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Microglial Sex-Differences in the Context of Alzheimer's Disease

by
Lay Kodama

DISSERTATION

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Neuroscience

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GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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For a long time, I believed science was an unbiased endeavor to discover the truth, done in a vacuum of its own world. These past four years, and especially this last year (i.e. 2020 – the year of personal reflection for the world), have very much challenged and dismantled that notion. Scientists are humans, and as humans, we bring with us our past experiences, our emotions, and our outside relationships, into the laboratory. They influence the cells in our incubators, the mice we breed for behavioral studies, and the data we carefully collect and analyze. The people we encounter, the environment in which we live, and the culture that support us, create our science. Here are the people who made the science in this dissertation happen.

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L.G., F.A.S., and L.K. conceived and planned experiments. F.A.S., L.K., J.C.U., T.T., S.G., and L.G. designed experiments. F.A.S., L.K., J.C.U., L.F., D.L., G.K.C., H.M., X.J., Q.L., L.Z., S.G., W.L., M.Y.W., X.W., Y.Z., Y.Q.L., and T.T. performed experiments. L.K., H.M., Q.L., L.Z., X.N., F.G., M.T., Y.M.L., G.F., G.C., Z.C., G.Y., M.W., B.Z., L.X., M.B., L.M., and F.W. contributed experimental and analytical tools. F.A.S., L.K., J.C.U., L.F., D.L., T.T., H.M., F.G., M.T., G.C., Q.L., M.W., B.Z., M.B., G.K.C., X.W., Y.Q., S.G., and X.N. analyzed data. J.H. and D.W.D. provided human samples. Y.Z., D.L., M.Y.W., and Y.Q.L. helped maintain the mouse colony. L.K., F.A.S., and L.G. wrote the manuscript with input from all other authors.

Abstract

Microglial Sex-Differences in the Context of Alzheimer's Disease

by

Lay Kodama

Sex differences have been clinically documented in numerous neurodegenerative diseases, including in Alzheimer's disease (AD), and yet the reasons for these sex differences are not well understood. Recent studies have found that microglia, the innate immune cells of the central nervous system, are a key cell type involved in AD. Interestingly, this cell type displays sex differences in their expression profiles and function. Could these sex differences in microglia explain the sex differences seen in AD? How can we further probe these differences to better understand disease mechanisms?

In Chapter 1 of this dissertation, we broadly set the landscape of sex-differences in AD and focus specifically on the emerging evidence of microglial sex differences in both the healthy and diseased brain. We also support these previous findings through our own preliminary single-nuclei transcriptomic analysis of human AD brain tissues and characterize microglial subpopulations differentially enriched in female versus male samples.

Cellular sex differences are largely mediated through sex chromosomes and sex hormones. In Chapter 2, we focus on the sex chromosomes by studying microglial microRNAs (miRNAs) since immune-related miRNAs are highly-enriched on the X chromosome compared to the autosomal chromosomes. In Chapter 2.2, we show that microglial miRNA expression differs in male and female mice and that loss of microglial miRNAs leads to sex-specific changes in the

microglial transcriptome and tau pathology. These findings suggest microglial miRNAs influence tau pathogenesis in a sex-specific manner. In Chapter 2.3, we further focus on individual microglial miRNAs by establishing a primary microglia screen to study the effects of highly-expressed microglial miRNAs on phagocytosis of *E. coli* particles. We found that removal of mature miRNAs in primary microglia reduced their uptake capacity, which was largely reversed by expressing individual miRNAs of the miR-17~92 cluster and its paralog, miR-106a~363 cluster encoded on the X chromosome.

Chapter 3 focuses on the sex-specific transcriptomic changes associated with microglia in AD patients with and without the R47H-*TREM2* mutation, one of the strongest immune-specific risk factors for late-onset AD. We also utilized a newly-generated tauopathy knock-in mouse model expressing one allele of human *TREM2* (*hTREM2*) with either the R47H mutation or the common variant and found R47H induced and exacerbated tau-mediated spatial memory deficits in female mice. Single-cell transcriptomic analysis of microglia from these mice also revealed transcriptomic changes induced by R47H that had significant overlaps with R47H microglia in human AD brains, including robust transcriptomic sex-differences, increases in proinflammatory cytokines, activation of Syk-Akt-signaling, and enrichment of disease-associated microglia.

Finally, in Chapter 4, we conclude on the ways these studies uncovered novel molecular pathways involved in AD pathogenesis through the lens of sex differences, and comment on future lines of investigation with the hope that such studies will lead to potential therapies that can benefit patients of both sexes.

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Chapter 1: Introduction

1.1 Clinical sex-differences in Alzheimer's Disease

For decades, the scientific community has recognized sex differences in the prevalence, incidence, symptomology, and pathology of diseases, including those affecting the central nervous system (CNS). For this dissertation, I will be focusing on the sex-differences observed in Alzheimer's Disease (AD). AD is the number one cause of late-onset dementia, characterized by progressive loss of memory and cognition, and currently has no cure. Epidemiological studies have found there are more women than men with AD, with a ratio of 2:1^{1,2}. Sex-differences also exist in the clinical manifestation and progression of the disease. For instance, most studies testing cognition in AD dementia find women score lower than men in verbal memory and fluency tasks³⁻⁵. The rate of cognitive decline and the conversion from mild cognitive impairment (MCI) to AD is also higher in women compared to men⁶⁻⁹. Though there are discrepancies in whether the measured pathological biomarkers are different between men and women, studies found that the same pathological burden in neurofibrillary tangles and amyloid plaques can have more detrimental effects on dementia in women than men¹⁰⁻¹². Moreover, studies have found an interaction between sex and the genetic risk factor *APOE4*, where female *APOE4* carriers had a higher conversion rate from MCI to AD, especially in younger age cohorts, suggesting female *APOE4* carriers have a higher susceptibility to AD¹³⁻¹⁵. In the case of mortality rate, however, a study found that men with AD have higher mortality than women, though women have more disabilities¹⁶. Together, these studies support the idea that sex differences are prevalent in AD, and prompt the need for further molecular investigations that take sex into account during data collection and analysis.

1.2 Transcriptomic sex-differences in Alzheimer's Disease

With the development and evolution of single-cell RNA sequencing technology and their diverse use in studying neurodegenerative diseases, many groups have now dissected the transcriptomic profiles of the human CNS to understand the molecular characteristics of AD. A handful of these studies have also reported the contributions of sex on these single-cell transcriptomic profiles. Mathys and colleagues were one of the first groups to comprehensively perform single-nuclei RNA-sequencing (snRNA-seq) on a large cohort of AD and control prefrontal cortex brain tissues (48 individuals – 24 with no/low AD pathology, 24 with high-levels of pathology, with equal numbers of male and female patients in each group)¹⁷. They found pathology, especially early-stage pathology, resulted in large changes in the transcriptome of all CNS cell types isolated. Further sub-clustering of each cell type revealed specific cell states were more enriched in pathology versus non-pathology conditions. Interestingly, these pathology-associated subpopulations were highly-enriched in female samples whereas non-pathology-associated subpopulations were more enriched in male samples. When correlated with disease traits, females and males also showed significantly different transcriptional responses. For instance, females showed downregulation of genes expressed in excitatory and inhibitory neurons while males showed upregulation of genes expressed in oligodendrocytes with increased tau and amyloid pathology. Intriguingly, they also found females had a significant association between white matter lesions (white matter hyperintensities measured by MRI) and lower cognition, but not in males, suggesting a potentially meaningful sex-specific response of oligodendrocytes to AD pathology¹⁷.

Another study using snRNA-seq of the entorhinal cortex reported some of the data variability could be explained by sex, though this variability was lower than the variability explained by individual samples¹⁸. The number of female and male patient samples, however, was

low (6 male, 2 female), making it challenging to establish sex-specific conclusions. We have also done preliminary studies analyzing transcriptomic sex difference at the single-nuclei level (3 male, 5 female, please reference methods section in Chapter 3), with samples matched for age, pathology, *APOE* genotype (E3/E4), and dementia rating scores (**Fig. 1.1 a,b**). We found significant numbers of differentially expressed genes located on the autosomal chromosomes in every cell type sequenced, with microglia having the most robust differences (**Fig. 1.1 c**). Although the number of single-nuclei and single-cell RNA sequencing studies on human and mouse tissues is increasing, usually the numbers of male and female samples are skewed, too few, and/or studies do not report the contribution of sex in their data analysis, making it difficult to extrapolate sex-specific findings. More studies are, therefore, necessary to dissect these molecular sex-differences in AD.

1.3 Microglial contributions to neurodegenerative diseases

In healthy brains, microglia play critical roles during development as well as during the aging process of the brain. They serve many functions in the CNS including surveillance of the environment through dynamic movements of their processes documented *in vivo*^{19,20}; phagocytosis and clearance of debris such as protein aggregates in disease models²¹⁻²⁴, synapses during development and neurodegeneration to modulate neuronal circuits²⁵⁻²⁸, and apoptotic cells in neurogenic niches^{29,30}; and signaling to other microglia and other cell types through cytokine release^{23,31,32}. Several groups have also published studies to transcriptomically and proteomically characterize microglia and their heterogeneity in normal adult mice and human brain tissues³³⁻³⁶.

The observation that microglia play a role in non-autoimmune neurodegenerative diseases was made by Alois Alzheimer in 1911, when he documented glial changes in post-mortem brain tissue in the first descriptions of AD patients³⁷. Similar findings were further shown in a study

published in 1987 that described microglia with amoeboid morphologies surrounding amyloid plaques in AD patient brains^{37,38}. GWAS and co-expression gene network studies also pointed to involvement of the innate immune system in late-onset AD, such as *BINI*, *TYROBP*, *CD33*, and *GRN*³⁹⁻⁴¹. The role of microglia in AD was further strengthened through whole genome sequencing studies revealing that mutations in a gene only expressed by microglia in the CNS, Triggering Receptor Expressed on Myeloid Cells 2 (*TREM2*), increased the risk of late-onset AD by two- to four-fold^{42,43}.

Microglia's involvement in other non-autoimmune neurodegenerative diseases have also been elucidated through GWAS and other genetic studies. For instance, mutations in *GRN*, which are highly expressed in microglia, cause Frontotemporal Dementia (FTD), although the protein is expressed in non-microglial cells as well^{44,45}, and a GWAS meta-analysis found enrichment of immune genes specifically in FTD⁴⁶. More recently, mouse models of these neurodegenerative diseases show microglial dysfunction and manipulation of microglial pathways alter disease pathology, including in mouse models of Amyotrophic Lateral Sclerosis (ALS)⁴⁷⁻⁴⁹, and Parkinson's Disease (PD)^{30,50,51}. Furthermore, microglia share common transcriptional profiles in many neurodegenerative diseases that distinguish them from their non-disease counterparts⁵²⁻⁵⁴, suggesting common pathways of microglial response in the diseased brain.

These studies have pushed the field to better understand microglial biology, but how microglia contribute to disease pathogenesis in neurodegeneration is still debated. Though many studies have illustrated the importance of the neuroimmune pathway in neurodegeneration, broad anti-inflammatory therapies have not been shown to be efficacious for non-autoimmune diseases such as AD, ALS, and PD⁵⁵. Therefore, there is a need to focus on the disease-specific mechanisms of microglial function and their heterogeneity in order to better target these cells for therapeutic

purposes. One intriguing aspect of microglia biology that may underlie some of the heterogeneity of these cells is their differences in male and female brains.

1.4 Microglial sex-differences in rodents

Previous studies highlighting sex differences in microglia show that microglia number, morphology, and immune molecule load is dependent on the age of the animal, brain region, as well as hormonal and environmental factors (**Fig. 1.2**). For instance, sex differences in number and morphology do not emerge until the testosterone surge at postnatal day 4 (P4) when males have more microglia with activated morphology, while this pattern shifts at P30 prior to the onset of adult circulating hormones⁵⁶. These findings seem to vary by brain region, and sex differences are especially prominent in the hippocampus and amygdala⁵⁶. Bulk microglia number through flow cytometry analysis, however, did not show differences in microglia number due to age or sex⁵⁷. Microglia can also influence sex-specific maturation of the brain, as seen in Lenz *et al.* (2013), where inactivation of microglia through minocycline prevents masculinization of the Preoptic Area (a region responsible for reproductive behaviors) assessed through microglia number, the masculine pattern of dendritic spines, and adult sexual behavior⁵⁸.

Microglia also have sex differences in their transcriptome. In Thion *et al.* (2018) and Hanamsagar *et al.* (2017), the authors show that during development, microglia isolated from male and female embryos and pups as well as during puberty do not display dramatic transcriptomic differences between the sexes^{59,60}. Both studies profiled microglia isolated from rodents over the course of development up to P60 and found that only in adulthood do microglia show sex-divergence in their gene expression^{59,60}. In Kang *et al.* (2018), the authors isolated microglia from 3-, 12-, and 24-month-old male and female mice and found sex differences at all time points, but

the transcriptome was especially divergent at 24-months of age⁶¹. This suggests sex differences could emerge more prominently in aging-related diseases, such as neurodegenerative diseases, as the microglia also become more sexually-divergent.

Although it is agreed upon that microglia have transcriptional sex-differences in adulthood, which sex has a more proinflammatory phenotype is debated. In Hanamsagar *et al.*, microglia from female mice were found to express more proinflammatory genes, especially genes involved in lipopolysaccharide (LPS) stimulation⁵⁹. An earlier study by Schwarz *et al.* (2012) also found that in bulk tissue, immune factors analyzed, including interleukin-1 beta, were more highly expressed in females compared to males⁵⁶. However, two studies published in 2018 by Villa *et al.* and Guneykaya *et al.* found that microglia isolated from 12-week-old male mice were more reactive and responsive to stimuli than those isolated from female mice^{62,63}. These disparate findings could be due to technical differences in the isolation protocol, the age of microglial isolation, microbiome and environmental factors, and/or the brain regions studied. Nevertheless, these studies suggest microglia from male and female animals exhibit transcriptome differences that become more distinct with aging.

1.5 Microglial sex differences in AD patients

Sex differences are prominent in the non-diseased brains as shown in many mouse studies. There is also mounting evidence that male and female microglial responses to pathology at the functional and transcriptomic level are also different in mice. For instance, disease-associated microglial signatures seem to be sex-specific in SOD1 mice, a mouse model of ALS⁶⁴, and microglial coverage and interaction with amyloid plaques is sex-specific in amyloid mouse models⁶⁵.

As mentioned previously, snRNA-seq has enabled the deep dissection of transcriptomic changes in AD patient brain tissues. These studies have shown sex-differences in the enrichment of disease-associated subpopulations in response to AD in multiple cell types, including microglia¹⁷. Yet many of the sex-specific conclusions for microglia specifically are not well-documented in AD, largely due to the fact that human studies have few sex-matched samples, not enough numbers of microglia sequenced, or regressed out sex as a factor during downstream analysis.

To better characterize microglial sex differences in AD brain tissues, we performed a preliminary study using snRNA-seq and transcriptomic analysis of microglia from 11 female and 11 male AD patient brain tissues. The samples were matched in age and pathology (**Fig. S1.1 a**). Following an established human snRNA-seq protocol^{18,66}, we sequenced single-nuclei, removed potential multiplets using DoubletFinder⁶⁷ and filtered for low-quality nuclei determined by gene counts, UMI counts, and percent mitochondrial genes per nuclei (**Fig. S1.1 b-d**). Microglia were subsetted by using the microglial makers *CD74*, *CSF1R*, and *C3*, resulting in 10,122 nuclei that were then used for downstream analysis. Nine distinct transcriptomic clusters were identified, with all clusters contributed by all samples at varying proportions (**Fig. 1.3 a, Fig. S1.1 e**).

Clusters 0-3 and cluster 9 cells had high expression of canonical microglial markers such as *P2RY12* and *TMEM119* (**Fig. 1.3 b**). Clusters 0-2 were the most abundant microglia (**Fig. S1.1 e**), suggesting they are likely to be homeostatic microglial states, while other microglial clusters may be more distinct activation states. Clusters 4 and 6 were likely neurons or potential doublets of neurons and microglia, while clusters 7 and 8 had high expression of peripheral myeloid cell markers such as *PTPRC*, *RUNX3*, and *CD163* (**Fig. 1.3 b**).

To further characterize these subclusters, we performed gene set enrichment analysis (GSEA) against previously-published single-cell microglial signatures from human AD patient brain tissues^{17,68} and 5XFAD mice⁵². As expected, clusters 7 and 8 were anti-enriched in most microglial reference datasets, as they are likely peripheral myeloid cells, not microglia (**Fig. 1.3 c**). For the microglia subclusters, cluster 3 cells were most reminiscent of previously-characterized disease-associated microglia⁶⁹ (DAM, **Fig. 1.3 c**), expressing DAM signature genes such as *SPPL*, *LPL*, and *GPNMB* (**Fig. 1.3 d**). Meanwhile, cluster 5 cells were most similar to a cluster found in Olah et al. (Olah 3, **Fig. 1.3 c,e**), which the authors claimed to be a subtype of homeostatic microglia based on assessment of inter-cluster relatedness⁶⁸. Cluster 9 cell were most similar to another cluster found in Olah et al. (Olah 9, **Fig. 1.3 c**), and was highly-enriched in genes involved in cell cycle and proliferation, such as *BRCA1*, *CENPP*, and *MND1* (**Fig. 1.3 f,g**).

To assess sex-differences in enrichment of these microglial clusters, we calculated the proportion of microglia in each cluster coming from female versus male patient samples. We found that many of the microglial subpopulations were enriched in a sex-dependent manner, with clusters 0, 3, 5, and 9 being more enriched in female than male samples, with the proliferative cluster 9 cells being most differentially enriched in female samples (**Fig. 1.3 h**). Differential expression analysis between male and female cells within individual subclusters largely yielded differences in genes encoded by the sex chromosomes but minimal differences in autosome genes (data not shown), suggesting similar microglial states exist in both male and female brains, though they vary in their proportions.

Together this preliminary analysis showed male and female AD brain tissues had distinct enrichment of microglial subpopulations, with female brains having more DAM-like microglia

and proliferative microglia, prompting further detailed investigations into sex-differences in microglial subpopulations and how they contribute to pathology in neurodegenerative diseases.

1.6 How are cellular sex differences established?

Sexual differentiation of the brain is achieved through the coordinated influence of the sex chromosomes and hormonal environment. The incomplete process of X-inactivation in females leading to approximately 15% of X-chromosome genes escaping inactivation⁷⁰ and the expression of genes on the Y-chromosome would suggest that potentially all brain cells are distinct between males and females, not just in traditionally sexually-dimorphic brain regions involved in reproduction and mating behaviors. How hormones contribute to brain sexualization on top of, and independently of, this differential genetic background is a major open question in the field of sex biology. Similarly, a characterization of the influence of sex hormones and sex chromosomes on microglia function needs to be further dissected to better understand how sex differences in microglia are established (**Fig. 1.4**).

Previous studies have shown that microglia are likely modulated by male and female sex hormones. Villa *et al.* (2018) demonstrated that hormones are necessary to initially establish microglial sex differences, as masculinizing the female brain by treating embryonic day 2 pups with benzoate resulted in transcriptionally “male” microglia in adult female mice⁶². Hormones not only serve to establish microglia identity during development but also continue to modulate microglial function in adulthood. For example, microglia are known to express steroid hormone receptors in normal and injured brains, and both estrogen and testosterone have anti-inflammatory effects on microglial cytokine production⁷¹⁻⁷⁴. In line with these findings, ovariectomized aged

mice show more microglia with inflammatory phenotypes and upregulation of inflammatory microglial and macrophage-associated genes^{75,76}.

