: AD control) or non-APP littermate controls that allow for conditional miR-155 deletion (Microglia miR-155 CKO). **B)** End-point PCR showing loss of miR-155 floxed alleles in DNA isolated from *ex vivo* FACS sorted microglia (CX3CR1-YFP^{+}/ CD45^{+} cells). qPCR of miR-155 levels in microglia from tamoxifen APP/PS1 MG miR-155 CKO mice and corn oil AD control. miR-155 is upregulated at 12 months in AD controls and not in APP/PS1 MG miR-155 CKO or littermate control mice in either experimental condition. Statistics: One-Way ANOVA with multiple comparisons: Microglia miR-155 CKO corn oil vs. APP/PS1 microglia CKO corn oil, p=0.0004. APP/PS1 microglia CKO corn oil vs. APP/PS1 microglia miR-155 CKO p=0.0002.

Since one microRNA can modulate expression of many effector genes, we focused on identifying changes in expression of genes that are confirmed or putative targets for miR-155 and are that genes associated with microglia anti-inflammatory functions, microglia survival and proliferation, and lysosomal biogenesis and turn over as these are important biological pathways that are dysregulated in AD. Using total RNA

extracted from FACS sorted microglia at 6 months of age we quantified changes in expression of miR-155 target genes: cMAF, SOCS1, SHIP1, and CSF1R. At 6 months of age, cMAF, SHIP1, SOCS1 were upregulated in the miR-155 deleted microglia of APP/PS1 mice, while CSF1R was not significantly upregulated (Figure 3.3; Two-way ANOVA $p<0.01$). These targets, along with CSF1R, were also upregulated in miR-155 deleted microglia from littermate controls which do not express the APP/PS1 transgene. Interestingly, the transcription factor that coordinates expression of lysosome biogenesis genes, TFEB, was significantly upregulated in littermate controls that received tamoxifen, and seemed to be trending, although not statistically significant in the AD model. TFEB is a putative target for miR-155 that we identified via a series of *in silico* alignments. Further experiments (eg. Binding sequence mutagenesis studies) need to be performed to validate TFEB as a target of miR-155. Taken together, we can conclude that in microglia, effector genes with known anti-inflammatory function are upregulated at 6 months of age, after microglia specific deletion of miR-155.

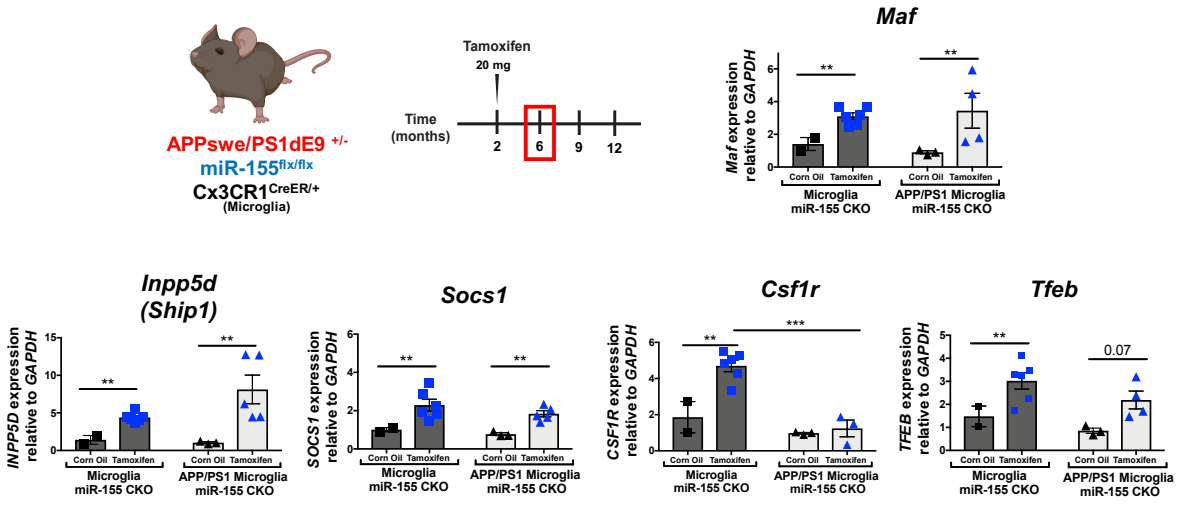

Figure 3.3: Targets for miR-155 are upregulated in microglia *in vivo* at 6 months of age. qPCR analysis of total RNA extracted form *ex vivo* FACS sorted microglia (CX3CR1-YFP^{+}/CD45^{+} cells). In the non-AD model, cMAF, CSF1R, TFEB, SOCS1 and SHIP1 are upregulated in microglia *in vivo* while CSF1R and TFEB are not significantly upregulated in the APP/PS1 mouse model of AD. Stats: 2-way ANOVA with multiple comparisons (** = p<0.005, *** = p<0.0005).

Conditional miR-155 knock-out from microglia leads to decreased $A\beta_{1\text{-}42}$ in the APP/PS1 mouse model of AD

We observed that conditional knock-out of miR-155 in microglia resulted in upregulation of anti-inflammatory targets of miR-155. Therefore, we asked if this specific modulation of microglia altered levels of $A\beta_{1\text{-}42}$ *in vivo.* We quantified total levels of $A\beta_{1\text{-}42}$ from insoluble protein fractions isolated from cortex of APP/PS1 mice post conditional knock-out of miR-155 at 6 months. We found that total levels of $A\beta_{1\text{-}42}$ were significantly reduced in cortex of APP/PS1 mice post miR-155 knock-out in microglia, as compared to APP/PS1 mice of the same cohort (Figure 3.4: two-tailed unpaired t-test, p<0.05). Taken together, conditional knock-out of miR-155 in the CNS specifically in microglia results in less $A\beta_{1\text{-}42}$ in cortex.

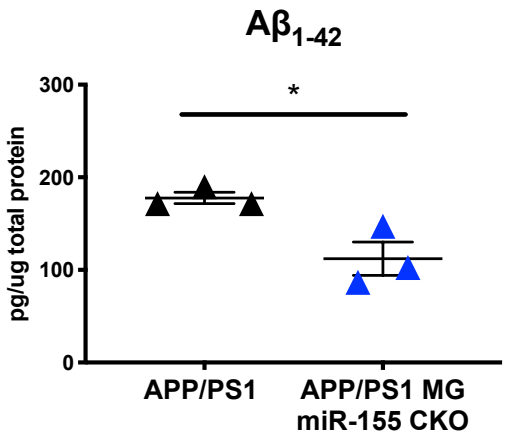

Figure 3.4: Conditional miR-155 knock-out from microglia leads to decreased $A\beta_{1-42}$ in cortex of APP/PS1 mice. Analysis of the insoluble protein fractions by Luminex (Millipore Sigma; St. Louis, MO) detected decreased levels of $A\beta_{1-42}$ in APP/PS1 microglia miR-155 conditional knock-out mice (APP/PS1 MG miR-155 CKO) compared to non-deleted controls (APP/PS1) (two-tailed unpaired t-test, * = $p<0.05$).

Conditional miR-155 knock-out from microglia causes increased mortality in the APP/PS1 mouse model of AD

We initially hypothesized that conditional knock-out of miR-155 in microglia in the APP/PS1 mouse model of AD would lead to increased anti-inflammatory profiles of microglia, and over all reduced pathology and survival of the mouse model. However, we observed that microglia specific miR-155 deletion lead to a significant increase in mortality of APP/PS1 mice. Conditional deletion of miR-155 in microglia in the littermate controls did not have an impact in survival (Figure 3.5). Due to the significant impact that miR-155 deletion in microglia had on mortality of APP/PS1 mice, we performed necropsies with the aid of Veterinarians in the Department of Comparative Medicine of animals found spontaneously dead in the cage after tamoxifen treatment. With these studies, the Veterinarians in the DCM were able exclude a peripheral inflammatory contribution to this spontaneous death phenotype.

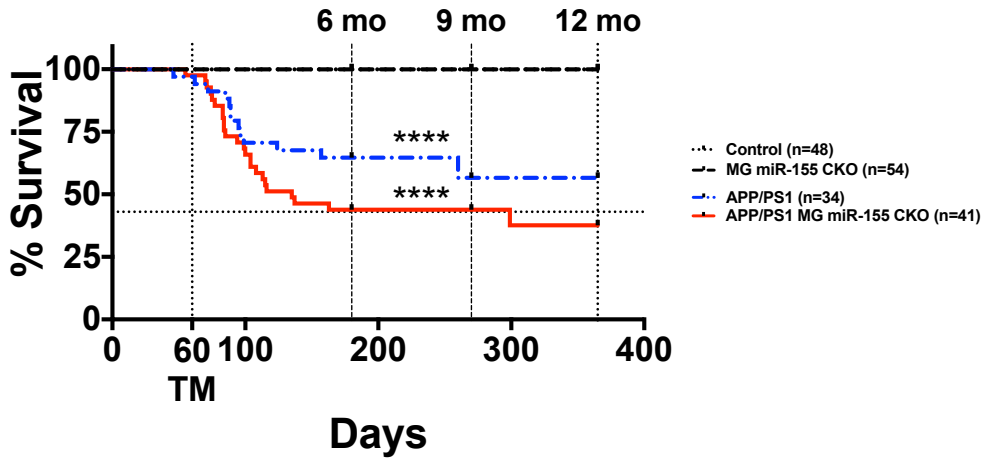

Figure 3.5: miR-155 deletion specific to microglia impacts the survival of APP/PS1 mice. Survival curve analysis (Gehan-Breslow-Wilcoxon test) of APP/PS1 microglia miR-155 CKO vs. APP/PS1, and control mice (**** p = <*0.0001*).

Conditional deletion of miR-155 specifically in microglia leads to increased interictal spikes, seizures and spontaneous death

The APP/PS1 line presents with spontaneous recurring seizures as early as 4 months of age. This spontaneous seizure phenotype contributes to mortality reported in the APP/PS1 line (Reyes-Marin & Nunez, 2017). We hypothesized that the significant impact on survival and spontaneous death phenotype we observed was due to recurring seizures that lead to death. To test this hypothesis, we implanted mice at 7 weeks of age with four electrodes to assess brain activity. We then recorded a baseline ECoG for one week prior to tamoxifen treatment at 8 weeks of age. After tamoxifen treatment, we recorded brain activity continuously for 3-5 weeks post treatment (Figure 3.6 A) then quantified the total number of epileptic events (interictal spikes). Prior to miR-155 deletion in microglia, we observed a minimal number of interictal spike events in EEG. Two weeks after miR-155 conditional knock-out of miR-155 in microglia we observed a significant increase in interictal spiking events in EEG channels (Figure 3.6 B; One-way

ANOVA with repeated measures with Greenhouse-Gessier corrections, F(1.283, 22.72), p<0.05). We also observed electrographic seizures in mice post miR-155 deletion (Figure 3.6 C) that were not observed prior to conditional knock out of miR-155. These seizures lead to sudden death of 2 out of 3 animals that were part of the initial cohort of the study. Therefore, we can conclude that microglia specific deletion of miR-155 contributes to increased mortality through increased hyperexcitability prior to the onset of histologically detectable plaque pathology in APP/PS1 mice.

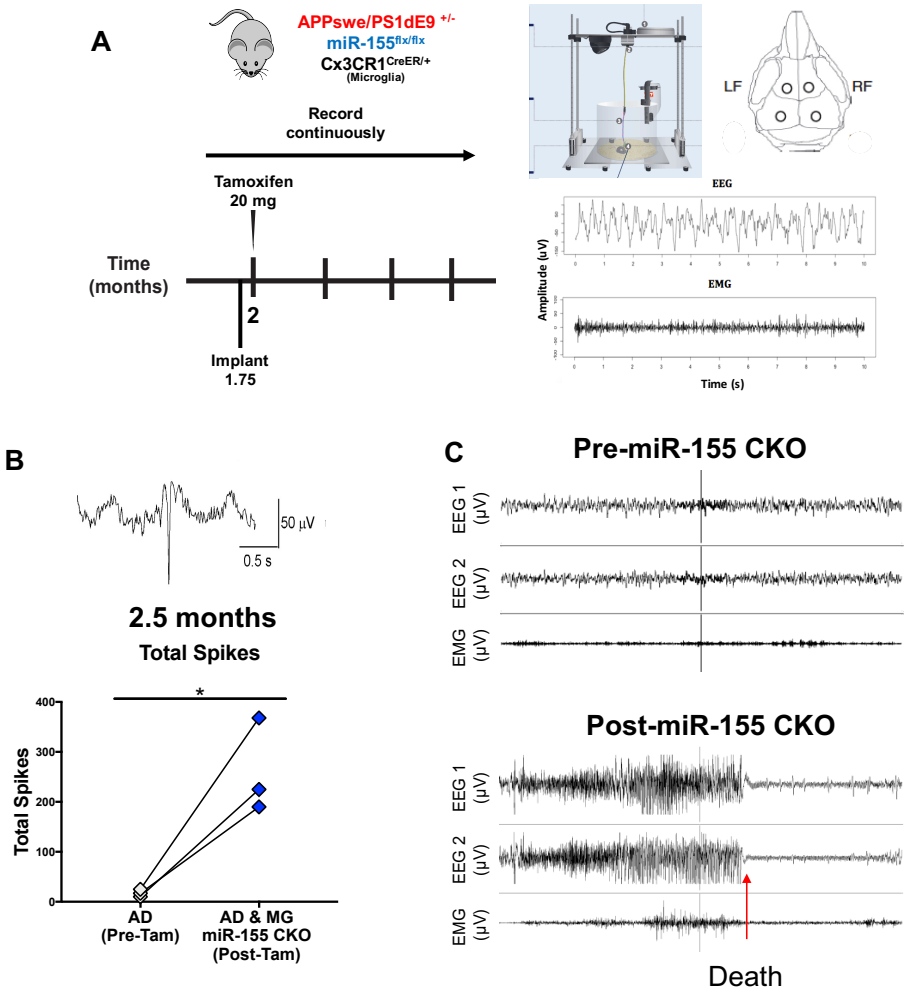

Figure 3.6: Conditional miR-155 deletion in microglia leads to recurring seizures at 2 months of age that lead to spontaneous death in APP/PS1 mice. A) APP/PS1 MG miR-155 flx/flx CX3CR1-CreER mice were implanted at 6 weeks of age with ECoG electrodes consisting of dental screws (Pinnacle Technology, Lawrence, KS; No. 8209:

0.10-in.). A midline incision was made above the skull. Recording electrodes were screwed through cranial holes as follows: over the left frontal cortex (1.5 mm lateral and 2 mm anterior to bregma) and over the right parietal cortex (1.5 mm lateral and 2 mm posterior to bregma), a ground electrode was placed over the visual cortex (1.5 mm lateral and 4.0 mm posterior to bregma), and a reference electrode was placed over the cerebellum (1.5 mm lateral and 6.5 mm posterior to bregma). Electromyogram (EMG) signals were obtained by placing a pair of silver wires into the neck muscles. Recordings occurred continuously for 3-5 weeks until death. B) Epileptiform interictal spikes were identified semi-automatically over a 24-hour recording session with the Sirenia Seizure software (Pinnacle Technology, Lawrence, KS) using a line length threshold[39] established for both ECoG channels, then confirmed visually by a manual scorer blind to experimental conditions. Spikes were then classified as occurring during either Wake, NREM or REM based on scores obtained through automated sleep classification. Stats: Paired two-tailed t-test (* = $p<0.05$). C) representative traces of two EEG, one EMG channel pre-miR-155 (left) and post-miR-155 deletion (right) specifically in microglia in the CNS.