Interestingly, young adult microglia are able to maintain their sex differences in the absence of hormones, as ovariectomized female mice did not drastically alter the microglial transcriptome⁶². These findings seem to be age-dependent, as Sarvari *et al.* found that ovariectomizing 2-month-old mice did not change the expression of inflammatory genes, while a similar treatment of 13-month-old mice had dramatic changes⁷⁶. Additionally, female microglia transplanted into male brains after microglia-depletion, using a colony-stimulating factor 1 receptor inhibitor, were found to retain their “female” transcriptomic profile⁶². These experiments suggest microglia are able to maintain some sex-specific signatures during young adulthood even in different hormonal environments. These sex-specific profiles could, therefore, be a result of genomic (i.e. sex chromosomal) and/or epigenetic regulation. This explanation is especially compelling since the X chromosome is enriched in many genes involved in the immune system, including Toll-like receptor pathways (*BTK*, *IRAK1*, *IKK γ*) and microRNAs (miR-18 and miR-19) involved in immune regulation⁷⁷⁻⁷⁹, suggesting sex chromosomes are an important sex-specific regulator of immune function.

Further studies are needed to establish how endocrine signaling changes microglial phenotypes specifically during aging when the estrous cycle becomes acyclic in female rodents and in post-menopause women. Changes in circulating hormone levels could also explain why the microglial transcriptome becomes more divergent as the mice age. Characterizing this would be especially important for understanding neurodegenerative diseases, as these diseases manifest in humans in the context of low circulating hormones⁸⁰.

1.7 Concluding remarks

Microglia from male and female rodents and humans exhibit sex-differences, likely owing to their ability to sense different hormonal environments and their differential sex chromosome composition. As the field of neurodegenerative diseases has come to appreciate the role of microglia in a myriad of diseases, we should also appreciate how these cells may be contributing to sex differences in the diseased brain. Further sex-specific studies need to be conducted with the mindset that sex differences in cells may be an adaptation to normalize the differences of the sexes. Moreover, these sex differences may be maladaptive in disease states and lead to differences in disease vulnerability and progression. Therefore, studying sex differences in microglia could shed light onto why sex differences exist in human neurological diseases.

In this dissertation, I focus on the contributions of microglial sex differences in the context of both healthy and tauopathy states. Specifically, in Chapter 2, I focus on how microRNAs, which are key modulators of immune cell function and are densely encoded by the X chromosome, regulate microglial phagocytosis function and microglial response to tau pathology. In Chapter 3, I explore sex-specific microglial transcriptomic changes in AD patients with and without the R47H mutation in the microglial-gene *TREM2*. Through these studies, I aim to use sex-differences as a lens to uncover novel aspects of microglial biology as well as illustrate the necessity to further understand sex-differences in neurodegenerative diseases in order to develop potential novel therapies for AD.

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1.8 Methods

Human Postmortem Samples

For methods used to generate Figure 1.1, please reference the methods section in Chapter 3. For Figure 1.4, tissues from the mid-frontal cortices from brains of AD donors (n = 11 females, 11 males) were used for single-nuclei RNA-sequencing, for a total of 22 samples. Samples were matched in age and tau pathology. Samples were obtained from the Mayo Clinic brain bank and University of Pennsylvania brain bank.

Additional information about the donors can be found in Figure S1.1 a.

Isolation of Nuclei from Frozen Postmortem Human Brain Tissue

The protocol for isolating nuclei from frozen postmortem human brain tissue was adapted from a previous study with modifications^{18,66}. All procedures were done on ice or at 4°C. In brief, postmortem brain tissue was placed in 1500 µl of Sigma nuclei PURE lysis buffer (Sigma, NUC201-1KT) and homogenized with a Dounce tissue grinder (Sigma, D8938-1SET) with 20 strokes with pestle A and 15 strokes with pestle B. The homogenized tissue was filtered through a 35-µm cell strainer, and were centrifuged at 600 g for 5 min at 4°C and washed three times with 1 ml of PBS containing 1% BSA, 20 mM DTT and 0.2 U µl⁻¹ recombinant RNase inhibitor. Then the nuclei were centrifuged at 600 g for 5 min at 4°C and re-suspended in 800 µl of PBS containing 0.04% BSA and 1x DAPI, followed by FACS sorting to remove cell debris. The FACS-sorted suspension of DAPI-stained nuclei were counted and diluted to a concentration of 1000 nuclei per microliter in PBS containing 0.04% BSA.

Droplet-based Single-nuclei RNA-seq of Human Brain Tissue

For droplet-based snRNA-seq, libraries were prepared with Chromium Single Cell 3' Reagent Kits v3 (10x Genomics, PN-1000075) according to the manufacturer's protocol. The snRNA-seq libraries were sequenced on the NovaSeq 6000 sequencer (Illumina) with 100 cycles.

Analysis of Droplet-Based Single-nuclei RNA-seq Data from Human Brain Tissue

Gene counts were obtained by aligning reads to the hg38 genome with Cell Ranger software (v.3.1.0) (10x Genomics). To account for unspliced nuclear transcripts, reads mapping to pre-mRNA were counted. Cell Ranger 3.1.0 default parameters were used to call cell barcodes. We further removed genes expressed in no more than 2 cells, cells with unique gene counts over 4,000 or less than 200, and cells with high fraction of mitochondrial reads ($> 5\%$). Potential doublet cells were predicted using DoubletFinder⁶⁷ for each sample separately with high confidence doublets removed. Normalization and clustering were done with the Seurat package v3.0.1⁸¹. In brief, counts for all nuclei were scaled by the total library size multiplied by a scale factor (10,000), and transformed to log space. A set of 2000 highly variable genes were identified with *SCTransform* from *sctransform* R package in the variable stabilization mode. This returned a corrected unique molecular identifiers (UMI) count matrix, a log-transformed data matrix, and Pearson residuals from the regularized negative binomial regression model. Principal component analysis (PCA) was done on all genes, and *t*-SNE was run on the top 20 PCs. Cell clusters were identified with the Seurat functions FindNeighbors (using the top 20 PCs) and FindClusters (resolution = 0.02). In this analysis, the neighborhood size parameter pK was estimated using the mean-variance normalized bimodality coefficient (BCMvn) approach, with 20 PCs used and pN set as 0.25 by default. To label the microglia cluster, the following markers were used: *CD74*, *CSF1R*, and *C3*.

Microglia Subclustering. The subset function from Seurat was used to subset microglia-only cells. Samples were integrated using the Seurat reciprocal PCA method using the first 30 PCs. Clusters were generated using FindNeighbors (using the top 30 PCs) and FindClusters (resolution = 0.4). Cluster markers were found using FindAllMarkers function and MAST differential expression⁸².

Gene Set Enrichment Analysis. Markers for each microglial cluster was passed through GSEA (v. 4.0.1)⁸³ using a pre-ranked gene list. Ranking was calculated by taking the sign of the log2 fold change value and multiplying by the inverse of the adjusted p value from the Seurat FindAllMarkers function. Reference datasets were taken from Mathys (M1 markers)¹⁷, Olah (Clusters 1-9)⁶⁸, and DAM⁶⁹ to compare all microglial markers and the Reactome dataset was used to compare with cluster 9 markers.

1.10 Main Figures

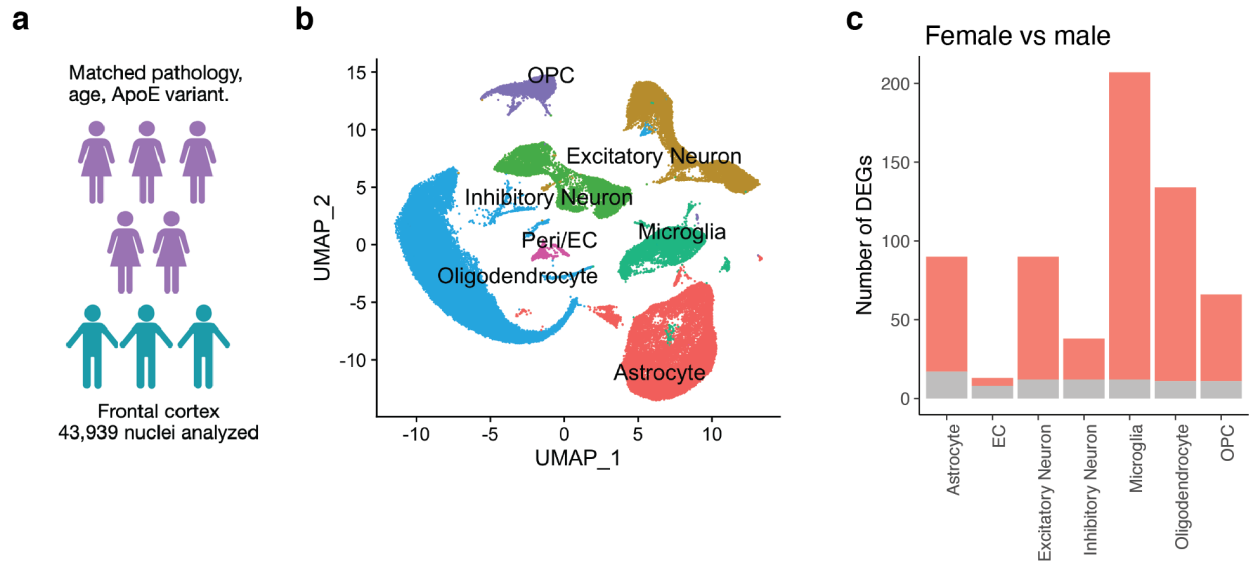


Figure 1.1. Male and female AD brains have significant sex-differences at the single-nuclei transcriptomic level. (a) Schematic showing the composition of AD patient donors used for single-nuclei sequencing. (b) UMAP of all nuclei sequenced colored by cell type. (c) Bar plot showing the number of DEGs between female and male nuclei (y-axis) for each cell type (x-axis). Grey bar denotes number of DEGs located on the sex chromosomes.

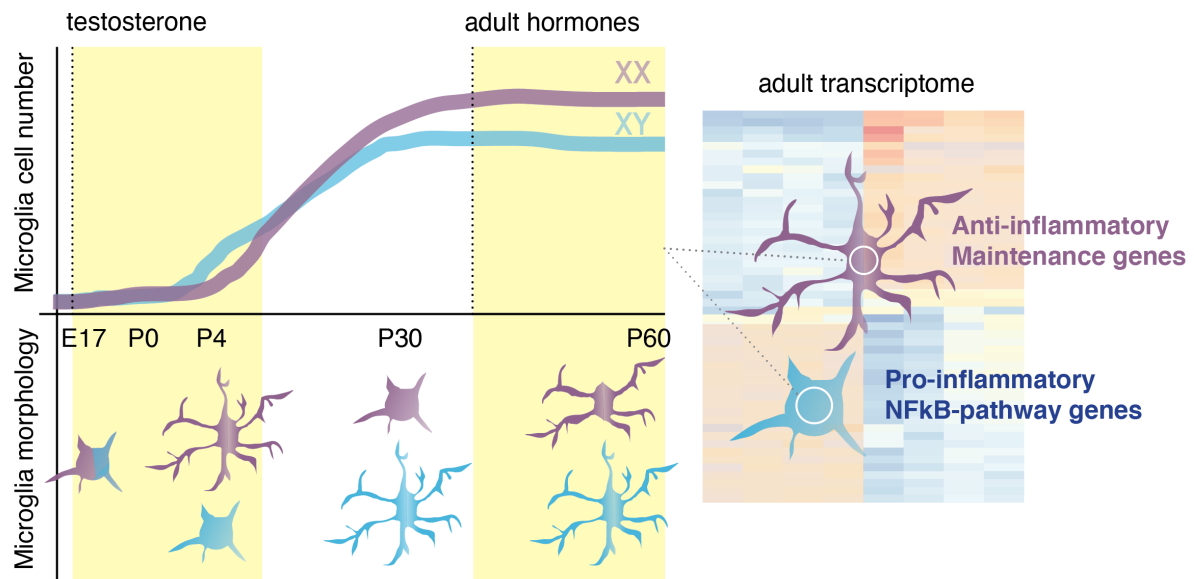


Figure 1.2. Overview of sex differences in microglia.

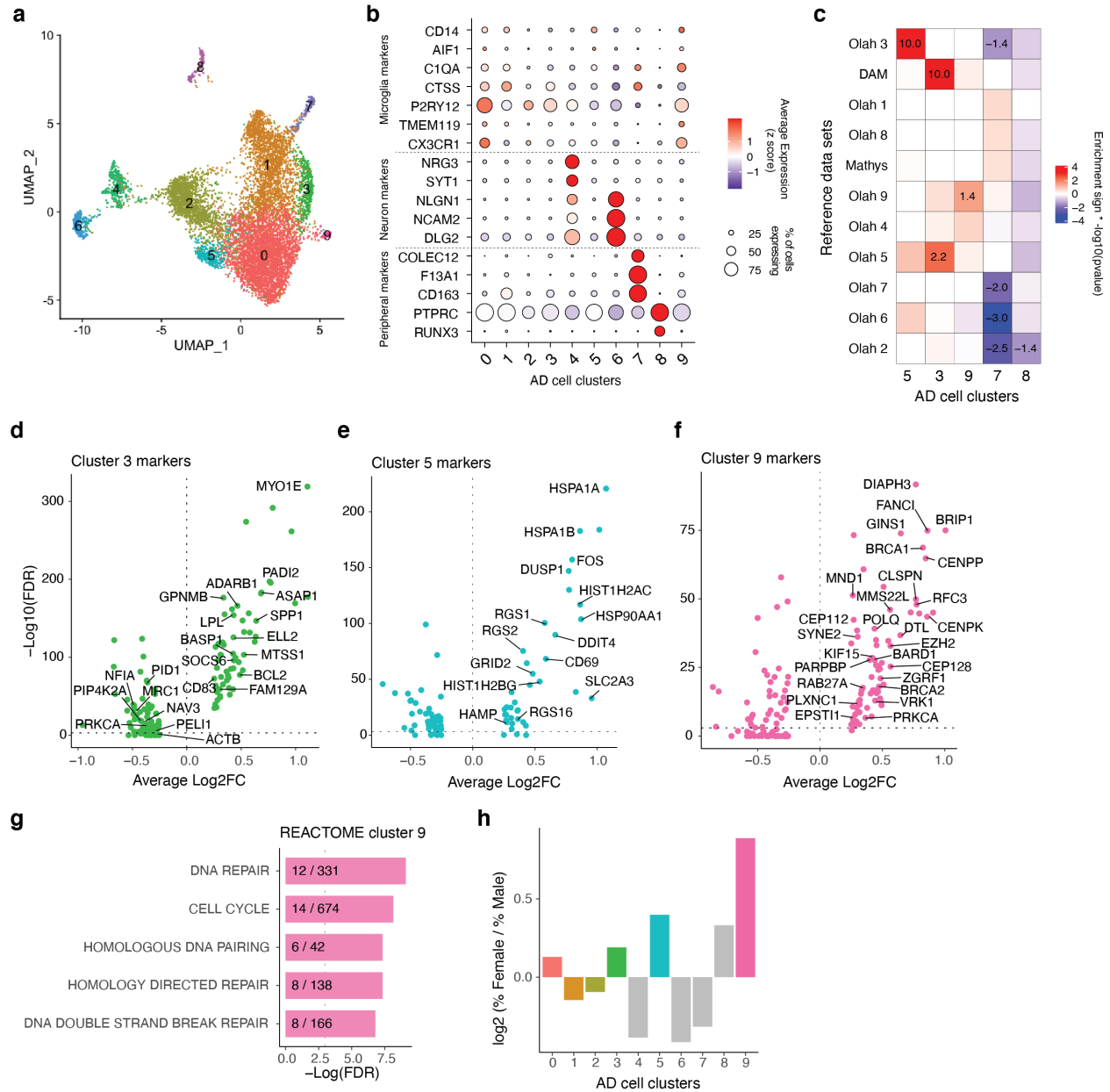


Figure 1.3. Sex-dependent enrichment of microglial subpopulations in male and female AD brains. (a) UMAP of 10,122 nuclei subclustered into 9 distinct transcriptomic clusters from 22 AD patient brain tissues ($n = 11$ samples / sex). (b) Dot plot showing average expression level as z scores for selected marker genes in each cell cluster. Color denotes z score and size of the circle denotes the percent of cells in which the gene was detected. (c) Heatmap showing gene set enrichment analysis results comparing marker genes of AD cell clusters against microglial signature genes identified in previously-published studies^{17,68,69}. Color denotes the sign of the enrichment score (+1 for positive enrichment, -1 for negative enrichment) multiplied by the $-\log_{10}(\text{p-value})$. (d-f) Volcano plots of DEGs between cluster 3 (d), cluster 5 (e), or cluster 9 (f) cells versus all other clusters. Highlighted genes are those that overlap with the DAM signature (d)⁶⁹, Olah 3 cluster (e)⁶⁸, and Olah 9 cluster (f)⁶⁸. Horizontal dotted line denotes $-\log_{10}(0.05)$. (g) Bar plot of pathways enriched in cluster 9 markers based on the Reactome data set. Numerator =

number of genes overlapping in the pathway; denominator = total number of genes in the pathway.
(h) Log2 ratio of cell fraction from female versus male samples. Grey bars for non-microglial clusters.

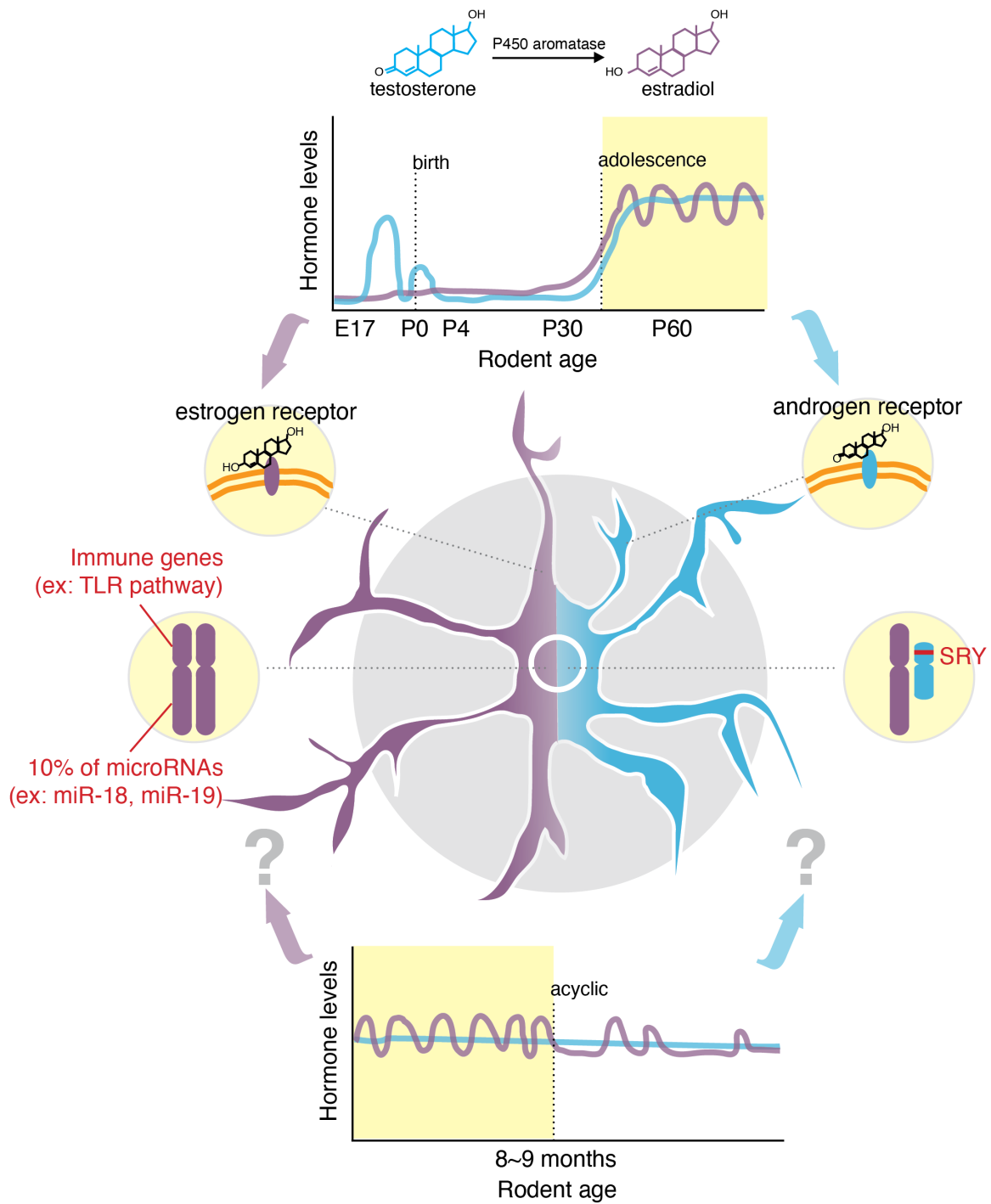


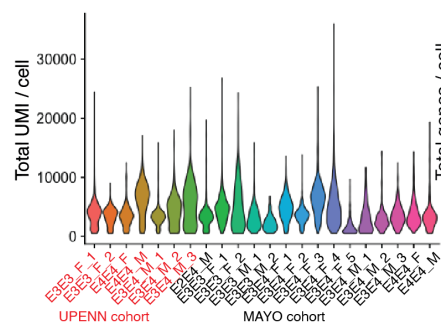
Figure 1.4. Model of how microglial sex differences are established.