Conditional knock-out of miR-155 from microglia in 8 month old APP/PS1 mice leads to increased epileptic events

We next asked if the seizure phenotype observed in AD models was sensitive to developmental timing or if miR-155 deletion would impact the seizure phenotype of APP/PS1 mice after the onset of Aβ plaque pathology. Briefly, after implanting APP/PS1 mice with ECoG electrodes as described above at 7.75 months of age we obtained a baseline recording of one week. Animals were then given tamoxifen to conditionally knock-out miR-155 in microglia. Continuous recording followed for two weeks (Figure 3.7 A). In adult APP/PS1 mice, conditional miR-155 deletion lead to an increase in interictal spiking events, although this increase in interictal spikes was not statistically significant (Figure 3.7 B-C). The trend in increased hyperexcitability observed after conditional miR-155 knock-out in APP/PS1 mice may not be limited to a precise age or time point in disease pathophysiology. Since 8-month-old APP/PS1 mice present with increased interictal spiking two-weeks post conditional knock-out of miR-155 we can

hypothesize that there may be increased microglia capacity for synaptic stripping that can develop at early or late disease stages in AD pathogenesis.

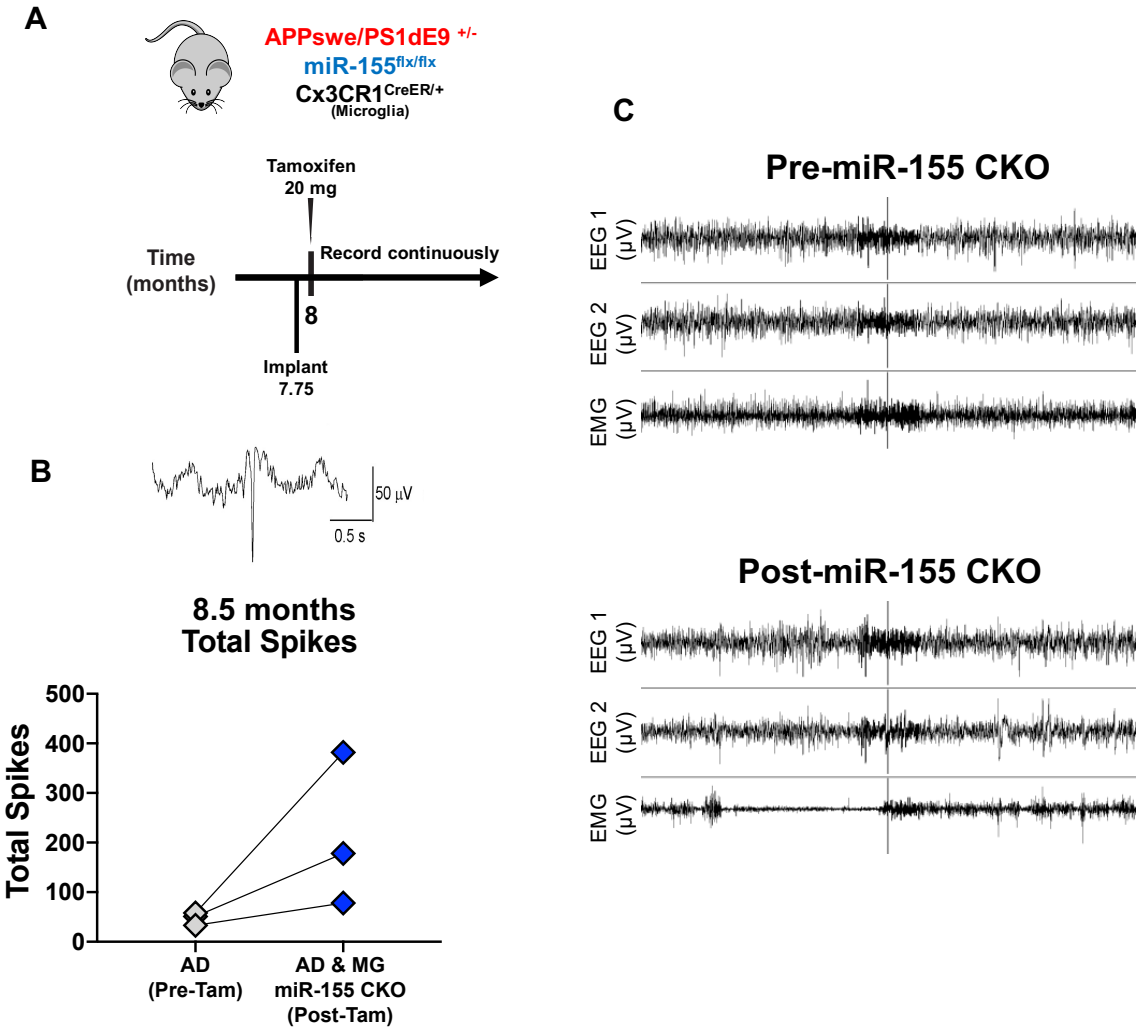

Figure 3.7: Conditional miR-155 deletion in microglia in adult APP/PS1 leads to increased epileptic events. A) Mice were implanted with EEG and EMG electrodes. Continuous recordings were made for 2 weeks (Pinnacle Technologies). B) Interictal spikes were quantified and C) electrographic seizures were monitored post miR-155 deletion from microglia (high-amplitude, synchronous spiking in the EEG).

Total plaque quantification in 8-month-old APP/PS1 miR-155 CKO mice showed decreased number of plaques.

Since miR-155 knock-out in microglia in 8-month-old mice lead to increase hyperexcitability in the adult APP/PS1 brain, and plaque is easily observed at this time point using antibodies directed against Aβ fibrils, we next asked if conditional knock-out of miR-155 in microglia would decrease plaque burden. We hypothesized that conditional miR-155 knock-out in microglia would lead to decreased plaque burden in older animals with established plaque pathology. We observed a global decrease of plaque burden in APP/PS1 microglia conditional miR-155 knock-out mice (Figure 3.8 A) two weeks post miR-155 deletion, compared to age matched AD mice (Figure 3.8 B; unpaired two-tailed t-test, * = $p<0.05$). In addition, APP/PS1 miR-155 CKO mice showed a reduction of plaques in hippocampus compared to APP/PS1 mice, although the area of microglia surrounding plaques remained unchanged (Figure 3.8 C-E). Therefore, miR-155 knock-out in microglia not only leads to increased hyperexcitability in the already vulnerable AD brain, but also leads to a global decrease in plaque pathology.