1.11 Supplementary Figure

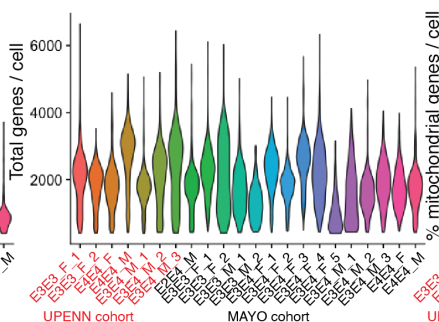
a

Gan Sort	ID #	Sex	Age	Age Onset	PMI	Clinical Diagnosis	Pathological Diagnosis	APOE	Braak	Thal phase	TDP-43	Brain Bank
Gan-43	108953	Female	83	77	4	Dementia with Lewy Bodies	AD	E3/E3	6			Upenn
Gan-44	105007	Female	87	78	16	Alzheimer's Disease Probable	AD	E3/E3	6			Upenn
Gan-45	102475	Female	72	66	5	Probable Alzheimer's Disease	AD	E4/E4	5			Upenn
Gan-47	122417	Male	64	60	6	Probable Alzheimer's Disease	AD	E3/E4	6			Upenn
Gan-48	111593	Male	71	62	4.5	Alzheimer's Disease Probable	AD	E3/E4	6			Upenn
Gan-49	107833	Male	78	64	6.5	Alzheimer's Disease Probable	AD	E3/E4	6			Upenn
Gan-50	100436	Male	78	70	6	Probable Alzheimer's Disease	AD	E4/E4	6			Upenn
Gan-2	142	M	83		10	AD	AD	E2E4	6	5		Mayo
Gan-4	121	F	85		5	AD	AD/DLBD	E3E3	5	5	0	Mayo
Gan-8	122	F	67		5	AD	AD	E3E3	5	5	0	Mayo
Gan-6	114	M	65		12	AD v FTD	AD/CAA	E3E3	6	5	0	Mayo
Gan-10	104	M	72		8	DLB	AD/DLBD	E3E3	5.5	5	0	Mayo
Gan-14	129	F	86		9	AD	AD/VaD	E3E4	5	5	1	Mayo
Gan-16	130	F	85		7	AD	AD/VaD	E3E4	4	3	0	Mayo
Gan-18	105	F	81		9	AD	AD/VaD	E3E4	6	5	0	Mayo
Gan-22	106	F	67		12	FTD	AD	E3E4	6	5	1	Mayo
Gan-24	137	F	81		10	AD	AD	E3E4	6	5	0	Mayo
Gan-20	138	M	80		6	CBD	AD	E3E4	6	5	0	Mayo
Gan-26	101	M	89		12	VaD	AD	E3E4	5	5	1	Mayo
Gan-30	119	M	79		7	AD	AD/TLBD	E3E4	6	5	0	Mayo
Gan-40	141	F	74		10	AD	AD/CAA/BLBD	E4E4	6	5		Mayo
Gan-38	115	M	80		9	AD	AD	E4E4	5.5	5		Mayo

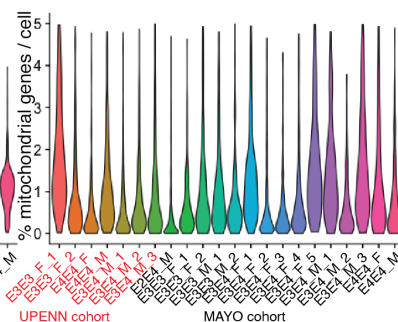
b



c



d



e



Figure S1.1. Single-nuclei sequencing of microglia from AD patient brain tissues. (a) Table with clinical information about the patient donors. (b-d) Violin plots showing total UMI counts /

cell (b), total genes / cell (c), and percent mitochondrial genes / cell (d) sequenced for each sample. Red samples from the University of Pennsylvania brain bank while black samples from the Mayo Clinic brain bank. (e) Proportion of each AD cell cluster in each sample.

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Chapter 2: Microglial Sex-Differences Mediated Through MicroRNAs

2.1 Introduction to microglial microRNAs

2.1.1 MicroRNAs in the context of neurodegeneration

First discovered in *C. elegans*, microRNAs (miRNAs) are ~22-23 nucleotide long, small-non-coding RNAs whose main known effect is suppression of target gene expression ¹. This suppression is largely mediated by complementary targeting of the 3'-untranslated-region (3'-UTR) of messenger RNAs (mRNAs) for degradation and translation suppression ^{2,3}. Although one miRNA has only modest suppression of target protein expression, one miRNA is able to target many downstream mRNAs, and one mRNA can be targeted by multiple miRNAs, giving miRNAs the ability to regulate diverse networks of molecules and cellular pathways. As such, miRNAs are known as functional fine-tuners, buffering protein level expression and adding a complex layer of translation control ⁴. Studies have also found that most 3'UTR sequences targeted by miRNAs are evolutionarily conserved, and it is speculated that almost all protein-coding genes may be targets of miRNAs, suggesting there is a strong functional pressure to maintain these complimentary binding sequences ^{2,5}.

Because of these robust and diverse roles of miRNAs, miRNA dysregulation can have profound effects on biological functions. Many human diseases, such as cancers, have been shown to be mediated by dysregulated miRNA expression ⁶⁻⁸. Changes in miRNA expression have also been studied in the context of neurodegeneration ^{9,10}. Various studies have shown that blunt-modulation of neuronal and glial miRNAs by knocking-out Dicer, an enzyme involved in the maturation of most known miRNAs, results in neurodegenerative phenotypes ¹¹⁻¹⁴. For instance,

one of the earliest studies knocked out Dicer (Dicer KO) in cerebellar Purkinje cells and found progressive Purkinje cell degeneration and ataxia in mice ¹¹. MiRNAs have also been studied in neurodegeneration as ways to understand how they may modulate known disease-risk gene expression. For instance, several studies have looked at miRNAs that target the 3'UTR sequences of AD risk genes such as *BACE1*, an enzyme involved in amyloid formation, and *APP*, the amyloid precursor protein, and found decreased expression levels of these miRNAs that correlated with increased expression of these risk-genes ¹⁵. Proteins that are dysregulated in diseases can also have downstream effects on miRNA biogenesis pathways, leading to changes in miRNA expression. Many studies have also profiled miRNAs in peripheral fluids as a way to identify novel disease biomarkers. Though these studies have shed light on the global changes of miRNAs in neurodegenerative diseases, patient-to-patient variability and disease stage variations have made it difficult to conclusively show which miRNAs may be specifically changed in disease conditions. A recent systematic review of all such studies found a select number of miRNAs from peripheral fluids from AD patients were changed in all studies, and many of the downstream targets of these commonly changed miRNAs were found to be immune-related ¹⁶. Together, these studies show that miRNA expression is dysregulated in the context of neurodegenerative diseases and may be involved in aspects of disease progression.

2.1.2 MicroRNAs in the immune system and microglia

MiRNAs have been extensively studied in many biological contexts, including in the regulation of the immune system, with many focusing on peripheral immune cells. They have been shown to be important for immune cell development, maturation, and tuning of cell responses to immune stimuli ^{17,18}. Immune cells constantly survey the environment and rapidly response to diverse

biological cues; as such, miRNAs may play a fundamental role in regulating and fine-tuning these dynamic responses. To globally understand whether miRNAs significantly contribute to immune cell function, studies have utilized the Dicer KO system to knockdown the expression of mature miRNAs. Most of these studies done in the early lineage cells have found that these cells fail to mature and undergo apoptosis, leading to significant reduction in cell number as well as having exacerbated immune responses, leading to autoimmunity¹⁹⁻²¹. Individual miRNAs can also have robust phenotypic consequences. For instance, miR-155 is an extensively-studied pro-inflammatory miRNA in the context of both adaptive and innate immune cells^{22,23}.

Microglia, as the resident innate immune cells of the central nervous system (CNS) and as a key immune regulator of CNS function during healthy and disease states, are also modulated by miRNAs. Several studies have performed comprehensive miRNA sequencing of isolated microglia versus peripheral tissue macrophages and monocytes, and found microglia from mice have a distinct miRNA profile from macrophages^{24,25}. Like in other cells, miRNAs have functional consequences on microglia biology. Removal of Dicer specifically in microglia using an inducible *Cx3cr1*-Cre system decreases microglia number, but does not significantly alter baseline microglial states. When exposed to immune stimuli, however, Dicer KO microglia have a hyper-active response, suggesting miRNAs are globally important for regulating proper immune response²⁵. Individual miRNAs have also been shown to modulate microglia function *in vivo* and *in vitro*²⁶. For instance, miR-124, primarily expressed by neurons in the CNS, was also found to be highly expressed in homeostatic microglia compared to peripheral macrophages and activated microglia in Experimental autoimmune encephalomyelitis (EAE) mice, a mouse model of Multiple Sclerosis, and could reduce microglial activation²⁷. Remarkably, intravenous injections of lipofectamine-coated miR-124 mimics could reduce EAE symptoms in mice even if treated after

symptom onset ²⁷. In this way, microglial miRNAs are able to robustly regulate microglial responses to pathology.

2.1.3 MicroRNA sex-differences

As outlined in Chapter 1 of this thesis, the aim of this dissertation is to uncover molecular pathways relevant to understanding the clinical sex-differences observed in AD. Why, then, do we focus on miRNAs? Microglia are genetically-implicated in AD and miRNAs are able to modulate microglia function. Meanwhile, miRNAs are also heavily-encoded by the X chromosome. According to miRbase, ~150 miRNAs are encoded by the X chromosome, while only 4 have been discovered on the Y chromosome in humans (in mice, 0 miRNAs are found on the Y chromosome) ²⁸⁻³³, giving the X chromosome a higher density of miRNAs when compared to autosomes ³⁴. Incomplete X-inactivation would presumably lead to sex-differences in the expression of these miRNAs, giving cells sex-specific miRNA expression, though a systemic study to look at which X-linked miRNAs escape inactivation has not been done. Aside from chromosomal differences, one study found that miRNAs can be regulated by sex hormones such as estrogen ³⁵.

Many X-linked miRNAs have been studied, including miR-221~222 cluster in the context of cancers as it can suppress *CDKN1B*, a tumor suppressor ^{36,37}, and therefore could be linked to the sex-differences in the incidence and prevalence of many cancers. X-linked miRNAs are also heavily-involved in the immune system, such as miR-18 and miR-19 ³⁸, as well as miR-223, which is involved in regulating neutrophil differentiation from myeloid precursors and is a negative regulator of LPS-response and sepsis ³⁹⁻⁴¹. Another X-linked cluster, miR-106a~363, which has two paralog clusters on autosomes, miR-17~92 and miR-106b~25 (and will be discussed further in Chapter 2.2), is also heavily-involved in immune cell regulation, such as monocyte

differentiation and maturation and downregulation of IL-10^{42,43}. Whether these X-linked miRNAs are involved in regulating sex-differences in cancer and immune-related diseases is unknown as most of these studies do not specifically report sex-comparisons^{38,44}. Given the evidence that X-linked miRNAs have robust cellular functions especially in regulating the immune system and the fact that the X-chromosome encodes a high-density of miRNAs, miRNAs may bridge the principle topics of discussion in this dissertation: Sex differences, AD, and microglia.

2.1.4 How to study microRNAs:

Clearly miRNAs are crucial for many diverse biological functions and cell types. Dicer KO studies give a comprehensive and global overview of how miRNAs may be modulating cellular biology, but do not shed light on which miRNAs may be important for which pathways. Some studies have demonstrated bioinformatic predictions of miRNAs based on the Dicer KO cell's transcriptome, which can lead to identifying the most influential miRNAs mediating these transcriptomic changes⁴⁵. MiRNA concentration has been shown to correlate with the level of reduction in target gene expression⁴⁶, and therefore these predicted miRNAs mediating the Dicer KO effect is likely miRNAs that are very highly expressed. This approach, however, is limited in only revealing those miRNAs with the most robust influence. Individual miRNAs, therefore, need to be targeted and studied to appreciate the full regulatory network of miRNAs.

Most studies that have taken this approach look for one or two downstream targets that may explain the miRNA-phenotype. These studies are largely done by first predicting the downstream targets computationally, doing luciferase assays to demonstrate binding of the miRNA to the 3'UTR of the target gene, and doing either KO or over-expression studies for the target gene and/or introducing a mutation in the 3'UTR sequence to prevent miRNA-binding.

These methods can lead to identification of novel genes involved in phenotypes of interest. This, however, does not take into consideration all the other targets of that miRNA. One of the powers of studying miRNAs is to leverage this molecular network approach to understand how biological pathways may be coordinated, but this can also be a daunting endeavor as miRNAs have many downstream target genes. Some studies have shown miRNA families with the same seed sequence can be functionally redundant and miRNAs from the same gene cluster or from the same transcript can target and coordinate the repression of functionally interrelated pathways ⁴⁷⁻⁴⁹. Therefore, studying miRNAs could shed light onto novel functional pathways and understand how they are coordinated to regulate common phenotypes.

In this chapter, I study miRNAs in the context of sex-specific effects on microglia function as a way to uncover novel molecular networks mediating cellular function in the context of health and neurodegeneration. I aim to take these approaches by first understanding how removal of mature miRNAs in microglia modulates cell function in health and in tauopathy (Chapter 2.2), as well as aim to study how individual miRNAs regulate microglial phagocytosis function (Chapter 2.3).

Chapter 2.2: Microglial microRNAs mediate sex-specific responses to tau pathology

2.2.1 Abstract

Sex is a key modifier of neurological disease outcomes. Microglia are implicated in neurological diseases and modulated by miRNAs, but it is unknown whether microglial miRNAs have sex-specific influences on disease. We show that microglial miRNA expression differs in males and females and that loss of miRNAs leads to sex-specific changes in the microglial transcriptome and tau pathology. These findings suggest microglial miRNAs influence tau pathogenesis in a sex-specific manner.

2.2.3 Introduction

Sex has heterogeneous effects on human disease pathogenesis, including in Alzheimer's disease (AD) and other neurological diseases^{50,51}. Dysfunction of microglia, the resident innate immune cells of the central nervous system, has been linked to many neurological diseases⁵²⁻⁵⁴. Sex differences in microglial gene expression and functions are seen in young adult mice^{55,56}, and may be especially pronounced in the aging brain^{57,58}. MicroRNAs (miRNAs) regulate immune networks in microglia^{25,27} and exhibit sex-specific expression in some cell types⁵⁹. However, whether microglial miRNAs are expressed and function in a sex-specific manner is unknown. A better understanding of microglial miRNA function could identify novel molecular networks that contribute to neurological diseases.

2.2.4 Results

Adult microglia have sex-dependent microRNA expression

We performed miRNA sequencing (miRNA-seq) on microglia isolated from brains of adult mice (B6C3F1/J). Unsupervised clustering grouped samples by sex (**Fig. 2.1 a**). Sixty-one miRNAs were enriched in males and 26 in females (**Fig. 2.1 b**, Supplementary Table 1). Thirteen of these (8 in males, 5 in females) were encoded by the X chromosome (Supplementary Table 2). qPCR analyses validated several differentially expressed (DE) miRNAs (**Fig. 2.1 c**). Despite differing miRNA profiles, male and female cortical microglia had similar branching complexities (**Fig. 2.1 d–f**) and did not show differences in phagocytic uptake of *E. coli* particles (**Fig. 2.1 g**), suggesting male and female microglia use different sets of miRNAs to achieve similar baseline phenotypes.

Loss of mature microRNAs affects microglia in a sex-dependent manner

To evaluate the transcriptional role of microglial miRNAs, we selectively depleted the miRNA-processing enzyme Dicer in adult microglia by crossing *Cx3cr1^{CreERT2/+}* with *Dicer^{fl/fl}* mice (Dicer KO). *Dicer* mRNA and Dicer protein levels were significantly reduced in CD11b⁺ cells, but not in CD11b[−] cells (**Fig. 2.2 a–d**), accompanied by depletion of several Dicer-dependent miRNAs that are highly-expressed in microglia (**Fig. 2.2 e**). Dicer removal resulted in decreases in microglial density (**Fig. 2.2 f**), consistent with previous studies²⁵. Interestingly, loss of mature miRNAs resulted in far greater changes in the transcriptome of male microglia than female microglia (**Fig. 2.2 g**, Supplementary Tables 3,4). Pathway analysis of male DE genes showed enrichment in immune system pathways, including genes involved in TNF α signaling through NF κ B activation, such as *Il1b*, *Cd69*, *Tlr2*, and *Vegfa* (**Fig. 2.2 h,i**). Upstream activators of this expression pattern included TNF α , NF κ B, and interleukins (Il2, Il1b) (**Fig. 2.2 j**), as predicted by Ingenuity Pathway

Analysis, suggesting an overall enhanced inflammatory state. Thus, removing miRNAs alters the expression of immune-related genes to a greater extent in male compared to female microglia.

Loss of microglial microRNAs increases DAMs and tau pathology in male PS19 mice

Microglia have been implicated in the spread of tau burden in the brain^{53,54,60}, but it is not known whether male and female microglia respond to tau pathology differently. To probe this, we used the P301S (PS19) tauopathy mouse model⁶¹ and labeled pathogenic forms of tau with the MC1 antibody⁶². The densities of MC1-positive tau inclusions and microglial densities were similar in male and female animals (**Fig. S2.1**). However, the miRNA and mRNA profiles were altered by tau pathology to a far greater extent in male microglia (**Fig. S2.2 a-c**, Supplementary Tables 5-7). We then identified a subset of the DE mRNAs predicted to be targets of DE miRNAs in male microglia, focusing only on miRNA and mRNA targets that have anti-correlated expression patterns (**Fig. S2.2 d**). Out of these, downregulation of miR-1249-5p explained the greatest number of upregulated targets in male PS19 microglia. These data suggest sex-specific microglial responses to similar levels of tau pathology.

Depleting Dicer in neurons results in tau hyperphosphorylation⁶³, but it is unknown how loss of microglial miRNAs alters tau pathology. We found that the density of MC1-positive tau inclusions was higher in male PS19 Dicer KO mice than in females, reflecting differences in accumulation in the cortex, amygdala, and piriform region (**Fig. 2.3 a-d**). Male PS19 Dicer KO mice also had more amoeboid-like microglia, consistent with increased pathology, while microglial densities were similar (**Fig. 2.3 e-g**, **Fig. S2.3 a**). Bulk sequencing of isolated microglia from these mice showed that aside from differential expression of sex-chromosome genes, such as *Xist*, *Ddx3y*, *Uty*, and *Eif2s3y*, male microglia had enrichment of genes involved in inflammation and phagocytosis, including *Spp1*, *Ccl6*, *Lpl*, *Il1b*, and *Cst7* (**Fig. 2.3 h**, Supplementary Table 8),

characteristic of disease-associated microglia (DAMs)^{64,65}. These differences may be exacerbated by age and tau pathology, as DAM genes were not upregulated in younger mice (**Fig. S2.3 b**). Transcriptional sex differences, however, were still observed in younger mice, highlighting the dynamic role of miRNAs in the sex-dependent adaptations to tau pathology at different stages of the disease.

To further probe these sex-specific effects in tauopathy, we did single-cell RNA-seq on PS19 Dicer KO microglia, gating on CD45⁺CD11b⁺ cells (**Fig. S2.3 c-e**). Of the 721 cells sequenced, 676 cells passed quality control and clustered into 5 distinct groups (**Fig. S2.3 f-k**). Female and male cells differentially distributed, such that females had more cluster 1 and 2 cells while males had more cluster 3 cells (**Fig. 2.3 i,j**). While the clusters were distinguishable by distinct markers (**Fig. S2.3 l**), expression of resident microglial markers was relatively uniform, except in cluster 5, which likely comprised of CD45^{hi} (*Ptprc*) peripheral macrophages (**Fig. S2.3 m**). Cluster 1 cells expressed higher levels of homeostatic genes such as *P2ry12*, *Tmem119*, *Hexb*, and *Sall1* compared to all other clusters (**Fig. 2.3 k**, Supplementary Table 9). Cluster 2 cells expressed higher levels of genes associated with microglia in human AD and experimental autoimmune encephalomyelitis mouse models^{66,67}, such as *Apoe*, *Ms4a7*, *Klra2*, *Clec12a*, and *Mrc1*, with a downregulation of homeostatic genes, such as *Sall1*, *P2ry12*, and *Gpr56* (**Fig. 2.3 l**, Supplementary Table 10). The male-enriched Cluster 3 cells expressed higher levels of genes involved in phagocytosis, exosome biology, and immune modulation seen in DAMs⁶⁴, such as *Lpl*, *Cst7*, *Spp1*, *Tyrobp*, *Cd9*, and *Cd63* (**Fig. 2.3 m**, Supplementary Table 11), consistent with the bulk RNA-seq (**Fig. 2.3 h**). IHC confirmed upregulation of lipoprotein lipase (Lpl) in male microglia (**Fig. 2.3 n,o**). This sex-dependent cluster enrichment supports the differential microglial response to tau

pathology, where male PS19 mice exhibited increased DAMs and decreased homeostatic microglia compared with females.

2.2.5 Discussion

In this study, we found that male and female microglia expressed different miRNAs, both at baseline and in tauopathy. These differences did not result in sex-dependent differences in microglia function, such as morphology and phagocytosis, nor in tau pathology load in tauopathy mice. However, loss of these miRNAs had sex-dependent consequences on the microglial transcriptome and tau pathogenesis. Therefore, microglial miRNAs are key contributors to sex-specific phenotypes and should be further studied to understand microglial biology in the context of neurodegenerative diseases.

We used Dicer knockout as a model to remove miRNAs in microglia. Although Dicer's main function is involved in miRNA-processing, several other non-canonical functions of this protein have been described. Dicer is primarily located in the cytoplasm of cells where it cleaves the hairpin loops of pre-miRNAs for subsequent loading onto the RNA-induced silencing complex (RISC). Studies have found that in some situations, Dicer can localize to the nucleus and have regulatory functions on transcription⁶⁸⁻⁷⁰. These non-canonical roles of Dicer cannot be ruled out in this study, making it especially important to further study how individual miRNAs and miRNA-specific regulatory mechanisms alter microglia function.

Sexual differentiation of the brain is achieved through the coordinated influence of the sex chromosomes and hormonal environment. The incomplete process of X-inactivation in females can lead to approximately 15% of X-chromosome genes to escape inactivation⁷¹. However, sex-dependent differential expression of miRNA expression in microglia could not be fully explained

by the sex chromosomal composition, as only 13 out of the 61 DE miRNAs were encoded by the X-chromosome, and contributed to both male and female differentially expressed miRNAs (8 in males, 5 in females, Supplementary Table 2). A study looking at transcriptomic sequences of multiple tissue types from multiple organisms has shown conservation of sex-biased genes across mammalian species, and 85% to 95% of those genes are autosomal, suggesting sex chromosomes can only explain a fraction of the sex-specific DEGs⁷². Previous studies specifically in microglia have also shown that microglia can be modulated by male and female sex hormones^{55,73,74}. How sex hormones modulate microglial miRNA expression is unknown. Several studies have uncovered estrogen's regulation of miRNA expression in other cell types and tissues, especially cancer cells. For instance, estrogen bound to estrogen receptors translocate to the nucleus to promote the transcription of estrogen-responsive miRNAs, such as miR-21, miR-23a, and miR-221/222^{75,76}. Estrogen can regulate expression of transcription factors that can then promote miRNA expression downstream. For instance, estrogen can induce expression of c-Myc, which can in turn increase expression of the miR-17~92 cluster⁷⁷. Some studies have also found estrogen can act directly on the miRNA-processing machinery, such as Dicer and Drosha⁷⁸. Much less is known about how androgens and testosterone regulate miRNA-expression in somatic cells. Such hormone-mediated regulatory mechanisms could also be at play for microglial miRNA expression. To fully differentiate the effects of sex hormones and chromosomes on the transcriptome, one would need to use the Four-Core Genotype mouse model^{79,80} (discussed in Chapter 4) and assess miRNA and mRNA expression in microglia from these mice.

Despite differences in miRNA expression at baseline and in tauopathy models, male and female microglia exhibit similar baseline phenotypes such as microglial morphology and phagocytosis, and do not have sex-dependent tau pathology levels. How do microglia function

similarly in sex-specific environments given differences in underlying genetics, hormone responsiveness and gene expression patterns? One possibility is that each sex uses different mechanisms to achieve similar baseline functional states i.e. aim to make the sexes functionally similar in sex-specific environments^{81,82}. This idea has been seen in other CNS cells. For example, knocking down expression of the Y chromosome gene, *Sry*, resulted in a decrease in Tyrosine Hydroxylase (TH) expression in the substantia nigra, leading to functional motor deficits in male mice⁸³. Notably, even though females have lower numbers of TH-positive neurons due to the lack of *Sry*, they have comparable motor function to healthy males. This suggests compensatory pathways may be at play in females to maintain motor function at a similar level to healthy males. In this way, sex differences could be sex-specific mechanisms for obtaining the same function.

In response to non-homeostatic disease states, sex differences in microglia may have important functional consequences for disease progression. In a model of ischemic stroke (permanent middle cerebral artery occlusion model), transplanting female microglia into male mice reduced the progression of ischemic damage compared to transplanting male microglia, suggesting a protective role of female microglia in ischemia⁵⁵. Interestingly, altering the maternal microbiome using a germ-free environment resulted in sex-specific effects on microglia after birth and later in adulthood⁸⁴. The unmasking of functional sex differences in non-homeostatic states suggests that differences in disease susceptibility may partially result from sex-specific maladaptive responses, and therefore, studying sex differences could provide novel insights into disease mechanisms. In this study, therefore, it is possible that males are more reliant on their miRNA-regulatory function to maintain their baseline states, whereas females use other regulatory methods. Therefore, perturbations such as removal of miRNAs, leads to exacerbated responses in male microglia compared to female microglia. To understand whether these male-specific

transcriptional changes are driving the increase in pathology, a similar approach used in the *Villa et al.* study could be taken⁵⁵, by depleting the female microglia from tauopathy mice and transplanting and repopulating the brain with male microglia. These kinds of more specific studies will be needed to further investigate this hypothesis.

We found that Cluster 3, which expressed a DAM-like signature, was enriched in male compared to female Dicer KO microglia. However, apolipoprotein E (*ApoE*), which is also part of the DAM signature, had the opposite enrichment pattern and was more enriched in female compared to male microglia (**Fig. 2.3 I**). Interestingly, *APOE* genotype has a sex-specific phenotype in AD. Humans with the $\epsilon 4$ variant of the *APOE* gene have a higher risk of developing late-onset AD, and this risk factor is correlated more strongly in females compared to males⁸⁵⁻⁸⁸. Emerging evidence suggests that ApoE is a modulator of the CNS immune system and can have differential outcomes on microglial function depending on the variant⁸⁹⁻⁹¹. A recent study also showed microglia are a major source of plaque-associated ApoE and that *ApoE* expression is modulated by Trem2 function in AD mouse models⁹². Interestingly, *ApoE* is highly upregulated in disease-associated microglia (DAM) and has a differential expression in male versus female SOD1 mice, a mouse model of ALS^{64,65,93}. Transcripts driven by the APOE network also seem to be upregulated in microglia isolated from female 24-month-old mice compared to those isolated from male mice⁵⁸. Similarly, knockout of miR-155, a pro-inflammatory miRNA acting downstream of ApoE, was found to decrease these disease-associated signatures in spinal cord microglia and had sex-specific effects on survival and rotarod performance⁹³. These studies suggest, DAM signatures are likely to be sex-dependent in various disease models, and alterations in these signatures can have sex-specific consequences on disease phenotypes, consistent with our findings.

2.2.6 Methods

Animals

For RNA-seq and histological analyses, *Cx3cr1*^{CreERT2/+} (<https://www.jax.org/strain/021160>) were crossed with *Dicer*^{fl/fl} (<https://www.jax.org/strain/006366>) and P301S transgenic (<https://www.jax.org/strain/008169>) mice to obtain *Cx3cr1*^{+/+;Dicer}^{fl/fl}, *Cx3cr1*^{+/+;Dicer}^{fl/fl};*P301S*⁺, *Cx3cr1*^{CreERT2/+;Dicer}^{fl/fl}, and *Cx3cr1*^{CreERT2/+;Dicer}^{fl/fl};*P301S*⁺ mice. For miRNA-seq, P301S transgenic mice (<https://www.jax.org/strain/008169>) and their background control mice (<https://www.jax.org/strain/100010>) were used. Mice of both sexes were studied. For animal studies, sample size was determined by using power analysis with type 1 error = 5%, power = 80%, and effect size and standard deviation based on previous studies using the P301S (PS19) transgenic mouse line. Randomization of groups was not relevant for this study as all animals in the study were treated similarly. Mice had free access to food and water and were housed in a pathogen-free barrier facility with a 12-h light/12-h dark cycle. All mouse experiments were approved by the Institutional Animal Care and Use Committee, University of California, San Francisco and complied with all relevant ethical regulations.

Drug administration

Tamoxifen (Sigma-Aldrich, T5648) was prepared in corn oil at 20 mg/ml and given by intraperitoneal injection (2 mg/day) for 10 consecutive days, starting when mice were 3–3.5 months of age.

Brain tissue harvest

Mice were anesthetized with avertin and transcardially perfused with phosphate-buffered saline (PBS). Whole brains were fixed in 4% paraformaldehyde for 48 h and transferred to 30% sucrose

for 48 h. 30- μ m thick coronal sections were prepared on a sliding microtome (Leica, SM2010R), immersed in cyroprotectant, and stored at -20 °C before staining.

Immunohistochemistry

Free-floating coronal sections were washed in PBS, permeabilized in PBST buffer (0.5% Triton X-100 diluted in PBS), and immersed in 5% normal donkey serum at room-temperature for 1 h. For anti-Dicer and anti-Lpl primary antibodies, antigen retrieval was performed using Reveal Decloaker (BioCare, RV1000M) for 10 min at 90 °C then cooled to room temperature before blocking. Primary antibodies were diluted in PBST containing 5% normal donkey serum. Brain sections were incubated in primary antibodies at 4 °C overnight and then with secondary antibodies at room temperature for 1 h. Tissues were mounted on glass slides with antifade mounting medium (Life Technologies, P36935). Primary antibodies and dilutions used for staining were the following: Anti-Iba1 (Abcam, ab5076, 1:500 and Wako, 019-19741, 1:500), anti-Dicer (Abcam, ab167444, N167/7, 1:100), anti-Lpl (Abcam, ab21356, LPL.A4, 1:50) and anti-MC1 (a kind gift from P. Davies, 1:500)⁶². Secondary antibodies used were the following at 1:500 dilution: Cy3 AffiniPure Donkey Anti-Goat (Jackson ImmunoResearch, 705-165-147), Alexa Fluor 488 Donkey Anti-Mouse (Jackson ImmunoResearch, 715-545-150), Alexa Fluor 488 Donkey Anti-Mouse (Thermo Fisher Scientific, R37114). Validation information for primary antibodies included in “Life Sciences Reporting Summary”.

Epifluorescence fluorescence microscopy

Each coronal hemibrain slice was scanned at 10x magnification with an epifluorescence microscope (BZ-9000, Keyence). The images were stitched together with Keyence BZ-X Analyzer software (v1.3.0.3).

Confocal fluorescence microscopy

Images of hemibrain slices were acquired using an LSM880 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY) at 1 μm focal plane intervals at 40x magnification. Images were examined by maximum intensity Z-projection.

Image analyses

All images were analyzed with ImageJ 1.51⁹⁴. For MC1 density quantification, tiff images were processed with the auto local threshold Phansalkar plugin⁹⁵, regions of interest were hand-traced, and MC1+ puncta were counted with the “Analyze Particles” function. For morphological analysis, confocal images of the lower cortical region were analyzed with Imaris software (v9.0.2, Bitplane) using the “Filament Tracer” function for microglial processes. Investigators were blinded during image acquisition and analysis.

Adult microglia isolation

Adult microglia were isolated by magnetic activated cell sorting as previously described⁹⁶. Briefly, mice were anesthetized with avertin and transcardially perfused with PBS to remove circulating blood cells in the central nervous system. Dissected brains were digested with 3% collagenase type 3 (Worthington, LS004182) and 3 U/ml dispase (Worthington, LS02104) and incubated at 37 °C for 45 min. Digestion was stopped with inactivation buffer containing 2.5mM EDTA (Thermofisher, 15575020) and 1% fetal bovine serum (Invitrogen, 10082147). Tissue was then triturated in a serological pipette several times and passed through a 70- μm filter. Myelin in the homogenate was depleted with myelin removal beads (Miltenyi Biotec, 130-096-733) and a magnetic LD column (Miltenyi Biotec, 130-042-901). Microglia were isolated from the elutant

with CD11b magnetic beads (Miltenyi Biotec, 130-049-601) and a magnetic MS column (Miltenyi Biotec, 130-042-201).

High-throughput miRNA-sequencing

Freshly isolated microglia from 2 mice were pooled, and RNA enriched in miRNAs was extracted with the miRvana miRNA Isolation Kit and phenol (Invitrogen, AM1560) as recommended by the manufacturer. Total RNA (100 ng) was used for adapter ligation with the Total RNAseq Kit v2 (Life Technologies, 4475936) and subsequently run on a 1% Tris-borate-EDTA acrylamide gel followed by gel extraction of small RNA (45–60 bp). The small RNA fraction was used for downstream reactions to build a small RNA library. Libraries were sequenced in multiplex on an Ion Proton (ThermoFisher Scientific, 4476610) and reads were mapped to mature miRNA from mirBase with Bowtie2 (v2.3.4)⁹⁷.

miRNA-seq data analyses

Batch effects were removed with the R package RUVSeq⁹⁸ (v1.16.1) and normalized to *in silico* control genes. 1 male and 1 female sample were excluded from the miRNA-seq analysis based on principle component analysis. Differences in gene expression were calculated with the R package DESeq2⁹⁹. Counts were normalized with the trimmed mean normalization method¹⁰⁰. Genes with <15 counts across all samples were excluded from analysis. The false discovery rate (FDR) was calculated by the Benjamini-Hochberg method¹⁰¹. Predicted miRNA targeting of mRNA transcripts was done using Ingenuity Pathway Analysis (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>)¹⁰². Results were filtered for those with opposing log fold change values between mRNA and miRNAs and were either experimentally observed or highly predicted to be miRNA-mRNA target interactions.

High-throughput bulk RNA-sequencing

Freshly isolated microglia from 2 mice were pooled, and total RNA was extracted with the RNeasy mini kit (Qiagen, 74104). RNA quality was examined with a Bioanalyzer 2100 (Agilent Genomics). RNA samples with RNA integrity numbers >8 were used to construct a cDNA library. Oligo(dT) beads were used to enrich for mRNA. cDNA library generation was done using the QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (Lexogen, 015.96). The quality of the cDNA library was assessed by using a Nanodrop spectrophotometer to determine concentration and a Bioanalyzer 2100 to determine insert size. cDNA library samples were then sequenced with the HiSeq 4000 system (Illumina).

RNA-seq data analyses of bulk RNA-seq

RNA-seq reads were mapped using the Bluebee Genomics Platform and using the GENCODE mouse genome GRCm38 (Lexogen QuantSeq 2.2.3). Differential gene expression was calculated with the R package DESeq2⁹⁹, using the Wald test. Counts were normalized with the trimmed mean normalization method¹⁰⁰. Genes with <15 counts across all samples were excluded from analysis. The FDR was calculated with the Benjamini-Hochberg method¹⁰¹.

Real-time quantitative PCR

For miRNA qPCR, 10 ng of total RNA was reverse transcribed with the miRCURY LNA RT Kit (Qiagen). RT-qPCR was done with Power SYBR Green PCR master mix (Thermo Scientific) and miR-specific LNA primers (Qiagen) according to the Q5 Quantstudio system protocol. A small ribosomal RNA, 5S, was used for normalization across all samples, and relative differences were calculated with the $2^{-\Delta\Delta Ct}$ method. To validate *Dicer* mRNA reduction, RNA was converted to cDNA with iScript Reverse Transcription Supermix (BioRad). Quantitative RT-PCR was done

with an ABI 7900 HT sequence detector (Applied Biosystems) and SYBR Green PCR master mix (Applied Biosystems). GAPDH was used for normalization, and relative differences were calculated with the $2^{-\Delta\Delta C_t}$ method. The following primers were used for quantitative RT-PCR. Dicer: forward, CCTCGGGCCTTGAGGATTTT; reverse, CGACGCCACAGTTCTCTTCT. GAPDH: forward, GGGAAGCCCATCACCATCTT; reverse, GCCTTCTCCATGGTGGTGAA.

Gene network and functional analyses

Gene network analyses of RNA-seq data were done with gene set enrichment analysis (GSEA)¹⁰³ and the Hallmark gene dataset¹⁰⁴. Networks were visualized with Cytoscape (v3.6.1)¹⁰⁵, the STRING database¹⁰⁶, and perfuse force directed layout.

Preparation of tissue for scRNA-seq

Brain tissue was prepared as described¹⁰⁷. Briefly, 9-month-old mice were anesthetized with avertin and transcardially perfused with phosphate-buffered saline (PBS). The brain without the cerebellum was harvested and collected into cold medium containing 15mM HEPES, 0.5% glucose in Hank's balanced salt solution without Phenol Red; the entire procedure was done on ice. Brains were minced with a razor blade and homogenized with a 2ml douncer containing 2ml of medium A with 80 μ l DNase (12500 units/ml) and 5 μ l of recombinant RNase inhibitor (Takara Bio 2313B). Homogenized tissue was filtered through a 70- μ m strainers to obtain a single cell suspension. Cells were washed with medium A and resuspended in 850 μ l of MACS buffer containing 1.8 μ l of RNase inhibitor (sterile-filtered 0.5% bovine serum albumin, 2 mM EDTA in PBS). Cells were incubated with 100 μ l of myelin removal beads (MACS Miltenyl Biotec) for 10 min, and loaded onto LD columns (Miltenyi Biotec). Cells were collected and washed for FACS staining.

Single cell sorting for scRNA-seq

Cells were blocked in 5 μ l of mouse Fc block for 5 min on ice then incubated with primary antibodies for 10 min and washed with FACS buffer (sterile-filtered 1% fetal calf serum, 2mM EDTA, 25 mM HEPES in 1XPBS). Cells were incubated with secondary antibodies for 10min then washed with FACS buffer. Cells were resuspended in 500 μ l of FACS buffer with RNase inhibitor (Takara Bio 2313B, 1:500) and 0.5 μ l of propidium iodide (Thermo Fisher Scientific P3566, 1:1000) for single cell index sorting. Cell sorting/flow cytometry analysis was done on the cell sorter (BD InFlux) at the Stanford FACS Facility. The following gates were used for sorting microglia: (1) forward scatter-area (FSC-A)/side scatter-area (SSC-A) (2) Trigger Pulse Width/FSC (3) Live- Dead negative using propidium iodide (4) CD45^{low}CD11b⁺ and CD45^{hi}CD11b⁺. Single cells were sorted into 96-well plates containing 4 μ l of lysis buffer (4U Recombinant RNase Inhibitor (Takara Bio 2313B), 0.05% Triton X-100, 2.5mM dNTP mix (Thermo Fisher Scientific R0192), 2.5 μ M Oligo-dT30VN (50-AAGCAGTGGTATCAACGCAGAGTACT30VN-30, ERCC Spike-ins (Thermo Fisher Scientific 4456740) diluted at 1:2.4x10⁷). Plates were vortexed, spun down and frozen on dry ice, and plates were stored at -80°C freezer. Antibodies used for FACS: rabbit anti-mouse Tmem119 (Abcam ab210405, ~200 μ g/ μ l, 106-6, 1:400 dilution), CD45-PE-Cy7 (Thermo Fisher Scientific 25-0451-82, 30-F11, 1:300), CD11b-BV421 (BioLegend 101236, M1/70, 1:300), goat anti-rabbit Alexa 488 (Thermal Fisher Scientific 11034, 1:300).