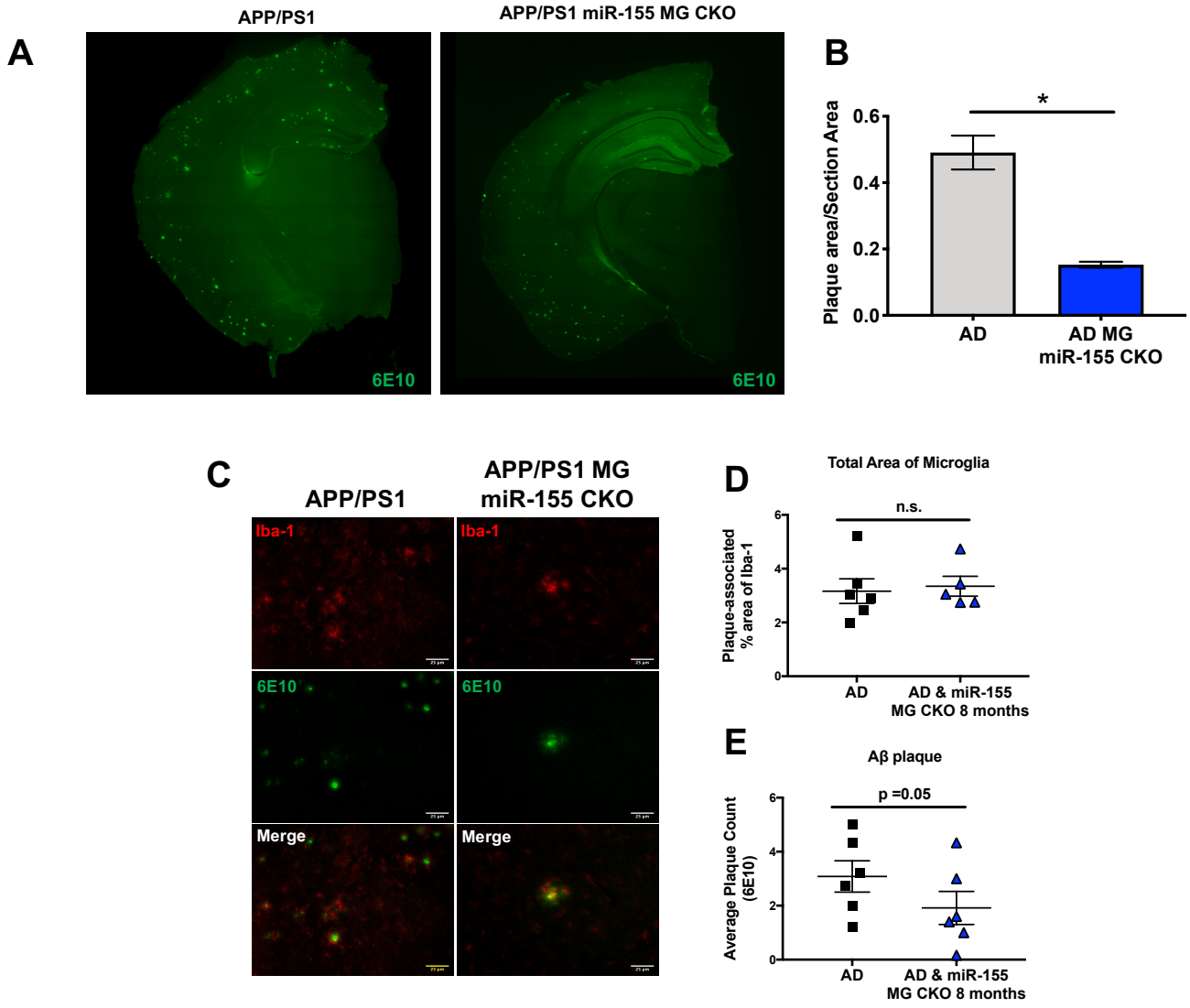

Figure 3.8: Conditional miR-155 deletion from microglia in adult APP/PS1 mice leads to a reduction in Aβ-plaque number. Two weeks post conditional miR-155 deletion mice were sacrificed and brains were post-fixed, then stained for A) Montage of Aβ-plaques (6E10) in control animals (APP/PS1) and microglia miR-155 conditional knock-out (APP/PS1 miR-155 MG CKO) and B) Aβ-plaque area quantification based on 6E10 immunostaining (Unpaired two-tailed t-test, $p<0.05$). C) Representative images of Aβ-plaques of control animals (APP/PS1) and microglia miR-155 conditional knock-out (APP/PS1 mIR-155 MG CKO) in hippocampus (CA3). D) Percent area of microglia associated with Aβ-plaques and E) total plaque count were quantified using Fiji and analyzed using Prism 8 (Unpaired two-tailed t-test, n.s. = not significant).

Conditional deletion of miR-155 from microglia in 5xFAD mice leads to increased interictal spikes and seizures

To investigate if the seizure phenotype observed in the APP/PS1 line post miR-155 deletion was observed in a second AD model, we crossed the 5xFAD model of AD to obtain 5xFAD x miR-155f$^{lx/flx}$ x CX3CR1-Cre$^{ER/+}$ mice that would allow us to conditionally delete miR-155 from microglia. As described above, we implanted mice with ECoG electrodes and recorded a baseline EEG for one week prior to miR-155 conditional knock-out at 8 weeks of age. We recorded from mice continuously for two weeks post deletion of miR-155 in microglia (Figure 3.9 A). As we had previously observed in the APP/PS1 mice, there was an observed increase in interictal spikes in 5xFAD mice two weeks after miR-155 deletion in microglia. In addition, we observed a higher interictal spike baseline than in APP/PS1 mice of the same age prior to deletion of miR-155 in microglia (Figure 3.9 B). Although these data require further quantitation and assessment of statistical significance, the observations we made thus far suggest that the loss of miR-155 in microglia impacts epileptogenesis in the 5xFAD mouse, an additional amyloidosis model of AD.

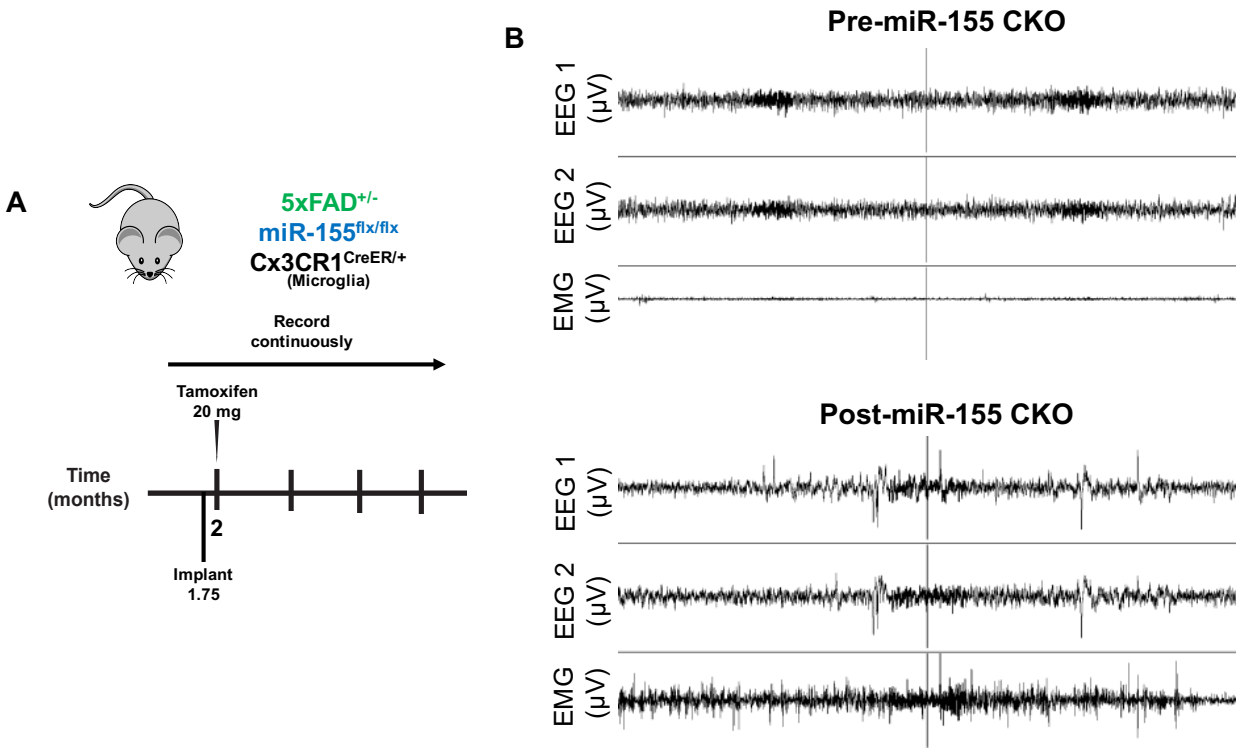

Figure 3.9: Conditional miR-155 deletion in microglia leads to recurring epileptic events at 2 months of age in 5xFAD mice. A) 5xFAD miR-155$^{flx/flx}$ CX3CR1-Cre$^{ER/+}$ mice were implanted at 7 weeks of age with ECoG electrodes consisting of dental screws (Pinnacle Technology, Lawrence, KS; No. 8209: 0.10-in.). A midline incision was made above the skull. Recording electrodes were screwed through cranial holes as follows: over the left frontal cortex (1.5 mm lateral and 2 mm anterior to bregma) and over the right parietal cortex (1.5 mm lateral and 2 mm posterior to bregma), a ground electrode was placed over the visual cortex (1.5 mm lateral and 4.0 mm posterior to bregma), and a reference electrode was placed over the cerebellum (1.5 mm lateral and 6.5 mm posterior to bregma). Electromyogram (EMG) signals were obtained by placing a pair of silver wires into the neck muscles. Recordings occurred continuously for 3-5 weeks until death. **B)** Epileptiform interictal spikes were identified semi-automatically over a 24-hour recording session, representative traces of two EEG, one EMG channel pre-miR-155 and post-miR-155 deletion specifically in microglia in the CNS in 5xFAD mice.

miR-155 CKO microglia internalize more Aβ fragments *in situ* and inhibitory synaptic markers detected *ex vivo* in the context of AD

Conditional miR-155 deletion lead to increased processing of fibrillar forms of Aβ by primary neonatal microglia *in vitro* and conditional knock-out of miR-155 in microglia significantly exacerbated the seizure phenotype observed in APP/PS1 and 5xFAD mice. Therefore, we hypothesized that conditional miR-155 knock-out *in vivo* lead to upregulation of Aβ clearance pathways, contributing to excessive synaptic stripping of inhibitory synapses, leading to hyperexcitability. To initially test this hypothesis, histological sections of 5xFAD mice post miR-155 knock-out in microglia were analyzed to assess the extent of microglia interactions with Aβ fragments. Although this quantification is ongoing, we initially observed that miR-155 CKO microglia in 5xFAD mice internalized Aβ. We did not observe as readily that microglia interacted with Aβ fragments in 5xFAD mice that retained miR-155 expression (Figure 3.10).