ScRNA-seq library preparation

Sequencing libraries were prepared following the Smart-seq2 published protocol¹⁰⁸. Briefly, plates were thawed and incubated at 72°C for 3 min in order to anneal RNAs to the Oligo-dT30VN primer. The 6 μ l of the reverse transcription mixture was added to each well: 95U SMARTScribe Reverse Transcriptase (100U/ μ l, Clontech 639538), 10U RNase inhibitor (40U/ μ l), 1XFirst-Strand

buffer, 5 mM DTT, 1M Betaine, 6mM MgCl₂, 1 μM TSO (Exiqon, Rnase free HPLC purified). RT was performed at 42°C for 90 min, followed by 70°C, 5 min. 15 μl of PCR amplification mix containing the following reagents was added to each well: 1X KAPA HIFI Hotstart Master Mix (Kapa Biosciences KK2602), 0.1 μM ISPCR Oligo (AAGCAGTGGTAT CAACGCAGAGT), 0.56U Lambda Exonuclease (5U/μl, New England BioLabs M0262S). cDNA was amplified using the following PCR program: (1) 37°C 30 min; (2) 95°C 3 min; (3) 23 cycles of 98°C 20 s, 67°C 15 s, 72°C 4 min; (4) 72°C 5 min. cDNA samples were purified using PCRClean DX beads (0.7:1 ratio, Aline C-1003-50), and resuspended in 20 μl EB buffer. cDNA quality was examined with a Fragment Analyzer (AATI, High Sensitivity NGS Fragment Analysis Kit: 1 bp - 6000 bp). To make libraries, all samples were diluted down to 0.15 ng/μl in 384-well plates using Mantis Liquid Handler (Formulatrix) and Mosquito X1 (TTP Labtech) with customized scripts. Nextera XT DNA Sample Prep Kit (Illumina FC-131-1096) was used at 1/10 of recommendation volume, with the help of a Mosquito HTS robot for liquid transfer. Tagmentation was done in 1.6 μl (1.2 μl Tagment enzyme mix, 0.4 μl diluted cDNA) at 55°C, 10 min. 0.4 μl Neutralization buffer was added to each well and incubated at room temperature for 5 min. 0.8 μl Illumina Nextera XT 384 Indexes (0.4 μl each, 5 μM from 4 sets of 96 indexes) and 1.2 μl PCR master mix were added to amplify whole transcriptomes using the following PCR program: (1) 72°C 3 min; (2) 95°C 30 s; (3) 10 cycles of 95°C 10 s, 55°C 30 s, 72°C 1 min; (4) 72°C 5 min. Libraries from one 384 plate were pooled into an Eppendorf tube and purified twice using PCRClean DX beads. Quality and concentrations of the final libraries were measured with Bioanalyzer and Qubit, respectively. Libraries were sequenced on the Illumina HiSeq 4000 at the Weill Cornell Medicine Genomics and Epigenomics Core Facility.

Processing of scRNA-seq raw data

Prinseq¹⁰⁹ v0.20.4 was first used to filter sequencing reads shorter than 30 bp (`-min_len 30`), to trim the first 10 bp at the 5' end (`-trim_left 10`) of the reads, to trim low-quality reads from the 3' end (`-trim_qual_right 25`) and to remove low-complexity reads (`-lc_method entropy, -lc_threshold 65`). Then, Trim Galore v0.4.3 (<https://github.com/FelixKrueger/TrimGalore>) was applied to trim the Nextera adapters (`-stringency 1`). The remaining reads were aligned to the mm10 genome by calling STAR¹¹⁰ v2.5.3a with the following options: `-outFilterType BySJout,-outFilterMultimapNmax 20,-alignSJoverhangMin 8,-alignSJDBoverhangMin 1,-outFilterMismatchNmax 999,-outFilterMismatchNoverLmax 0.04,-alignIntronMin 20,-alignIntronMax 1000000,-alignMatesGapMax 1000000,-outSAMstrandField intronMotif`. Picard was then used to remove the duplicate reads (`VALIDATION_STRINGENCY = LENIENT, REMOVE_DUPLICATES = true`). Finally, the aligned reads were converted to counts for each gene by using HTSeq (`-m intersection-nonempty, -s no`)¹¹¹.

Quality control for scRNA-seq data

The following criteria were used to filter out cells with low-quality sequencing. The distribution of total reads (in logarithmic scale) was fitted by a truncated Cauchy distribution, and data points in two tails of the estimated distribution were considered to be outliers and eliminated. Fitting and elimination were then applied to the remaining data. This process was run iteratively until the estimated distribution became stable. The threshold was set to the value at which the cumulative distribution function of the estimated distribution reaches 0.05. Cells with small numbers of detected genes and poor correlation coefficients for ERCC (low sequencing accuracy) were dropped. 676 cells were retained for downstream analysis after filtering from 721 cells.

Clustering analysis of scRNA-seq data

The Seurat v3.0.1 R package was used to perform unsupervised clustering analysis on the filtered scRNA-seq data^{112,113}. Gene counts were normalized to the total expression and log-transformed. Principal component analysis was performed on the scaled data using highly variable genes as input. The JackStrawPlot function was used to determine the statistically significant principal components. These principal components were used to compute the distance metric and generate cell clusters. Non-linear dimensional reduction (t-SNE) was used to visualize clustering results. Differentially expressed genes were found using the FindAllMarkers function that ran Wilcoxon rank sum tests.

Statistics

All experiments were done with at least two biological replicates. Mean values from each mouse were used for computing statistical differences. Data distribution was assumed to be normal but this was not formally tested. Individual data points are shown when possible. Statistical analyses were done with Prism 7.0e (Graphpad, San Diego, CA) and R v3.5.1 (F Foundation for Statistical Computing, Vienna, Austria). Data were visualized with Prism or the R package ggplot2¹¹⁴. Two-tailed *t* tests were used to compare two groups and two-sided Fisher's exact tests were used to analyze contingency tables. Differential expression analysis for bulk-sequencing and single-cell sequencing was done using the Wald test and the Wilcoxon rank sum test, respectively, with *P* values corrected by Benjamini-Hochberg. $P < 0.05$ was considered statistically significant.

2.2.7 Data Availability

The bulk RNA-seq and miRNA-seq data that support the findings of this study were deposited in the Gene Expression Omnibus repository (<https://www.ncbi.nlm.nih.gov/geo/>). Bulk RNA-seq

data was deposited under accession number GSE122663, and single-cell RNA-seq data was deposited under accession number GSE135330.

2.2.8 Acknowledgements

We thank the Gladstone Histology & Light Microscopy Core and the Weill Cornell Medicine CLC Microscopy and Image Analysis Core Facility for help with imaging, and the Gladstone Bioinformatics Core for help with sequencing data analysis. Bulk RNA-seq was done at the Center for Advanced Technology, University of California, San Francisco. Single-cell RNA-seq was done at the Weill Cornell Medicine Genomics and Epigenomics Core Facility. FACS was done at the Stanford FACS Facility. We thank S. Ordway and K. Claiborn for editing the manuscript, K. M. Ansel and his lab members for comments on the manuscript and discussions on miRNA biology, and Y. Fu (University of California, San Francisco) for the *Dicer^{fl/fl}* mice. We apologize that many relevant publications are not cited due to space limitations. This work was supported by NIH grants 1R01AG054214-01A1, U54NS100717, R01AG051390, and Tau Consortium grant (to L.G.), NIH grants 1F30AG062043-02 and T32GM007618 (to L.K.), NIH grant F31AG058505 (to F.A.S.), NIH grant U54NS100717, Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, Larry L. Hillblom Foundation, and the Edward N. & Della L. Thome Memorial Foundation (to K.S.K.), Alzheimer's Association AACSF 17-531484, NIH grant R25 R25NS070680, and UCSF Clinical and Translational Science Institute 5TL1TR0018 (to C.D.C.), IRACDA Postdoctoral fellowship (to J.C.U.), and R01MH110504 (to G.Y.). Gladstone Institutes received support from the National Center for Research Resources Grant RR18928.

2.2.9 Main Figures

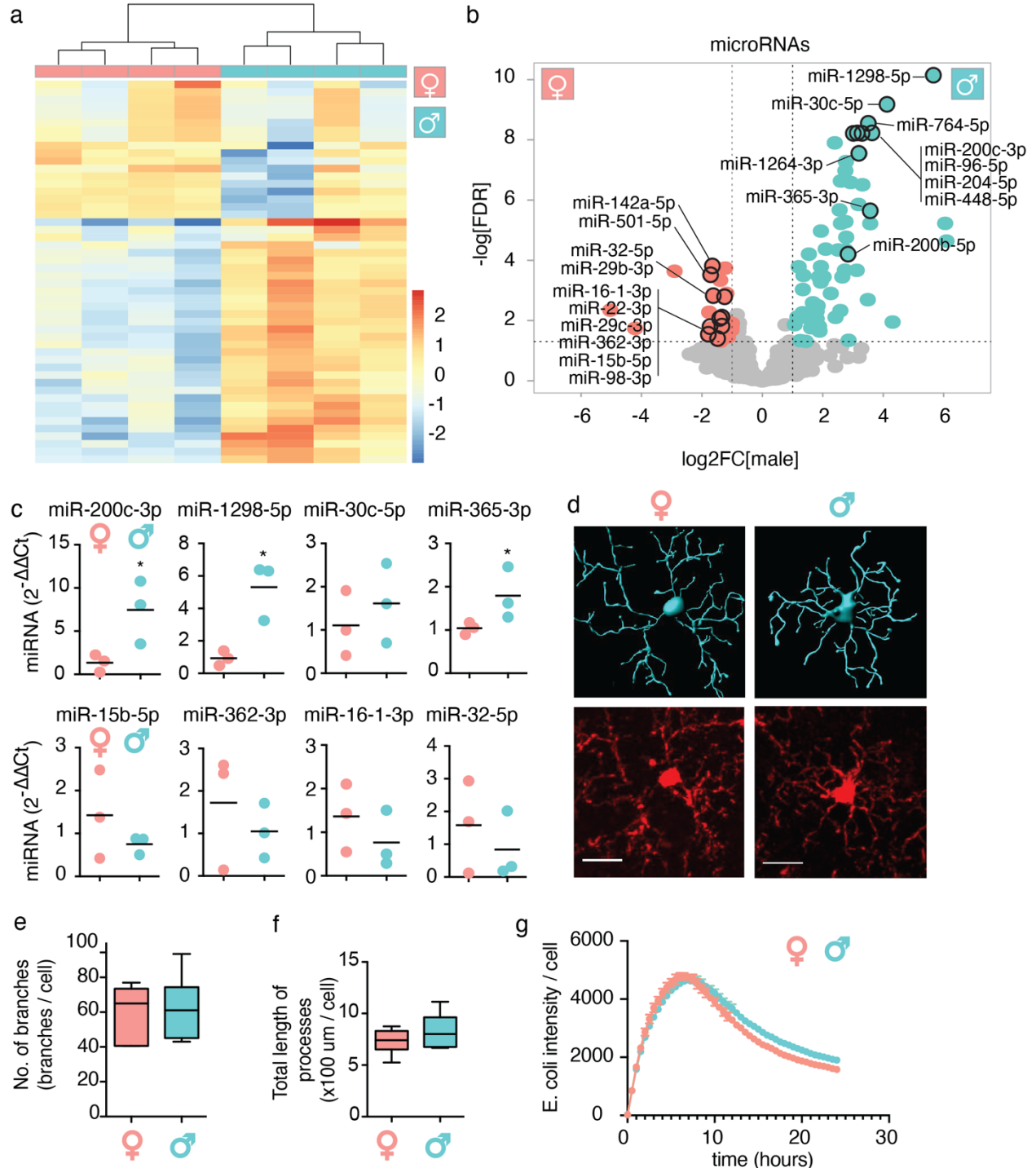


Figure 2.1. Adult microglia have sex-dependent microRNA expression.

(a) Heatmap of 50 miRNAs measured by miRNA-seq of microglia isolated from 6-month-old mice. Counts were log-transformed, normalized, and centered. $n = 4$ biologically independent samples/sex, 2 animals/sample. (b) Volcano plot of data from (a). Turquoise, enriched in males (61 miRNAs; $P \leq 0.05$ by Benjamini-Hochberg correction and $\log_2FC \geq 1$); pink, enriched in

females (26 miRNAs; $P \leq 0.05$ and $\log_2\text{FC} \leq -1$); grey, not significantly different. Top 10 miRNAs in each sex with highest $\log_2\text{FC}$ values and normalized counts over 100 are labeled. Vertical dashed lines indicate $\log_2\text{FC} \pm 1$. Horizontal dashed line indicates $-\log_{10}(0.05)$. Full list of DE miRNAs in Supplementary Table 1. Wald test used. (c) qPCR validation of differentially expressed miRNAs from (b). Each dot represents one mouse. Bar denotes mean. $n = 3$ biologically independent samples/sex, 2 animals/sample. * $t = 2.784$, $\text{df} = 4$, $P = 0.0496$ (miR-200c-3p), * $t = 4.119$, $\text{df} = 4$, $P = 0.0146$ (miR-1298-5p), * $t = 2.864$, $\text{df} = 4$, $P = 0.05$ (miR-365-3p), unpaired, two-tailed t test. (d) Representative 3D Imaris morphology reconstruction (top) and corresponding Iba1 immunostaining (bottom) of cortical microglia from 9-month-old mice. Image acquired and analysis repeated for every mouse quantified in (e,f). Scale bar, 15 μm . (e,f) Number of branch points (e) and total length of processes (f) per cell, determined by Imaris-based automatic quantification. Error bars represent SEM. Bar denotes mean. Each dot represents one mouse. ~60 cells/sex. (g) Bulk fluorescence intensity quantification of pH-rodo conjugated to *E. coli* normalized by primary microglia cell number ($n = 3$ wells/sex).

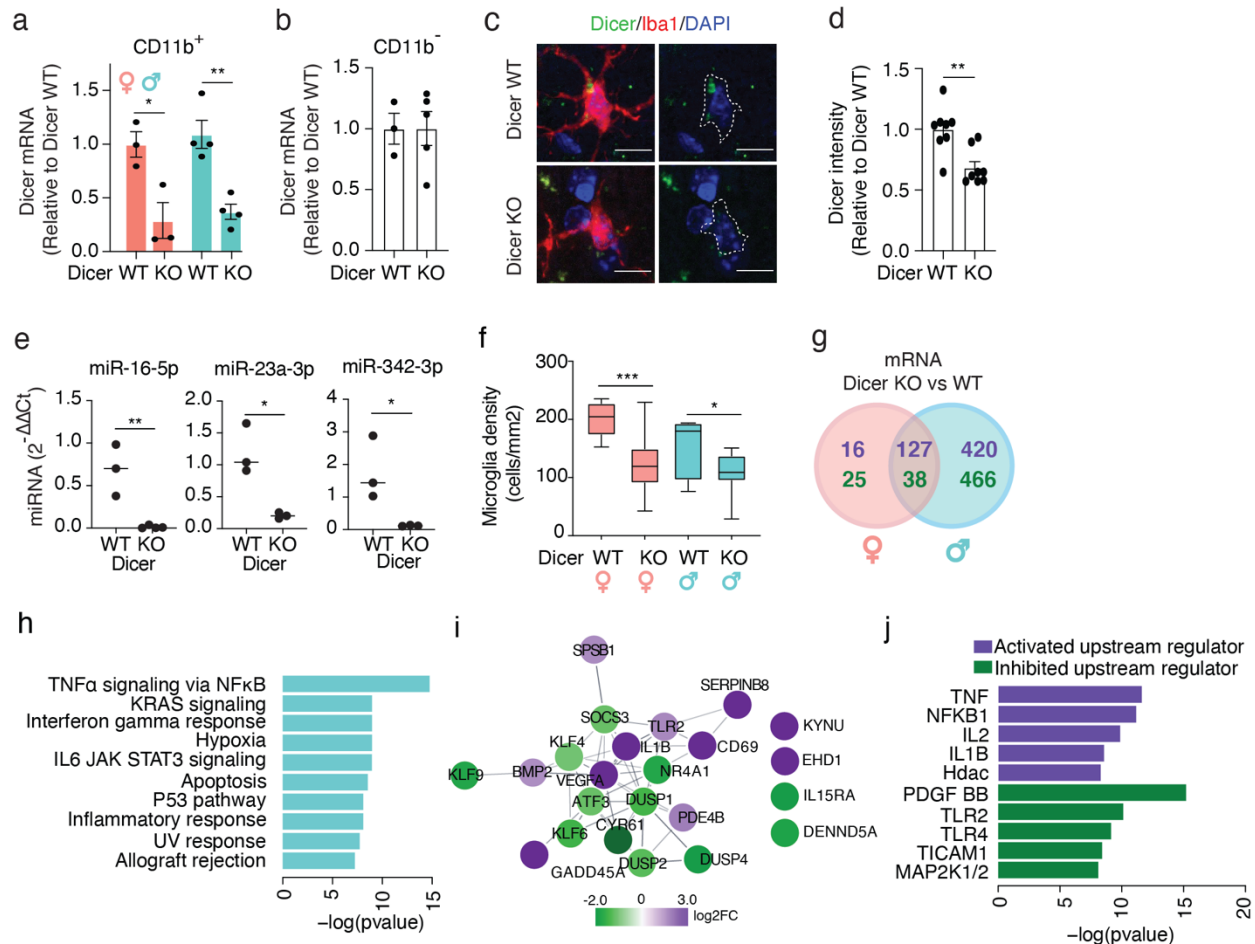


Figure 2.2. Loss of mature microRNAs affects microglia in a sex-dependent manner. (a,b) qPCR of Dicer mRNA in CD11b⁺ (a) and CD11b⁻ (b) cells isolated from female and male 9-month-old Dicer WT and KO mice. Error bars represent SEM. Bar denotes mean. Each dot represents one mouse. $n = 3$ biologically independent animals/genotype for females, 4 for males (a). $n = 3$ biologically independent animals for Dicer WT, 5 for Dicer KO (b). * $t = 3.464$, $df = 4$, $P = 0.025$, ** $t = 4.855$, $df = 6$, $P = 0.0028$, unpaired, two-tailed t test. (c) Representative images from 9-month-old Dicer WT and KO mice. Iba1⁺ cells outlined in white. Image acquired and analysis repeated for every mouse quantified in (d). Scale bar, 10 μ m. (d) Relative fluorescence intensity of cortical Dicer immunostaining. Error bars represent SEM. Bar denotes mean. Each dot represents one mouse. $n = 8$ biologically independent animals/genotype, ~40 cells/genotype. ** $t = 3.740$, $df = 14$, $P = 0.0022$, unpaired, two-tailed t test. (e) qPCR of miRNAs in CD11b⁺ cells isolated from 9-month-old Dicer WT and KO mice. Each dot represents one mouse. Bar denotes mean. $n = 3$ biologically independent samples/genotype, 2 animals/sample. ** $t = 4.597$, $df = 4$, $P = 0.0059$ (miR-16-5p), * $t = 4.328$, $df = 4$, $P = 0.0124$ (miR-23a-3p), * $t = 2.961$, $df = 4$, $P = 0.0415$ (miR-342-3p), unpaired, two-tailed t test. (f) Microglial density quantification from Iba1 immunohistochemistry. $n = 11$ Dicer WT females, 9 Dicer KO females, 4 Dicer WT males, 9 Dicer KO males. *** $t = 4.3$, $df = 18$, $P = 0.0004$, * $t = 2.734$, $df = 11$, $P = 0.0195$, unpaired, two-tailed t test. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. (g) Venn diagram of DE mRNAs comparing microglia isolated from Dicer WT and KO females and males. Purple, genes up-regulated in Dicer KO; green, genes down-regulated in Dicer KO. $n = 3$ Dicer WT female samples, 2 Dicer KO female samples, 5 Dicer WT male

samples, 4 Dicer KO male samples with 2 mice/sample. Full list of DEGs in Supplementary Tables 3,4. (h) Pathway analysis with GSEA Hallmark gene set on DE genes uniquely changed in male Dicer KO compared to Dicer WT microglia identified in (f). Significance calculated by a phenotypic-based permutation test, $P \leq 0.05$ by Benjamini-Hochberg correction. (i) STRING network analysis of genes overlapping with the “TNF α signaling via NF κ B” pathway identified in (g). Purple, $\log_2FC \geq 1$; green, $\log_2FC \leq -1$. (j) Ingenuity Pathway Analysis for predicted upstream regulators of genes overlapping with the “TNF α signaling via NF κ B” pathway identified in (g). One-sided Fisher’s exact test based on a Hypergeometric distribution.

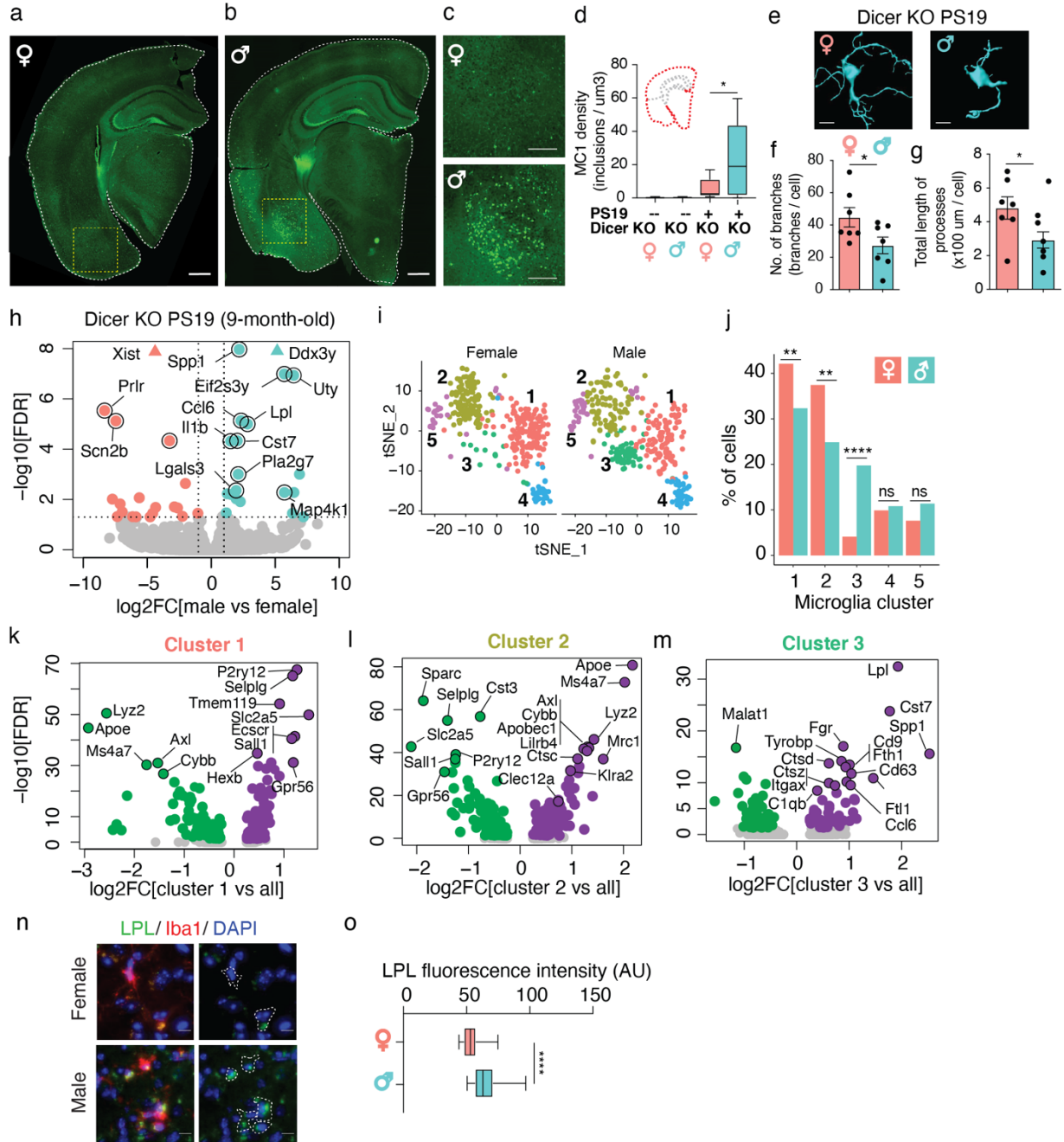


Figure 2.3. Loss of microglial microRNAs increases DAMs and tau pathology in male PS19 mice. (a-c) Representative images of MC1 immunostaining of hemibrains from 9-month-old Dicer KO PS19 female (a) and male (b) mice. Scale bar, 600 μ m. Yellow dashed box is magnified in (c). 2 independent experimental cohorts were used. Scale bar, 300 μ m. (d) MC1 density of hemibrain from Dicer KO nontransgenic (--) and transgenic (+) PS19 mice. $n = 8$ Dicer KO females, 6 Dicer KO males, 12 Dicer KO PS19 females, 10 Dicer KO PS19 males. * $t = 2.578$, $df = 20$, $P = 0.02$, unpaired, two-tailed t test. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. (e) Representative 3D Imaris morphology reconstruction of cortical microglia from 9-month-old Dicer KO PS19 mice. Scale bar, 10 μ m. Image acquired and analysis repeated for every mouse quantified in (f,g). (f,g) Number of branch

points (f) and total length of processes (g) per cell in Dicer KO PS19 mice, determined by Imaris-based automatic quantification. Error bars represent SEM. Bar denotes mean. Each dot represents one mouse. $n = 7$ biologically independent animals/sex, ~ 70 cells/sex. * $t = 2.200$, $df = 12$, $P = 0.0481$ (f), * $t = 2.319$, $df = 12$, $P = 0.0388$ (g), unpaired, two-tailed t test. (h) Volcano plot of RNA-seq data from Dicer KO microglia from 9-month-old male and female PS19 mice. Pink, enriched in females; turquoise, enriched in males; grey, not significantly different. Vertical dashed lines indicate $\log_2FC \pm 1$. Horizontal dashed line indicates $-\log_{10}(0.05)$. $n = 4$ biologically independent samples/sex, 2 animals/sample. Wald test used. (i) t-SNE plots of cells from female or male mice plotted based on clustering from Supplementary Fig. 2.3 h. 2 biologically independent animals/sex. (j) Percent of cells in each microglial cluster for each sex from (i). ** $P = 0.0088$ (cluster 1), ** $P = 0.0021$ (cluster 2), **** $P < 0.0001$ (cluster 3), ns $P = 0.6139$ (cluster 4), ns $P = 0.1145$ (cluster 5), two-sided Fisher's exact test. (k-m) Volcano plot of DEGs for cluster 1 (k), cluster 2 (l), and cluster 3 (m) as defined in (i). Purple, up-regulated genes; green, down-regulated genes; grey, not significantly different in each cluster compared to all other clusters. Wilcoxon rank sum test. DEGs defined as $P \leq 0.05$ by Benjamini-Hochberg correction. Full list of DEGs in Supplementary Tables 9-11. (n) Representative images of Lpl immunostaining of 9-month-old PS19 female and male cortical Dicer KO microglia. Scale bar, 15 μm . Iba1⁺ cells outlined. Image acquired and analysis repeated for every mouse quantified in (o). (o) Average Lpl fluorescence intensity per Iba1⁺ cell in each sex. $n = 43$ female cells from 3 mice, 78 male cells from 4 mice. **** $t = 7.413$, $df = 118$, $P = 2.04 \times 10^{-11}$, unpaired, two-tailed t test. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range.

2.2.10 Supplementary Figures

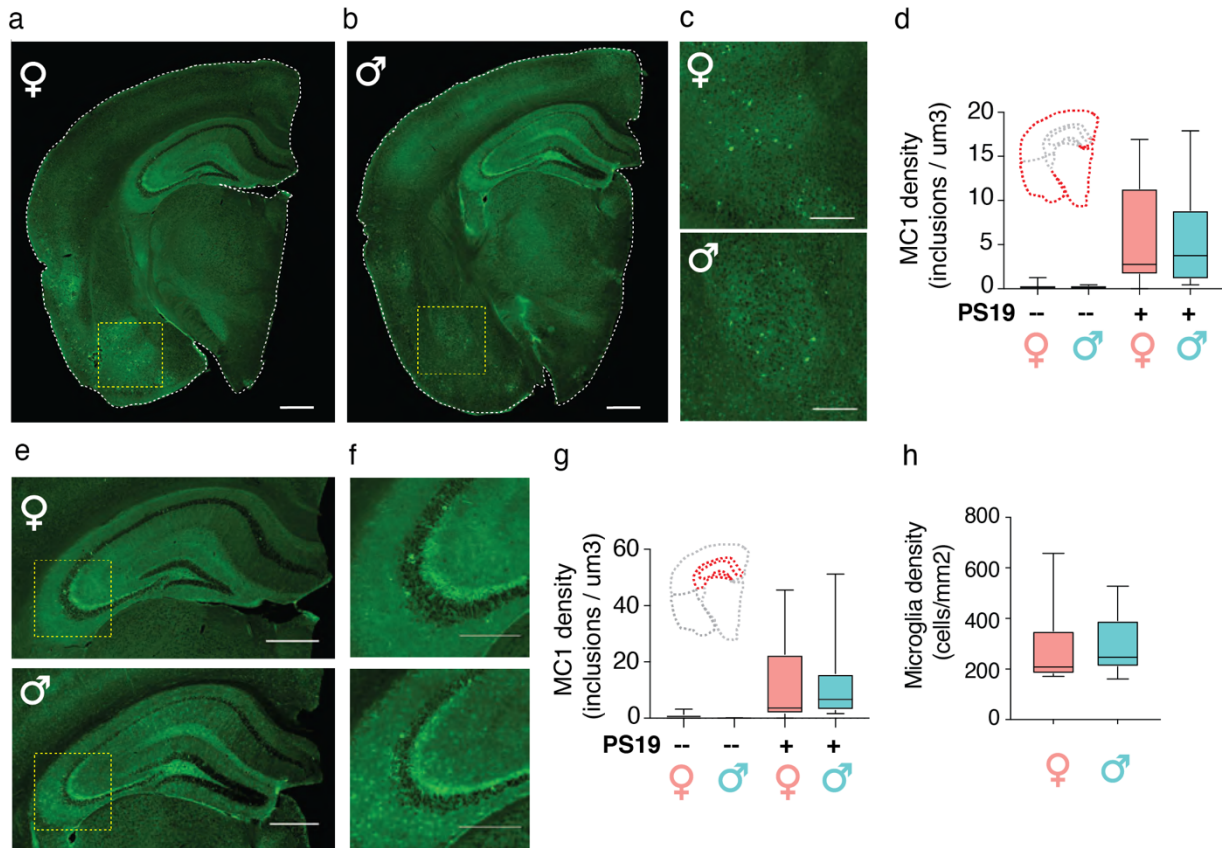


Figure S2.1. Male and female PS19 mice have similar tau pathology. (a–c,e,f) Representative images of MC1 immunostaining of 9-month-old PS19 female (a) and male (b) hemibrains. Scale bar, 600 μm . Yellow dashed boxes magnified in (c). Scale bar, 300 μm . (e) Representative image of hippocampus. Scale bar, 600 μm . Yellow dashed boxes magnified in (f). Scale bar, 150 μm . 2 independent experimental cohorts were used. (d,g) MC1 density of entire hemibrain (d) and hippocampus (g) of nontransgenic (–) and transgenic (+) male and female mice. $n = 11$ nontransgenic females, 5 nontransgenic males, 10 PS19 females, and 9 PS19 males. (h) Microglial density quantification from Iba1 immunohistochemistry. $n = 12$ female PS19, 10 male PS19 mice. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range.

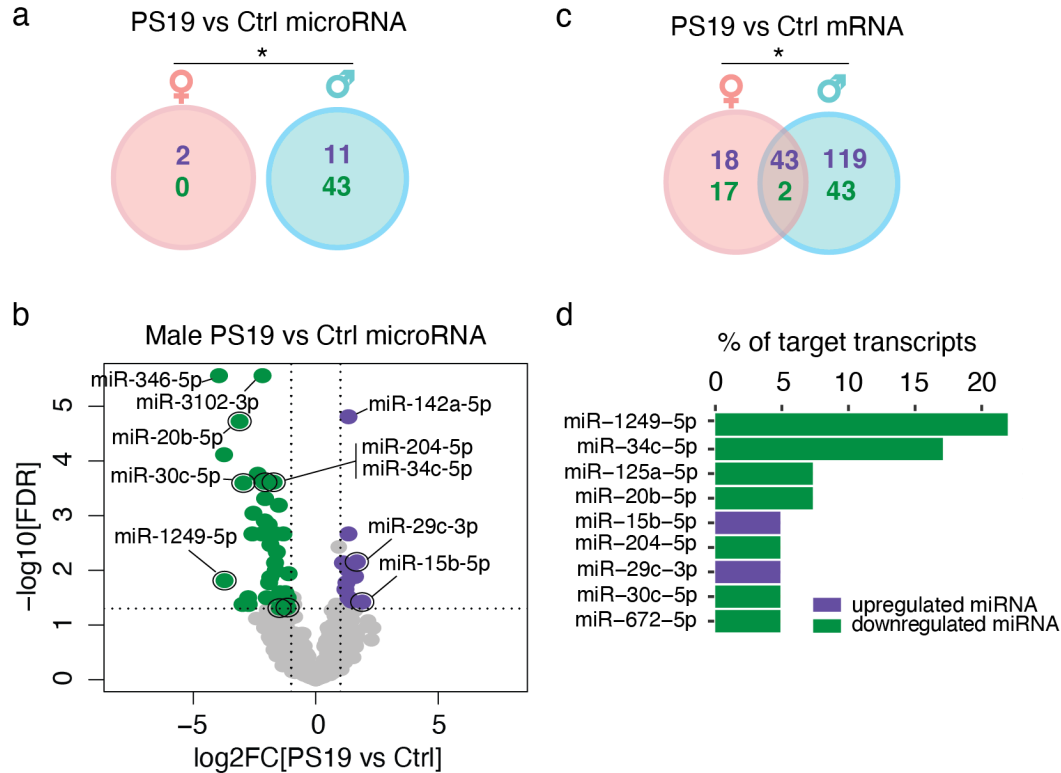


Figure S2.2. Male and female microglia have differential transcriptional responses to tau pathology. (a,c) Venn diagram of differentially expressed (DE) miRNAs (a) and mRNAs (c) comparing microglia from PS19 vs nontransgenic control (Ctrl) mice. DE genes defined as those with $\log_2\text{FC} \geq 1$ or ≤ -1 and $\text{FDR} < 0.05$. Purple numbers, up-regulated DE genes; green numbers, down-regulated DE genes. $n = 4$ Ctrl samples/sex, 5 PS19 samples/sex, 2 mice/sample (a). $n = 5$ male and 3 female Ctrl samples, 3 male and 4 female PS19 samples, 2 mice/sample (c). * $P = 0.05$ (a), * $P = 0.0146$ (c), two-sided Fisher's exact test. Full list of DEGs in Supplementary Tables 5–7. (b) Volcano plot of male miRNA-seq data from (a). Purple and green dots represent miRNAs upregulated in PS19 samples (11 miRNAs; $P \leq 0.05$ by Benjamini-Hochberg correction and $\log_2\text{FC} \geq 1$) and downregulated in PS19 samples (43 miRNAs; $P \leq 0.05$ and $\log_2\text{FC} \leq -1$), respectively. Grey dots are miRNAs not significantly different. Dots with black circles represent those that were analyzed in (d). Vertical dashed lines indicate $\log_2\text{FC} \pm 1$. Horizontal dashed line indicates $-\log_{10}(0.05)$. Wald test was used. (d) Bar graph showing 9 Ingenuity Pathway Analysis predicted target coverage of DE mRNAs from male PS19 vs Ctrl microglia (c) by DE miRNAs from male PS19 vs Ctrl microglia (a). Results were filtered for those with opposing miRNA and mRNA log fold changes (i.e. focusing on miRNA and mRNA targets that have anti-correlated expression patterns) and were either experimentally observed or highly predicted to be miRNA-mRNA target interactions.

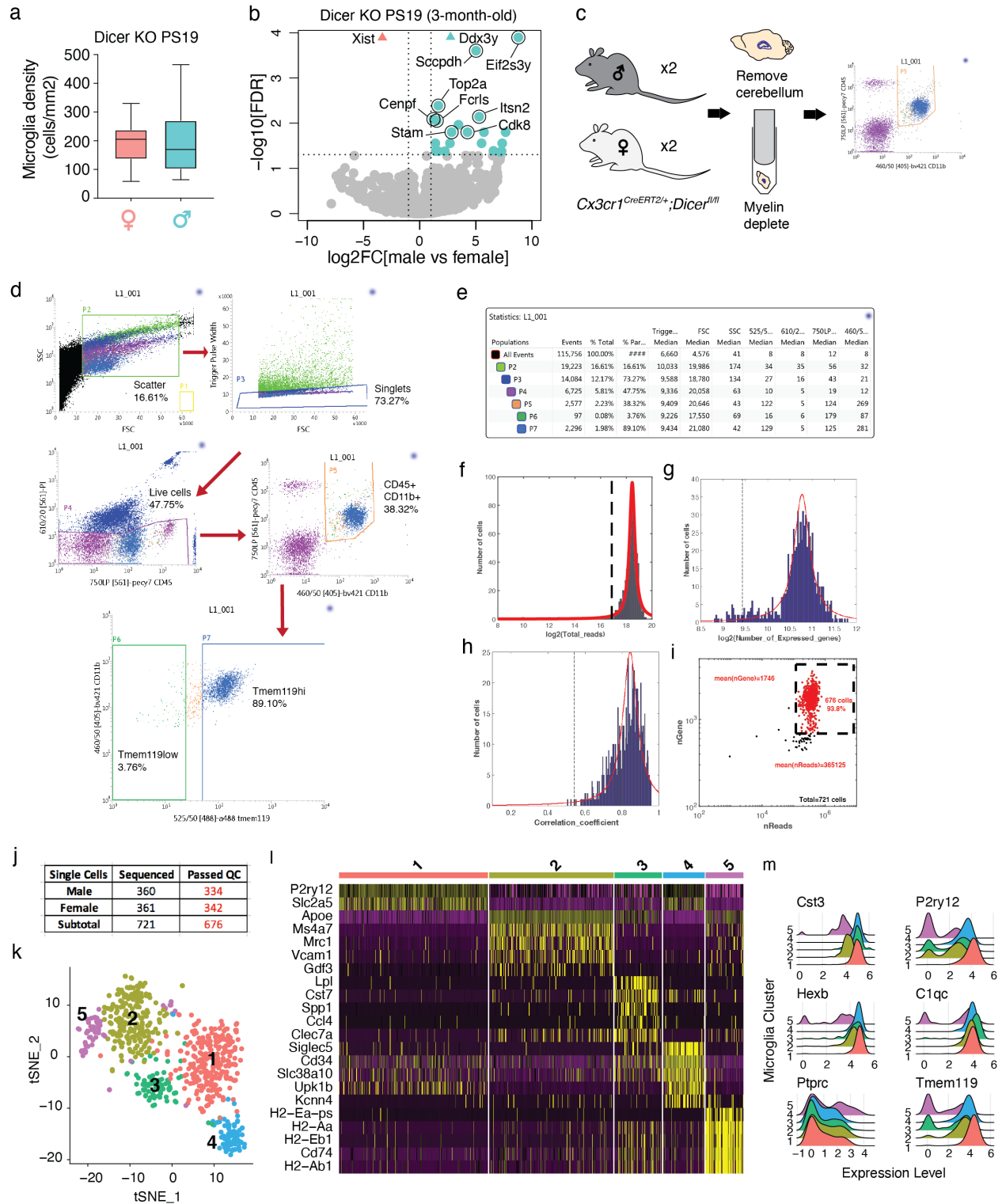


Figure S2.3. RNA sequencing of Dicer KO microglia from PS19 mice. (a) Microglial density quantification from Iba1 immunohistochemistry. $n = 16$ female Dicer KO PS19, 18 male PS19 mice. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. (b) Volcano plot of RNA-seq data from Dicer KO microglia from 3-month-old male and female PS19 mice. Pink, female-enriched; turquoise, male-enriched; grey, not

significantly different. Vertical dashed lines indicate $\log_2FC \pm 1$. Horizontal dashed line indicates $-\log_{10}(0.05)$. $n = 4$ male samples, 2 female samples, 2 mice/sample. Wald test was used. (c) Schematic of the single-cell isolation method. Brains without the cerebellum were harvested from 9-month-old Dicer KO PS19 female and male mice and homogenized. After myelin depletion, cells were sorted using flow cytometry and gated by CD45⁺;CD11b⁺ expression. (d) Representative FACS plots showing gating strategy and the cells sequenced. Similar gating strategy was used for all samples sequenced ($n = 2$ biologically independent animals/sex). (e) Number of cells, proportion and statistics for FACS plots from (d). (f-h) Quality control criteria for the single-cell sequencing data. Fitted curves for histograms of mapped reads (f), numbers of detected genes (g) and ERCC correlation coefficient (h) are labeled in red. Dashed lines are statistical cutoffs. Cells that passed all three criteria were retained for analysis. (i) Scatter plot highlighting cells that passed QC (red) among all cells sequenced. Each dot is a cell. (j) Summary of the numbers of cells sequenced and cells that passed QC (red). (k) t-SNE plot of microglia clusters from 9-month-old Dicer KO PS19 female and male mice. $n = 2$ biologically independent animals/sex. (l) Heatmap of top genes defining each microglial cluster. (m) Ridge plots of microglial marker expression levels by each microglial cluster.