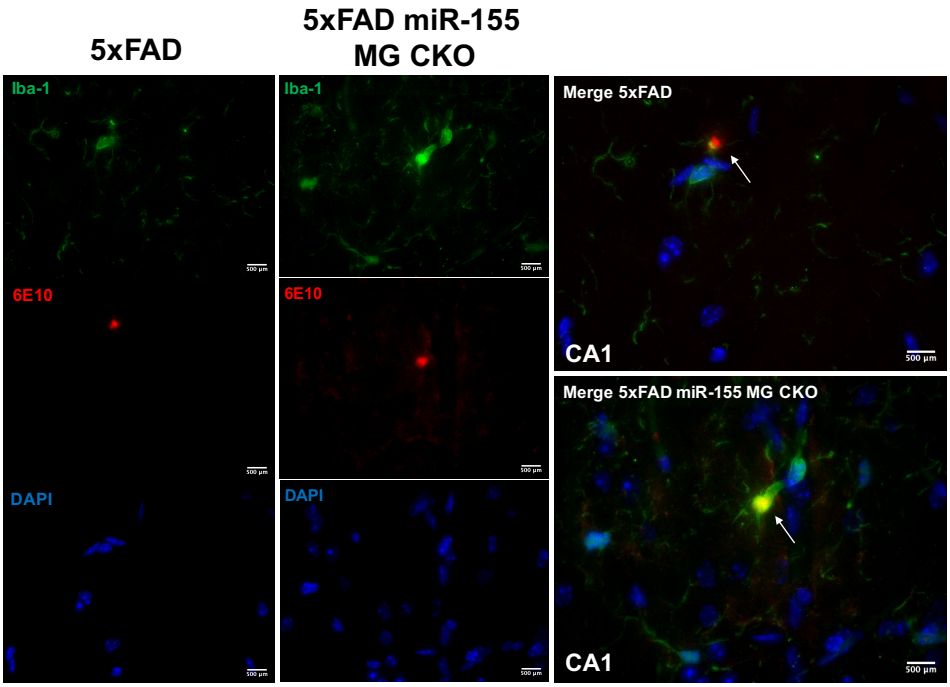

Figure 3.10: *In situ* analysis of conditional miR-155 deletion from microglia in 5xFAD mice suggests microglia internalize more Aβ fragments compared to controls. Two weeks post conditional miR-155 deletion mice were sacrificed and brains were post-fixed, then stained for Aβ (6E10) microglia (Iba-1).

To assess if microglia lacking miR-155 in 5xFAD mice had upregulated the uptake of inhibitory pre-synaptic markers along with potential internalization of Aβ, microglia were isolated from 5xFAD mice at 10 weeks of age (two weeks post miR-155 deletion) using a percoll density gradient. Cells were then fixed and stained to detect total synapses (synaptophysin; Figure 3.11 A-B) and inhibitory synapses (VGAT; Figure 3.11 C-D) using flow cytometry. We observed that microglia specific miR-155 deletion in WT mice lead to significantly higher levels of internalized synaptophysin relative to 5xFAD and 5xFAD miR-155 microglia knock-out cells (Figure 3.11 A-B). In addition, we observed that microglia isolated form 5xFAD mice two-weeks following miR-155 deletion internalized more VGAT compared to 5xFAD animals that retained miR-155 expression in microglia, and relative to miR-155 CKO littermate transgene controls

(Figure 3.11 C-D). Although, further samples are needed in important control groups to assess the significance of the extent of VGAT internalization post miR-155 deletion in microglia *ex vivo*, the data collected thus far suggests that in the context of Aβ, miR-155 deletion in microglia may lead to increased internalization of inhibitory synapses as compared to a general synapse marker.

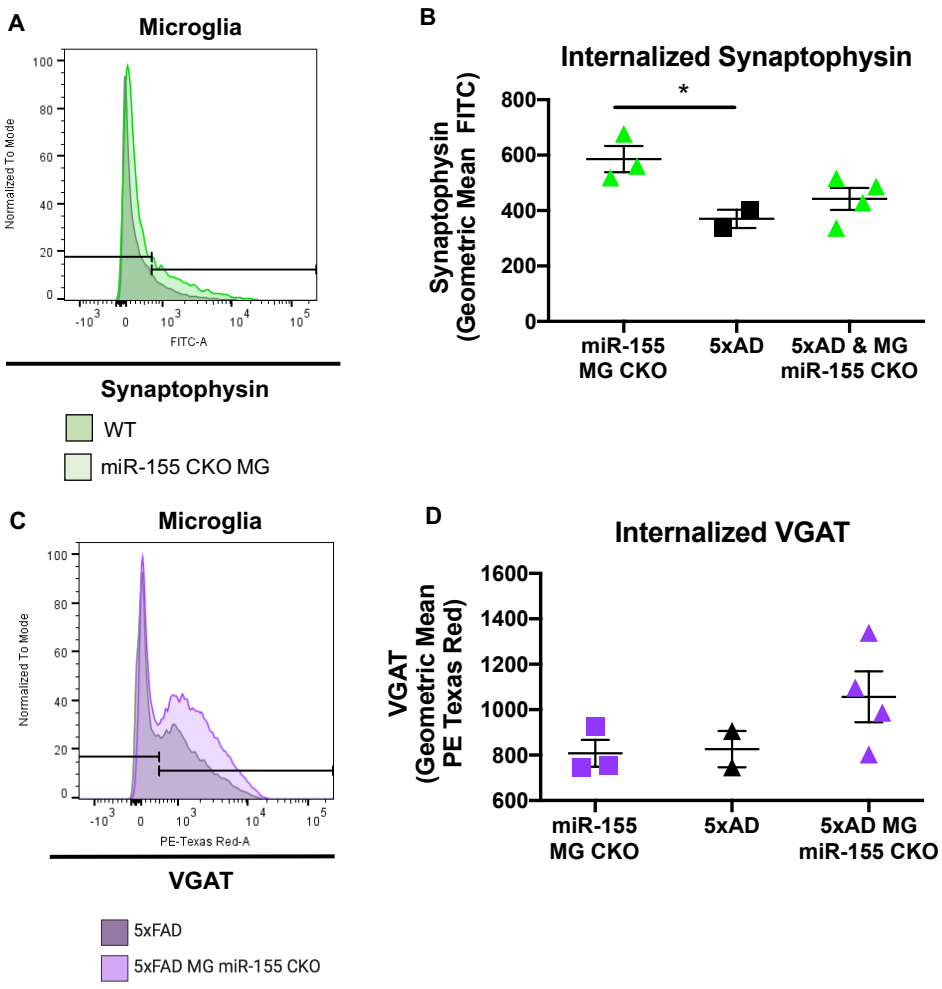

Figure 3.11: Conditional miR-155 deletion from microglia in 5xFAD mice suggests microglia internalize inhibitory synapses than controls. Microglia were *ex vivo* isolated using percoll, then they were fixed and stained for a total synaptic marker (synaptophysin) or a pre-synaptic marker associated with inhibitory synapses (VGAT). Total levels of internalized synaptophysin or VGAT were detected by cytometry (LSRII) and data were analyzed using FlowJo and Prism7 (One-way Ordinary ANOVA with multiple comparisons $p<0.05$).

Discussion

Alzheimer's disease (AD) is the most common form of age-related dementia and is characterized by progressive memory loss and cognitive decline. The AD pathogenic process begins well before diagnosis. As a result, there is a window for therapeutic intervention before irreversible neurodegenerative changes occur is potentially lost. AD etiology involves multiple factors and our current understanding of AD pathogenesis does not allow for the design of preventative or disease modifying therapeutics. In this work, we identified a novel role for a microRNA that is a driver of microglia pro-inflammatory responses, miR-155, in the pathogenesis of two independent mouse models of AD.