Chapter 2.3: Microglia screen uncovers candidate microRNA networks involved in phagocytosis

2.3.1 Abstract

Microglia are the innate immune cells of the central nervous system, and their dysfunction has been linked to many neurodegenerative diseases. As immune cells, they constantly survey the brain environment and modulate brain health through various functions. One of their key functions is phagocytosis of diverse substrates such as apoptotic cells, neuronal synapses, and protein aggregates. MicroRNAs (miRNAs) are small non-coding RNAs that can modulate cell fate and response, and have also been shown to be important regulators of microglial functions. Dysregulated expression of various miRNAs has been found in many neurodegenerative diseases, but a systematic approach to understand how individual miRNAs regulate microglia function has not been done. In this study, we establish a primary microglia screen to study the effects of highly-expressed microglial miRNAs on microglial phagocytosis of *E. coli* particles. We found that removal of mature miRNAs in primary microglia reduced their phagocytosis uptake capacity, which was largely reversed by expressing individual miRNAs of the miR-17~92 cluster. These findings suggest individual microglial miRNAs are capable of having significant effects on microglial phagocytosis function and the platform can be leveraged to explore novel downstream molecular networks controlling microglial phenotypes.

2.3.2 Introduction

Microglia, the innate immune cells of the brain, have emerged as a key cell type involved in the pathogenesis of Alzheimer's disease (AD). As outlined in Chapter 1 of this thesis, microglia have

many fundamental roles in the central nervous system, one of which is phagocytosis. Simply put, phagocytosis is the uptake of particulates greater than 0.5 μm within a plasma-membrane envelope. Once internalized, the cargo undergoes a complex and dynamic process of interactions with intracellular organelles, leading to fusion, fission, and membrane recycling events ^{115,116}. Microglia are able to use phagocytosis to take up diverse substrates, fine-tuned by environmental cues such as damage-associated molecular patterns and the cell's internal status. For example, microglial phagocytosis is crucial during development and early homeostasis of the brain, including but not limited to sculpting brain circuitry through phagocytosis of synapses and apoptotic neurons in neurogenic niches ¹¹⁷⁻¹²². These homeostatic functions can go awry in the context of disease, where microglia have been found to upregulate complement proteins such as C1q and C3 that tag synapses and lead to over-elimination of synapses that correlate with functional deficits in AD and other neurodegenerative mouse models ¹²³⁻¹²⁵. Microglial phagocytosis is also essential for clearance of debris from the brain, including protein aggregates in the context of neurodegenerative diseases. Dysfunction of microglial uptake of protein aggregates in AD mouse models have been shown to exacerbate AD pathology and neurotoxicity ¹²⁶⁻¹²⁸. Together, these studies established the importance of microglial phagocytosis in the pathogenesis of AD and therefore, dissecting the molecular regulators of this function is key to understanding the disease.

MiRNAs are one such molecular regulator involved in modulating phagocytosis. MiRNA-regulation of phagocytosis has been extensively studied in the context of macrophages, the peripheral “sister-cell” of microglia ¹²⁹. Individual miRNAs have been identified to regulate pathogen phagocytosis, such as miR-15a/16 ¹³⁰. Several groups have also done systematic screens of several miRNAs individually as well as in a combinatorial manner to look at the contribution of miRNAs on macrophage phagocytosis. One group, for example, focused on miRNAs expressed

during monocyte to macrophage differentiation (miR-24, miR-30b and miR-142-3p) and found several of these miRNAs can regulate pathogen and antibody-mediated uptake as well as TNF- α and other pro-inflammatory cytokine secretion levels ^{131,132}.

Although select miRNAs have been studied to understand miRNA-regulation of microglial phagocytosis ^{26,133}, systematic approaches to understand how individual miRNAs highly-expressed by microglia modulate phagocytosis function has not been done. Such an approach is important as global miRNA-depletion experiments only reveal how miRNAs in general are important for cell function, but does not give us the resolution to see how individual pathways are altered by miRNAs and which miRNAs are most influential in modulating certain cellular phenotypes. Previous studies have taken an approach of screening for individual miRNAs in miRNA-depleted cells to rescue their functions. For instance, in one study, T helper cells without the miRNA-processing enzyme, Drosha/Dgcr8, showed increased production of IL-4. Individual miRNA mimics were then transfected into these miRNA-depleted cells and IL-4 levels were measured. This assay revealed miR-24 and miR-27 reduced IL-4 production and, when coupled with an siRNA-screen to identify downstream targets of these two miRNAs, the authors were able to identify novel molecular networks involved in IL-4 production ⁴⁹.

Using a similar screening approach, we chose miRNAs highly-expressed in mouse microglia to probe their individual effects on microglia phagocytosis function using both miRNA-intact WT and miRNA-depleted Dicer KO cells. We found that removing miRNAs resulted in decreased microglial phagocytosis. When we transfected these cells with individual miRNAs, we found that miRNAs could robustly regulate phagocytosis, with some miRNAs behaving in similar ways in both Dicer WT and KO cells. The miR-17~92 miRNA cluster and their paralogues were able to partially rescue the Dicer KO phagocytosis deficit. This study established a robust assay

platform for testing individual miRNAs and their effects on microglial function, enabling the foundation to further investigate the downstream pathways modulated by these miRNAs of interest.

2.3.3 Results

Dicer KO microglia have reduced accumulation of *E. coli* compared to Dicer WT microglia.

Phagocytosis is a key function of microglia and alterations in this function can have significant consequences on brain health. To assess how miRNAs regulate phagocytosis in microglia, we generated mice with miRNA-depleted microglia by crossing *Cx3cr1^{CreERT2/+}* with *Dicer^{fl/fl}* mice (Dicer KO, also used in Chapter 2.2). We then cultured mouse primary microglia from Dicer KO and Dicer WT control pups (P3-P5) and induced efficient Dicer-deletion by incubating cultures with 4-Hydroxytamoxifen (Sigma-Aldrich) at day 10 of culture (**Fig. 2.4 a,b**). This manipulation was able to efficiently decrease microglial miRNAs, but did not affect miR-451 expression, a Dicer-independent miRNA (**Fig. 2.4 c**). Loss of microglial miRNAs decreased the number of Iba1+ microglia in culture, but did not significantly alter their morphological area compared to control cells (**Fig. 2.4 d-f**).

To monitor their phagocytic capacity and kinetics, we incubated them with *E. coli* particles conjugated with pHrodo (ThermoFisher), a pH-sensitive dye, and took time-lapse live-images using Incucyte (EssenBio). Primary microglia were able to efficiently take up these *E. coli* particles, with the peak accumulation (average intensity normalized by cell number) occurring between 7.5 and 10 hours after incubation with *E. coli* (**Fig. 2.4 g,h**). Single-cell tracking of microglia also revealed the reduction of cell number in the Dicer-depleted condition, and a large heterogeneity in uptake kinetics even within the same imaging well (**Fig. 2.4 i**). Dicer KO microglia showed reduced accumulation of *E. coli* compared to control cells at both the bulk and single-cell level

(**Fig. 2.4 g-i**). Cytochalasin D, an actin polymerization inhibitor, decreased the fluorescence level in both genotypes, suggesting the fluorescence signal is phagocytosis-mediated (**Fig. 2.4 h**).

Microglia are also known to interact with tau pathology and uptake tau ^{60,128}. We therefore tested phagocytosis of fluorescently-tagged K18 tau fibrils, a truncated form of human tau containing only the microtubule binding domain (**Fig. S2.4 a**). Microglia are able to efficiently take up these tau fibrils over time (**Fig. S2.4 b**). Dicer KO cells had increased accumulation of tau fibrils compared to controls at 2 and 6 hours after incubation, with similar accumulation at 24 hours (**Fig. S2.4 c,d**), suggesting loss of microglial miRNAs may have substrate-specific alterations in the phagocytosis phenotype.

Individual miRNAs are able to modulate microglial uptake of *E. coli*.

To understand how individual microglial miRNAs are able to regulate microglia function, we adapted a previously established platform to study the role of miRNAs in Th cells ⁴⁹ by transfecting individual miRNA mimics into primary microglia using lipofectamine (**Fig. 2.5 a**). We selected the top 50 most abundantly-expressed miRNAs in microglia based on miRNA sequencing data of adult mouse microglia (**Fig. 2.5 b, Fig. 2.1**) ¹³⁴. We chose the most-highly expressed miRNAs, as previous studies have found that highly-abundant miRNAs often mediate the most significant target suppression within cells ¹³⁵⁻¹³⁷. Microglia from male and female mice were combined to generate this ranked list, as no sex-differences were observed in the phagocytosis capacity *in vitro* (**Fig. 2.1 g**). Transfection efficiency using this platform was first determined using a positive control mimic targeting *Gapdh* mRNA, which resulted in efficient knockdown of the mRNA (**Fig. 2.5 c**). To determine the targeting activity of these miRNA mimics, we used a miRNA-GFP-sensor against miR-27a established previously with four perfectly complementary binding sites for the

miRNA of interest in the 3'UTR (**Fig. 2.5 d**)^{49,138}. hCD25 expression level was used to gate for cells transduced by the viral construct (**Fig. 2.5 e**). Dicer KO cells showed increased GFP expression (i.e. reduced miRNA-binding activity to the sensor) compared to control cells, while transfection of the miR-27a mimic was able to shift the miRNA-activity back to control levels (**Fig. 2.5 f**). The mimic was able to further reduce the GFP expression in control cells, suggesting this miRNA's binding activity was not saturated at baseline expression-levels.

We next transfected individual miRNA mimics into both Dicer KO and Dicer WT primary microglia and monitored *E. coli* uptake. We then calculated the height of the normalized uptake peak intensity for each miRNA. As expected, cytochalasin D treatment dramatically inhibited *E. coli* uptake (**Fig. 2.6 a,b**). 12 miRNAs increased the Dicer KO phagocytosis capacity by 0.5 standard deviations or greater, while 11 miRNAs decreased the capacity further (**Fig. 2.6 a**). miR-128-3p and miR-182-5p had the most extreme effects on modulating the peak phagocytosis. miR-128 is particularly of interest as it has been found to be a brain-specific miRNA in both mice and humans¹³⁹⁻¹⁴¹, studied primarily for its expression in neurons¹⁴²⁻¹⁴⁵, and has been predicted to bind to neurodegenerative disease risk genes such as *MAPT*, which encodes for Tau¹⁴⁶.

Transfection of these mimics had robust effects on modulating Dicer WT phagocytosis function as well, even though these cells already express baseline-levels of miRNAs. 17 miRNAs increased the cell's phagocytosis capacity by 0.5 standard deviations or greater, while 9 miRNAs decreased the capacity further (**Fig. 2.6 b**). As demonstrated with the miRNA-sensor, the activity of these miRNAs was not saturated in Dicer WT microglia at baseline. MiR-29b-3p and miR-23b-3p had the most extreme effects on modulating phagocytosis in these cells. MiR-23b could be interesting in the context of microglia as one of the upregulated disease-associate microglial genes

Spp1 is known to have a miR-23b binding site in humans, and miR-23b is downregulated in the context of Multiple Sclerosis^{146,147}.

Overall, most miRNAs had similar effects on phagocytosis in Dicer WT and Dicer KO cells, though this positive correlation was not statistically significant (**Fig. 2.6 c**). Some miRNAs had opposing effects depending on the Dicer-genotype, such as miR-30b-5p, which increased phagocytosis in Dicer WT but decreased phagocytosis in Dicer KO, suggesting miRNAs have context-dependent regulation of phagocytosis depending on what other miRNAs are being expressed at the same time (**Fig. 2.6 c**). Uptake z scores also had a significantly positive correlation with the cell number z scores in Dicer KO cells (**Fig. 2.6 d**) while no statistically significant correlation in Dicer WT cells (**Fig. 2.6 e**). Removal of Dicer results in a decrease in microglia number (**Fig. 2.4 d-f**), suggesting transfection of some miRNAs likely target pathways related to both cell cycle/proliferation and phagocytosis, such as miR-128-3p, miR-142a-5p, and miR-191-5p (**Fig. 2.6 d**). Meanwhile, some miRNAs had similar cell number z scores to the negative control, such as miR-140-5p, miR-93-5p, and miR-143-3p, likely altering phagocytosis function without altering cell number (**Fig. 2.6 d**).

MiR-17~92 cluster can rescue the reduced phagocytosis phenotype of Dicer KO microglia.

Dicer KO microglia showed reduced accumulation of *E. coli* compared to control cells (**Fig. 2.4 g-i**). To determine which miRNAs could rescue this phenotype, we focused on the miRNAs that could increase the phagocytosis capacity of Dicer KO cells and found over-representation of the miR-17~92 cluster (miR-19b-3p, miR-17-5p, miR-18a-5p, **Fig. 2.7 b**). MiR-106b-5p was also found to increase phagocytosis (**Fig. 2.6 a**), which is encoded by the miR-106b~25 cluster, a paralogue of the miR-17~92 cluster. Together, these four miRNAs are part of three miRNA

families: miR-17, miR-18, and miR-19. miRNA clusters are transcribed from physically adjacent miRNA genes and miRNA families have similar targeting seed sequences, and are therefore thought to coordinately regulate functionally related genes¹⁴⁸⁻¹⁵⁰, adding weight to their potential coordinated regulation of phagocytosis function in microglia. Because of this, we decided to focus on these miRNAs for further downstream analysis.

We tracked single-microglia phagocytosis uptake kinetics to understand how these miRNAs are regulating these cells at the single-cell level as well as study the heterogeneity of these cells. As seen in the bulk analysis, single-cell analysis showed rescue of phagocytosis with transfection of these mimics in Dicer KO cells, as well as a rescue in the number of cells, suggesting involvement in both phagocytosis and proliferation (**Fig. 2.7 b,c**). We then performed dimensionality reduction using the t-distributed Stochastic Neighbor Embedding (t-SNE) method to group these cells by their differential uptake dynamics (**Fig. 2.7 d**). This analysis also showed that miR-17-5p and miR-18a-5p were independently able to rescue the Dicer KO phenotype back to control levels by increasing the number of cells in clusters 0 and 1, which showed the most uptake capacity (**Fig. 2.7 e-g**). Dicer KO cells were largely composed of cluster 2 and 3 cells, which had extremely reduced or almost no uptake capacity (**Fig. 2.7 e-g**). These data suggest that at both the bulk and single-cell level, multiple miRNAs of the miR-17~92 cluster are able to increase the phagocytosis capacity of Dicer KO microglia.

Predicted targets of the miR-17~92 cluster are involved in innate immune pathways.

The miR-17~92 cluster is encoded on the murine chromosome 14 and human chromosome 13, with paralogues on two other chromosomes, and is composed of several miRNA families (**Fig. 2.8 a**)⁴⁸. It was first identified in the context of B-cell lymphoma, which showed amplification of the

region where this miRNA cluster is located and became the first oncogenic miRNA or “oncomir”^{151,152}. Many targets of this cluster have been identified including pathways involved in cell cycle regulation (*Pten* and the E2F transcription factor family members), TGF- β signaling, (*Tgfb β 1*, *Smad2*, *Smad4*), and apoptosis (*Bim/Bcl2l1*)¹⁵³. In the context of AD, miRNAs in this cluster have been found to target the 3'UTR of *APP* and their expression was downregulated in AD patient brains¹⁵⁴. This cluster has also been studied in the context of monocytes and macrophages and has been shown to be involved during macrophage differentiation as well as macrophage phagocytosis and inflammation through *Sirp α* ^{42,155,156}. To our knowledge, no study has looked at the effects of this cluster in the context of microglial biology.

To narrow down the targets of interest that may be relevant to microglia biology, we identified known downstream targets of the miR-17-5p miRNA family (composed of miR-17-5p, miR-20a, miR-20b, miR-106a, miR-106b, miR-93) using the TargetScan database¹⁵⁷. Many genes were predicted to be targets of this miRNA family at varying confidence scores, determined by the cumulative weighted score (**Fig. 2.8 b**). Genes with scores more negative than -0.2 (i.e. higher confidence) were used to do pathway enrichment to determine relevant biological functions. Pathways relevant to microglia were identified, including cytokine signaling, TGF- β signaling involved in the maturation of microglia²⁴, as well as phagocytosis-related pathways such as membrane trafficking and vesicle mediated transport (**Fig. 2.8 c**). 13 out of the 25 genes identified in these pathways were found to be highly-interconnected, with *Rab5b*, a marker of early endosomes and upregulated in lipid-droplet-rich microglia found in aged brains¹⁵⁸, and *Ubc*, involved in polyubiquitination, being the most connected with the other genes. *Rab30*, *Rab12*, *Rab8b* were other Rab-family genes also part of this network (**Fig. 2.8 d**). These predictions of

downstream targets suggest miRNAs in the miR-17 miRNA family may be good candidates to study gene networks regulating phagocytosis and microglial functions.

2.3.4 Discussion

MiRNAs are robust regulators of microglial functions, and studying how individual miRNAs regulate these cells is key to understanding the molecular networks that govern these cellular functions. In this study, we focused on miRNA-regulation of microglial phagocytosis by leveraging Dicer KO microglia to generate cells with low miRNA expression and re-introduced individual miRNAs into these cells to assess each miRNA's regulation of phagocytosis. Many miRNAs found to be expressed at high levels by microglia *in vivo* were able to regulate phagocytosis, with the miR-17~92 miRNA cluster robustly increasing phagocytosis in Dicer KO microglia. This experimental platform and our findings provide a framework to identify specific microglial miRNA candidates that can be explored to identify their regulatory effects *in vivo*.