We identified a novel, non-cell autonomous role for the hyperexcitability observed in AD models via the loss of an important regulator of microglia gene expression, miR-155. We had hypothesized that conditional knock-out of miR-155, a negative regulator of anti-inflammatory gene expression, would ameliorate the disease progression in the APP/PS1 mouse model of AD by downregulating the chronicity of the pro-inflammatory response mounted against the AD brain environment. However, we observed that one aspect of the disease phenotype, increased seizures, was exacerbated, not minimized by microglia specific deletion of miR-155. We identified a novel, non-inflammatory mechanism by which microglia may contribute to AD pathogenesis. Conditional deletion of miR-155 *in vivo* in two independent models of AD, the APP/PS1 and 5xFAD mouse model, lead to increased spontaneous epileptic events at 3 months of age, and increased spontaneous death in APP/PS1 mice. Furthermore, as animals aged, a unpursued hypothesis resulting from this study is that a compensatory mechanism to

ameliorate the early loss of miR-155 lead to downregulation of miR-155-target expression in animals that tolerated and survived the critical-hyperexcitability period post-miR-155 knock-out. These data provide further evidence to support the critical role of microglia in AD pathogenesis.

MiRNA regulation of microglia in Alzheimer's disease pathogenesis

Many lines of evidence support the hypothesis that AD pathogenesis involves a confluence of genetic, epigenetic and environmental factors. Excess accumulation of Aβ is a hallmark pathological feature of AD. AD pathology and animal models both exhibit altered CNS inflammation and this chronic inflammatory state in AD can promote neuronal dysfunction and contribute to AD progression. Inflammatory cells may contribute to neuronal injury directly through elaboration of cytotoxic factors, or indirectly through impairment of clearance or promotion of toxic peptide aggregation. Additionally, inflammatory homeostasis is disrupted with age and may contribute to accelerated aging and disease progression. Therefore, age-related impairment of innate immune regulation could be a modifiable pathogenic element contributing to neurodegeneration in AD.

MicroRNAs (MiRNAs) are powerful regulators of the timing and amplitude of innate immune responses. MiRNA profiles are altered in brain tissue, serum and peripheral blood mononuclear cells of patients with sporadic AD. Numerous miRNAS, including miR-155, are dysregulated in AD (Kiko et al., 2014; Mendell & Olson, 2012; Olive, Minella, & He, 2015; W. Su, Aloi, et al., 2016). MiR155 is a pro-inflammatory microRNA since its upregulation results in expression of IL-1, IL-6, TNFα and downregulation of anti-inflammatory mediators like IL-10 and Arginase (Faraoni et al.,

2009; R. Hu et al., 2014; Ryan M. O'Connell et al., 2009). We and others have demonstrated parallel modulatory roles for microglial polarization modulating miRNAs including miR-155, which directs microglia skewing through targeting of c-Maf (Guedes et al., 2014; Wei Su et al., 2014). In this work, we identified a novel mechanism by which miR-155 influences microglia functions. First, we confirmed that miR-155 expression is upregulated in microglia in the APP/PS1 mouse model of AD, and not in wild type aged animals. We then observed that conditional knockout of miR-155 in microglia prior to pathology onset in APP/PS1 mice lead to upregulation of mRNA targets for miR-155 that are known regulators of microglia functions and microglia survival. Furthermore, we found preliminary evidence that TFEB, transcription factor and modulator of lysosome biogenesis, may be modulated by miR-155 *in vivo* in microglia. Further experiments (eg. binding sequence mutagenesis studies) need to be performed to validate TFEB as a direct target of miR-155.

Epileptogenesis in Alzheimer's disease

AD is associated with an increased risk of seizures. It is estimated that 10% to 22% of patients with AD present with unprovoked seizures during the course of the disease. Familial and early-onset AD patients have a higher risk of developing seizures. Patients with AD and seizure disorders show greater cognitive impairment, faster progression of symptoms, and more severe neuronal loss at autopsy than those without seizures (Amatniek et al., 2006; Palop & Mucke, 2009; Vossel et al., 2013). Thus, it is important to investigate and understand the mechanisms leading to epilepsy in AD. Recently, this topic has gained further interests in the field. Several groups have shown that APP/PS1 mice have a lower seizure threshold compared to age matched wild-type

mice and present with a higher susceptibility to spontaneous seizures. Although the authors did not explore a specific mechanism by which APP/PS1 mice develop seizures, they identified a strong correlation between increased plaque number in mice and the increase of seizures (Reyes-Marin & Nunez, 2017).

We identified a novel link between inflammatory regulation and epileptogenesis in the APP/PS1 mouse model of AD. We found that conditional deletion of miR-155 in microglia in 3-month-old APP/PS1 mice lead to a significantly exacerbated spontaneous recurring seizures and death. This phenotype, although expected of APP/PS1 progression, is significantly impacted by innate immune cell regulation. Furthermore, conditional knock-out of miR-155 in microglia is sufficient to impact mortality in the model. Interestingly, the overall impact of microglia specific miR-155 deletion was not dependent on age. Conditional knock-out of miR-155 in 8-month-old APP/PS1 mice also lead to increased interictal spiking. While the impact was less severe in the older mice, the overall biological response was recapitulated. In addition, we observed a similar phenotype in a second model of AD, the 5xFAD mouse model. Previous reports have identified the 5xFAD mouse model as presenting with hyperexcitability in old age, at 18 months. In this study, we found again that conditional miR-155 deletion from microglia lead to increased epileptic events and interictal spikes that were not seen prior to deletion of miR-155 from microglia. These findings have not previously been reported and support the hypothesis that microglial expression of miR-155 impacts cellular functions that are restructuring the already vulnerable circuitry in the brain of these two AD mouse models. Future work needs to be done to further elucidate the role of microglia in promoting the hyperexcitability that is observed in mouse models of AD.

Microglia actively remove synapses in mouse models of AD

During AD progression, synapse loss significantly correlates with cognitive decline. Recent work supports the active role of microglia in removal of synapses during disease processes. During CNS development, the complement pathway is upregulated in order to eliminate synapses that are no longer required for stable connections of the network. This pathway is upregulated during disease, and it was characterized in the J20 mouse model of AD (Hong, Beja-Glasser, et al., 2016). The authors found that inhibition of C1q, knock-out of C3 or the microglial complement receptor CR3 leads to a reduction in phagocytic microglia and further maintains synaptic integrity. In addition, C1q is the initiating protein of the classical complement cascade, is expressed and localized at synapses before plaque deposition, promoting synaptic removal in the presence of oligomeric Aβ in this model (Hong, Beja-Glasser, et al., 2016). We hypothesized that the exacerbated seizure phenotype occurred due to microglia selective removal of inhibitory synapses post miR-155 deletion, potentially via modulation of complement, as one target for miR-155 is Complement Factor H, a down-regulator of C1q expression. In our work, we determined that miR-155 knock-out microglia from 5xFAD mice internalize more inhibitory synaptic terminals compared to normal 5xFAD mice or microglia from miR-155 CKO mice. In addition, miR-155 knock-out microglia from 5xFAD mice did not have significantly increased levels of synaptophysin, supporting our hypothesis that miR-155 influences epiletogenesis by influencing the role microglia play in synaptic striping. Further work should focus on the role of microglia regulation in synaptic engulfment via processing and removal of Aβ opsinized synapses during AD progression in models. This mechanism could help

elucidate therapeutic approaches to prevent or slow down loss of synaptic terminals through disease progression, and open new avenues of research related to the excitatory/inhibitory imbalance in the AD brain that leads to spontaneous recurring seizures.

Conclusions and Future Directions

Recent efforts in the field have focused on understanding the role of microglia in the progression of neurodegenerative disease. In Alzheimer's disease pathogenesis, microglia play a central role in mounting the pro-inflammatory response to injured and apoptotic neurons as well as to aggregated Aβ deposits (Jung, Keppler, Steinbach, Blazquez-Llorca, & Herms, 2015; Koenigsknecht-Talboo et al., 2008). In our earlier work, we focused on unveiling a role for miR-155 in modulating microglia metabolism of fibrillar forms of Aβ *in vitro*. In those studies, we determined that miR-155 overexpression reduced intracellular handling internalized Aβ into low-pH compartments, while miR-155 knock-out improved lysosomal transfer of Aβ. Therefore, we hypothesized that conditional knock-out of miR-155 from microglia *in vivo* would lead to decreased plaque pathology and an ameliorated phenotype in AD mouse models. We found instead that miR-155 knock-out specifically from microglia in 3-month-old mice caused increased hyperexcitability in APP/PS1 and 5xFAD mice. We determined that this was potentially mediated by excessive inhibitory synaptic stripping (Figure 3.12). Together, these findings identify miR-155 expression in microglia as a potential regulator of synaptic homeostasis and microglia responses to Aβ in models of AD.

As a result of the increased mortality, we were unable to test our hypothesis as originally planned in APP/PS1 mice. We did observe, however, that conditional miR-155

knock out in APP/PS1 mice at 8 months, an age when significant plaque pathology can be observed in the model, lead to decreased plaque burden only two weeks post miR-155 deletion from microglia. This decrease in plaque pathology was observed in conjunction increased hyperexcitability in APP/PS1 mice, a finding that is novel and contradicts current dogma in the field. Further studies should focus on elucidating the connection between microglia removal of synapses, different species of Aβ, and how this impacts the circuitry in the AD brain in efforts to identify novel mechanisms of epileptogenesis in the setting of to AD or other inflammatory disorders of the CNS.

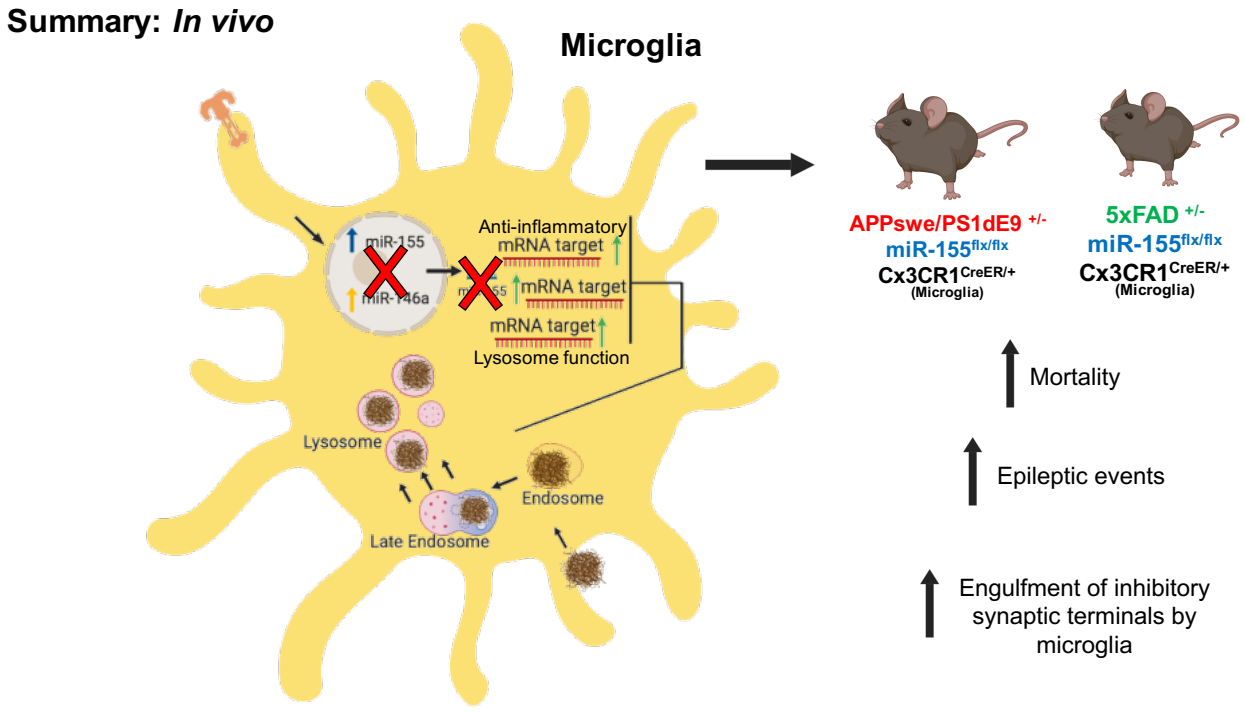

Figure 3.12. Summary of findings *in vivo*. Conditional knock-out of miR-155 in microglia in AD models leads to significant changes in pathology, including a great impact on survival and influence on epileptogenesis, at different ages.

Chapter 4

Discussion and Future Directions

Alzheimer's disease (AD) is a severe neurodegenerative disorder that clinically presents itself with significant memory loss as the disease progresses. Pathologically, the hallmarks of AD can only be thoroughly characterized post mortem at autopsy. At autopsy, amyloid beta (Aβ) plaques, loss of synapses, and dystrophic neurites can be observed throughout prefrontal and parietal cortices using histological stains to effectively diagnose the patient. The identification of altered miRNA levels, specifically in the post-mortem AD brain compared to age matched controls, has raised the strong possibility that these genetic modulators are a component of AD pathogenesis. In recent years, epigenetic modulation of the cellular players involved in AD pathogenesis has been a topic of interest. A growing number of studies have begun to highlight the significance of microglia and miRNAs as epigenetic modulators of cellular signaling processes in AD (Mizuno, 2012). These studies also suggest that dysfunctional miRNA regulation is associated with neurodegeneration, senescence and cellular aging. Thus, current efforts in the field are focused on understanding how miRNAs are regulated in AD, how their targets are further dysregulated by altered miRNA expression, and the factors that can be contributing to AD pathology and alter an AD phenotype *in vivo* models of the disease.

Mouse models of AD can highlight the significance of disrupted miRNA expression *in vivo*. Early upregulation of miR-155 is observed in the 3xTg model of AD is observed by in situ hybridization, prior to development of Aβ plaques (Guedes et al., 2014). Furthermore, expression of targets for microRNAs were dysregulated *in vivo*. The central aim of this thesis work was to elucidate the role of miR-155 in modulating

the microglia-mediated immune responses to disease associated molecular patterns (DAMPS), like fibrillar $fA\beta_{1-42}$ *in vitro*, and *in vivo* using the APP/PS1 and 5xFAD mouse models of AD. The central hypothesis is that miR-155 is a key modulator of microglia functions in response to inflammatory and disease associated stimuli.

Studies *in vitro* revealed a novel role of miR-155 modulation of microglia. We found that overexpression of miR-155 was sufficient to induce expression of miR-146a in primary microglia in the absence of a strong pro-inflammatory stimulus. Additionally, overexpression of miR-155 lead to decreased intracellular handling of $fA\beta_{1-42}$ by microglia, and this difference occurred downstream of $fA\beta_{1-42}$ internalization. We then asked if this deficit in $fA\beta_{1-42}$ handling to low-pH compartments was improved with conditional knock-out of miR-155 in primary microglia. We found that intracellular handling of $fA\beta_{1-42}$ to low-pH compartments was significantly increased in miR-155 knock-out microglia. Interestingly, We also observed that the impact of LPS exposure on microglia is a functional mimic of miR-155 overexpression with increased $fA\beta_{1-42}$ internalization but decreased intracellular handling to low-pH compartments, suggesting that our observations with overexpression and deletion of miR-155 are functionally relevant to microglia responses in the context of AD. Future work *in vitro* could focus on further teasing apart the mechanisms and kinetics of fAβ processing by microglia from plasma membrane binding to internalization into endosomes and endolysosomal processing of $fA\beta_{1-42}$. In addition, the role of miR-146a has yet to be elucidated in this process in microglia, therefore further studies should focus on the impact of miR-146a in the intracellular handling of $fA\beta_{1-42}$.

Microglia are important participants in AD pathogenesis and can influence pathology by their ability to internalize and catabolize Aβ. The findings, from the first section of the thesis supported an elemental role for miR-155 in modulating the ability of microglia to process Aβ. Therefore, we asked if conditional loss of miR-155 specifically from microglia lead to increased fAβ catabolism *in vivo* and an altered AD phenotype in mouse models of AD. Initially, we determined that miR-155 expression is significantly upregulated in aged APP/PS1 mouse microglia, and expression of miR-155 can be blocked in microglia by Cre-lox mediated deletion of miR-155 floxed alleles. Furthermore, we observed that targets for miR-155, modulators of anti-inflammatory gene expression, were upregulated at 6 months of age in microglia following Cre mediated deletion of miR-155 at 2 months.

Interestingly, we observed that conditional deletion of miR-155 lead to increased mortality in the APP/PS1 colony shortly after conditional loss of miR-155. The increase in mortality was unrelated to peripheral inflammatory mechanisms. Since the APP/PS1 mice are known to present with spontaneous recurring seizures that contribute to mortality, we hypothesized that conditional loss of miR-155 lead to exacerbation of this phenotype. We found that conditional loss of miR-155 form microglia increased the hyperexcitability of the vulnerable AD brain in two independent mouse models of AD, the APP/PS1 and the 5xFAD mouse models. Furthermore, we asked if this exacerbated seizure/death phenotype was dependent on developmental timing, since a large portion the colony was affected shortly after excision of miR-155 specifically from microglia. When we conditionally deleted miR-155 from microglia in 8 month old APP/PS1 mice, we observed that mice with advanced plaque pathology developed a similar

hyperexcitability phenotype. Therefore, we concluded that deletion of miR-155 from microglia affected epileptogenesis in the AD brain, and this process is not restricted to a specific age. In these mice, we also observed that the total number of plaques was greatly reduced only two weeks after miR-155 deletion from microglia. Further work needs to be done to elucidate the function of microglia in reducing plaque burden post pathology onset.

We have identified a novel pathway leading to epileptogenesis in AD models. We observed that deletion of miR-155 in microglia significantly impacts survival of APP/PS1 mice. Another interesting finding from this exacerbated mortality phenotype arose throughout the course of the study. We were unable to identify mice that survived the spontaneous seizure phenotype, or characterize if the surviving mice had occurrence of epileptic events. Therefore, the surviving population posited an interesting study on its own, where we could observe changes in APP/PS1 pathology in a selected percentage of the population that survived the spontaneous recurring seizure phenotype exacerbated with deletion of miR-155 in microglia. In these mice, we saw that expression of targets for miR-155 that were upregulated in their expression at 6 months of age, were normalized in their expression by 9 and 12 months, suggesting the existence of a compensatory mechanism mitigating the loss of miR-155, downregulating anti-inflammatory gene expression. This was observed in the APP/PS1 and in non-APP littermates. Compensatory mechanisms could include the upregulation of other miRNAs of inflammatory function, such as miR-146a or miR-125b. Further work in this topic should include further understanding the molecular compensatory mechanisms of

regulation of gene expression in the absence of microRNAs that target specific messages for downregulation or degradation.

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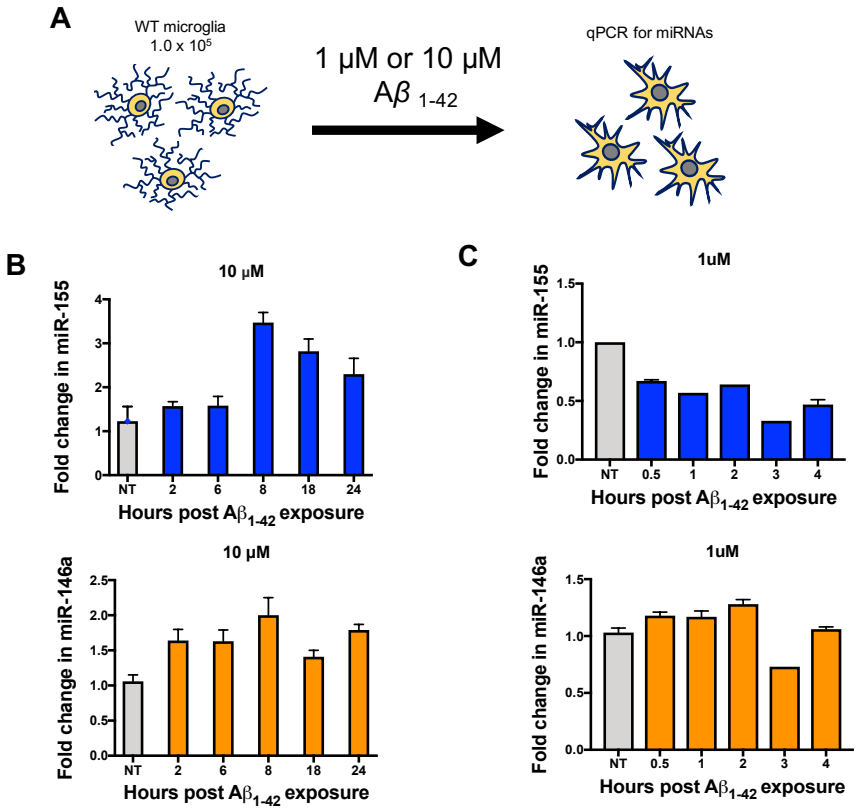
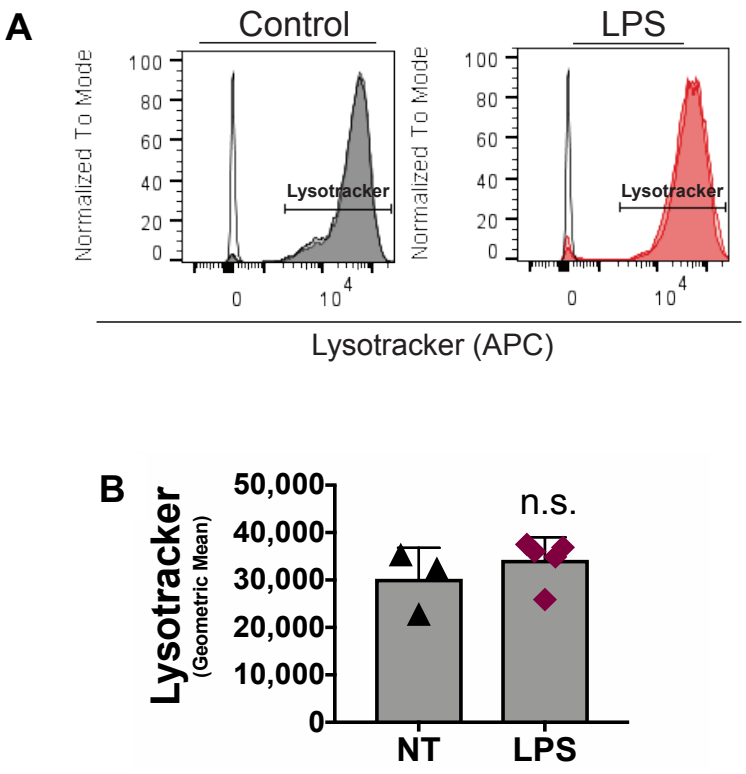
Appendix Figures



Appendix Figure 1: Microglia respond to concentrations of fAβ$_{1-42}$. Stimualtion of microglia with high concentrations of fAβ$_{1-42}$ leads to modulaiton of miR-155 and miR-146a, but miRNA levels remain undhange and not with low concentrations. A) Neonatal microglia were plated and exposed to 1 µM or 10 µM of fAβ$_{1-42}$, then total RNA was extracted and analyzed for chnaged in microRNA expression B) Quantification of miR-155 and mIR-146a expression over time 10 µM of fAβ$_{1-42}$ exposure. C) Quantification of miR-155 and mIR-146a expression over time 1 µM of fAβ$_{1-42}$ exposure. Data were analyzed with Prism 7.



Appendix Figure 2: LPS treatment does not lead to changes in total low-pH compartments. Neonatal microglia were plated and stimuilated with 100ng/mL for 24 hours, then cells were stained with Lysotracker Deep Red to observe total signal of low pH compartments (pH 4.0 - pH3.0). Cells were analyzed using flow cytometry. Data were analyzed using FlowJo and Prism 7 (Statistics: Welche's t-test, n.s. = not significant).