MiRNAs are heavily involved in cell proliferation. As expected, removal of Dicer resulted in a significant reduction in the number of microglia. The proliferative state of a cell can impact the cell's phagocytic capacity by, for instance, modulating the expression of phagocytic receptors such as Fc receptors and CR3¹⁵⁹⁻¹⁶¹. Therefore, it is conceivable that re-introduction of individual miRNAs is rescuing Dicer KO cell's phagocytic capacity indirectly by modulating the cell's proliferative capacity. Indeed, Dicer KO cell's phagocytic capacity was significantly correlated with cell number, even after the phagocytosis fluorescence was normalized by cell number, suggesting certain miRNAs are rescuing the phagocytosis phenotype by regulating both cell proliferation and phagocytosis. To effectively focus on miRNAs that specifically influence phagocytosis, it would be important to choose miRNAs that do not modulate cell number but still

alter the cell's phagocytic capacity. It would also be important to assess cell cycle markers such as staining for Ki67 to determine the cell's proliferative state after miRNA transfection. Studying how individual miRNAs are able to modulate both cell number and phagocytosis would also be interesting to identify molecular networks that converge to co-modulate both of these fundamental cellular functions.

In the brain, microglia are able to efficiently take up many diverse substrates, and environmental cues as well as their internal status dictate the specificity of what substrate is taken-up¹⁶². In this study, we focused on *E. coli* as a proxy for microglial phagocytic uptake, since many important biological pathways have been uncovered in macrophages and microglia using this substrate. However, Dicer KO phenotypes may behave differently for different substrates, as we found with tau fibril phagocytosis. Once candidate miRNAs are chosen based on the *E. coli* data, it would be important to assess phagocytosis of CNS-relevant stimuli such as tau fibrils, myelin debris, and synaptosomes.

In the murine adult brain, microglia have sex-specific expression of miRNAs that can have sex-dependent consequences on microglial function and tau pathology consequences (Chapter 2.2)¹³⁴. We hypothesized that although baseline functional states of microglia are similar between male and female mice, sex-dependent phenotypes can be uncovered when homeostatic microglial functions are disrupted. Similarly, in our miRNA screen, we found that primary microglia from male and female mice did not exhibit differences in phagocytosis of *E. coli*, consistent with previously-published findings in adult mice assessed through phagocytosis of fluorescent beads¹⁶³. However, it is possible disruption of miRNAs through removal of Dicer could undercover sex-differences in phagocytosis function. Moreover, the miR-17~92 miRNA cluster has a paralogue encoded by the X-chromosome (miR-106a~363 cluster). It would be important to assess whether

Dicer KO male and female cells have different phagocytosis dynamics and whether X-linked miRNAs are able to regulate these dynamics in a sex-dependent manner.

The same miRNA mimic transfected into Dicer WT or Dicer KO cells had differential effects on phagocytosis for some miRNAs. This would suggest that miRNAs can have distinct modulatory effects on function depending on what miRNAs are already present. Indeed, combinatorial miRNA regulation is perhaps more physiologically relevant as most individual miRNAs have low basal expression, but can act in a coordinated fashion with other miRNAs to regulate networks¹⁶⁴. Importantly, using overexpression of individual miRNAs may result in off-target effects¹⁶⁴. Therefore, computational models and experimental screens that consider combinations of miRNAs in their design and predictions of targets are necessary¹⁶⁴⁻¹⁶⁶. A similar approach could be taken for this study by decreasing the concentration of mimics to physiologic levels and transfecting combinations of miRNAs systematically.

As this study is currently incomplete, there are many potential future directions. Firstly, these candidate miRNAs from the initial screen, such as the miR-17~92 cluster in Dicer KO cells, need to be further validated. To more confidently test their relevant targets, we will also need to couple the bioinformatic prediction tools with RNA sequencing after transfection. Finally, these targets and/or the miRNA itself will need to be manipulated *in vivo* either through genetic studies or potentially through transfection, as lipofectamine-miRNA transfection is able to enter microglia *in vivo* as seen in a previous study²⁷. It would also be interesting to potentially couple this *in vivo* manipulation with a disease model such as tauopathy. The results from this current study will, therefore, serve as a platform to identify microglial miRNAs of interest for further study in the context of both health and disease.

2.3.5 Methods

Animals

For microglia primary cultures, *Cx3cr1^{CreERT2/+}* (<https://www.jax.org/strain/021160>) were crossed with *Dicer^{fl/fl}* (<https://www.jax.org/strain/006366>) mice to obtain *Cx3cr1^{+/+};Dicer^{fl/fl}* and *Cx3cr1^{CreERT2/+};Dicer^{fl/fl}* mice. Mice of both sexes were studied. Mice had free access to food and water and were housed in a pathogen-free barrier facility with a 12-h light/12-h dark cycle. All mouse experiments were approved by the Institutional Animal Care and Use Committee, University of California, San Francisco and Weill Cornell Medicine and complied with all relevant ethical regulations.

Primary Microglia Experiments

Isolating and culturing primary microglia: Cortices was harvested from postnatal day 3-5 pups with 3 pups per T75 flask. Pups were genotyped for the presence or absence of *Cx3cr1^{CreERT2}*. The meninges were removed, and the cortical tissue was finely chopped with a razor blade and digested in 0.25% trypsin (Gibco, Cat# 15090-45 100ml w/o phenol red, 2.5% stock) with DNase (Millipore, Cat# 260913-10MU, dissolved in 1xDPBS for 10U/uL stock) at 37°C for 25 min. Digestion was stopped with DMEM (with glucose and L-glucosamine without sodium pyruvate, Fisher Scientific, 10017CV) containing 20% FBS (Invitrogen, Cat# 10082147). The tissue was then triturated and spun at 200 g for 5 min, and the pellet was resuspended in DMEM with 10% FBS and 1% Penicillin-Streptomycin (10,000U / mL, Gibco) and plated in T75 flasks that had been pre-coated with 5 µg/ml poly-D-lysine (Millipore Sigma) and rinsed with water. After 10 days of maintaining cultures in flasks, 4-Hydroxytamoxifen (Ready Made Solution, Sigma Aldrich, 1:1000) was added directly into the flask. After 3 days of tamoxifen treatment, the flasks were shaken for 2 hours and the media was collected and spun at 200 g for 15 min. The cell pellet was

resuspended and plated at a density of 15,000 cells / well in a 96-well plate (Fisher Scientific) in DMEM with 10% FBS and 1% Penicillin-Streptomycin.

Tau fibril phagocytosis: 24 hours after plating the cells, media was removed and replaced with new media with 1 μ g of K18 tau fibrils conjugated with AlexaFluor 647 per well (generated by Dr. Jason Gestwicki's lab). After incubating with tau fibrils for 2, 6, 24 hours, media and tau fibrils were removed by adding 0.01% trypsin for 10 minutes then washed with media for 5 minutes to inactivate the trypsin. Cells were fixed using 1x Paraformaldehyde (Fisher Scientific) for 15 minutes. Cells were washed 3x with PBS with calcium and magnesium (Thermo Fisher), then permeabilized and blocked using blocking solution (5% normal donkey serum (NDS), Jackson ImmunoResearch in PBSTx (0.1% Triton X-100, Sigma Aldrich)) for 1 hour at room temperature. Blocking solution was removed and cells were incubated in PBSTx (0.01% Triton X-100) with 5% NDS and anti-Iba1 (1:500, Wako) primary antibody at 4°C overnight. The next day, cells were washed 3x with PBST (0.05% Tween-20, Sigma Aldrich) then incubated with PBSTx (0.01% Triton X-100), Hoechst (1:50,000), and donkey anti-rabbit AlexaFluor 546 secondary antibody (1:500, ThermoFisher Scientific) for 1 hour at room temperature. Cells were washed 3x in PBST and solution was replaced with PBS after the last wash. Plate was sealed using aluminum foil and imaged using the Cellomics Arrayscan XTI (Thermo Fisher) with the 20x objective. Images were quantified for Iba1+ cell number and tau fibril number using the High Content Screening Analysis software (Thermo Fisher).

microRNA mimic transfection: 24 hours after plating the cells, media was removed and replaced with new DMEM with 10% (no antibiotics). 500 nM miRNA mimics or negative scramble control

(Dharmacon) were transfected into each well using lipofectamine 2000 (Life Technologies) and Opti-MEM (Gibco).

miRNA sensor transfection: For testing the miRNA mimic transfection efficiency and mimic binding activity, miRNA sensors (generated by Dr. Mark Ansel's lab) were transfected using a method previously published ^{49,138}. Briefly, MSCV-PGK-hCD25 miRNA sensor miR-27a was constructed to express a GFP with four perfectly complementary binding sites for the miRNA in the 3'UTR. 24 hours after cells were plated, they were transduced with the sensor using 5 µg/mL polybrene (Sigma Aldrich). GFP and hCD25 expression was assessed 24 hours later using FACS. Cells were lifted by scrapping the bottom of the well and centrifuged at 200 g for 15 min. The pellet was resuspended and washed using FACS buffer (PBS + 0.5% FBS). Cells were blocked using Purified Rat Anti-Mouse CD16/CD32 (1:50, BD Biosciences, Cat# 553141) for 10 minutes at room temperature. Cells were then incubated with APC anti-human CD25 (1:100, BioLegend, Cat# 302609) for 1 hour at room temperature. After incubation, cells were washed 2x with FACS buffer then analyzed on the LSRII flow cytometer (BD Biosciences). FACS data analyzed using the FlowJo software (BD Biosciences)¹⁶⁷.

***E. coli* phagocytosis:** After 24 hours of miRNA mimic transfection, media and miRNA mimics were removed and replaced with imaging media (FluoroBrite DMEM, Thermo Fisher Scientific + 10% FBS). 0.015 mg pH-rodo *E. coli* (ThermoFisher; P35366) was added to each well. For cytochalasin D treatment, cells were incubated with 10 µM cytochalasin D (Sigma Aldrich) for 1 hour then media was replaced with imaging media. 4 fields of view were taken for each well every 30 minutes using Incucyte (Essen BioScience). Background fluorescence was subtracted and

population-level fluorescence information was determined through the Incucyte analysis software using Top-Hat and threshold based on negative control well with no pH-rodo *E. coli*.

Real-time quantitative PCR

For miRNA qPCR, 10 ng of total RNA was reverse transcribed with the miRCURY LNA RT Kit (Qiagen). RT-qPCR was done with Power SYBR Green PCR master mix (Thermo Scientific) and miR-specific LNA primers (Qiagen) according to the Q5 Quantstudio system protocol. A small ribosomal RNA, 5S, was used for normalization across all samples, and relative differences were calculated with the $2^{-\Delta\Delta C_t}$ method. To validate *Dicer* mRNA reduction after tamoxifen treatment and *Gapdh* mRNA reduction after treatment with positive-control mimic targeting *Gapdh*, RNA was converted to cDNA with iScript Reverse Transcription Supermix (BioRad). Quantitative RT-PCR was done with an ABI 7900 HT sequence detector (Applied Biosystems) and SYBR Green PCR master mix (Applied Biosystems). *Gapdh* and *Actb* was used for normalization for *Dicer* mRNA and *Gapdh* mRNA quantification, respectively. Relative differences were calculated with the $2^{-\Delta\Delta C_t}$ method. The following primers were used for quantitative RT-PCR. *Dicer*: forward, CCTCGGGCCTTGAGGATTTT; reverse, CGACGCCACAGTTCTCTTCT. *Gapdh*: forward, GGGAAGCCCATCACCATCTT; reverse, GCCTTCTCCATGGTGGTGAA. *Actb*: forward, GGCACCACACCTTCTACAATG; reverse, GGGGTGTTGAAGGTCTCAAAC.

Single-cell image tracking. Model Training: The single-cell analysis pipeline was adapted from previous work, using the Usiigaci framework ¹⁶⁸. A custom FIJI/ImageJ macro was written to manually annotate phase contrast images, such that a unique RGB value corresponded to an individual cell mask ¹⁶⁹. A preprocessing step converted the 8-bit RGB image into a 16-bit grayscale mask image (8-bit would yield max of 255 unique cells per image), such that each unique

RGB cell mask was a unique grayscale value. A Mask R-CNN model implemented in Tensorflow and Keras (open-source Matterport Inc. – MIT License), was trained to segment cells in phase contrast microscopy images ¹⁷⁰⁻¹⁷³. The model had a learning rate of 1×10^{-3} for training and used a Resnet-101 backbone ^{168,172}. The network heads were trained first for 50 epochs and then the full network was trained for another 100 epochs. All model training was performed using a GPU on Google Colab. The imgaug package was used for data augmentation during training, incorporating image rotations and flips, along with brightness augmentations and gaussian blur augmentations stochastically. Transfer learning was performed based on final weights from Usiigaci model. The average of two different models (epochs 130 and 134) were used for instance segmentation during this study. These models were chosen based on lowest validation loss during training. The model was trained on 20 images with ~50 cells per image to assess accuracy of the model on data that were not used during model training.

Analysis of Phase Contrast Microscopy Images: Phase contrast and fluorescent image were exported as TIFF images from the Incucyte system. Top-hat background subtraction was performed on the fluorescence image to remove autofluorescence. Time-lapse images were registered using a manual drift correction FIJI plug-in ¹⁷⁴. The output from the Mask R-CNN model was fed into a K-D tree-based cell tracking pipeline based on trackpy, from the Usiigaci pipeline. Once cells from each frame were annotated, a python script was used to measure fluorescence of each cell over the entire time-lapse. A threshold was manually set for each individual experiment. Average intensity measurements above the threshold were measured for each individual cell by taking the total intensity within a cell above the threshold divided by the area of the cell above the set threshold. Cells with more than 30 missing time points and cells with more than 5 missing time

points within the first 10 frames were excluded from the analysis and a linear regression was used to impute missing data from the time-lapse. Cells that contained enough datapoints to be included in the cut-off but missing datapoints at the beginning and end of the time-lapse were filled with zeros.

Clustering of Single-Cell Fluorescence Data: Clustering algorithms were adapted from the Seurat R package¹⁷⁵ for single-cell RNA sequencing analysis. Briefly, the fluorescence measurement for each cell at each time point was scaled such that the mean expression across cells is 0 and all time points were used to generate a PCA. For clustering and generation of the tSNE, FindNeighbors, FindClusters (resolution 0.15), and RunTSNE functions were used.

Gene network and functional analyses

Gene network analyses of RNA-seq data were done with gene set enrichment analysis (GSEA)¹⁰³ and the Hallmark gene dataset¹⁰⁴. Networks were visualized with Cytoscape (v3.6.1)¹⁰⁵, the STRING database¹⁰⁶, and perfuse force directed layout.

Statistics

Statistical analyses were done with Prism 7.0e (Graphpad, San Diego, CA) and R v3.5.1 (Foundation for Statistical Computing, Vienna, Austria). Data were visualized with Prism or the R package ggplot2¹¹⁴. Two-tailed *t* tests were used to compare two groups. $P < 0.05$ was considered statistically significant.

2.3.6 Acknowledgements

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2.3.7 Main Figures

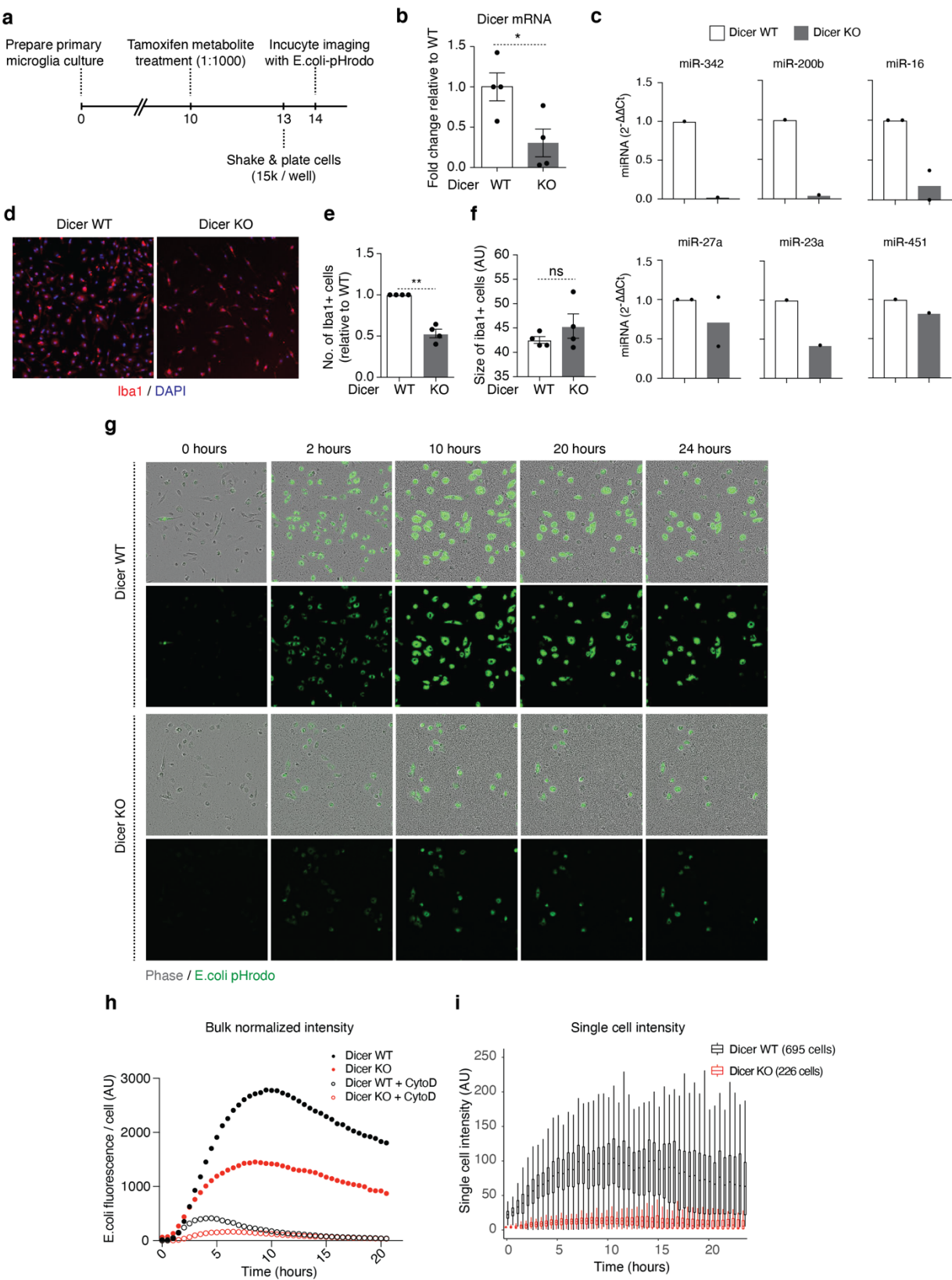


Figure 2.4. Dicer KO microglia have reduced *E. coli* accumulation. (a) Schematic of primary microglia culture and phagocytosis assay. (b) qPCR quantification of *Dicer* mRNA expression level normalized to *Gapdh* and using the $2^{-\Delta\Delta C_t}$ calculation. * $t = 2.836$, $df = 6$, $P = 0.0297$, unpaired t-test. (c) qPCR quantification of selected miRNA expression levels normalized to 5S. Each dot represents an independent experiment. (d) Representative images of immunostaining against Iba1 (red) in Dicer WT (left) and Dicer KO (right) primary microglia taken using the Cellomics Arrayscan XTI (Thermo Fisher). (e,f) Quantification of the number of Iba1+ DAPI+ cells (e) and Iba1+ area per cell (f) in Dicer WT and Dicer KO cells. ** $t = 6.352$, $df = 4$, $P = 0.0031$, unpaired t-test. (g) Representative Incucyte images of pHrodo-conjugated *E. coli* (green) and phase (grey) of Dicer WT (top) and Dicer KO (bottom) primary microglia at selected time points over the course of 24 hours. (h) Quantification of the average fluorescence across the entire field of view normalized by the number of cells for Dicer WT (black), Dicer KO (red), Dicer WT with cytochalasin D (open black), and Dicer KO with cytochalasin D (open red). (i) Quantification of the fluorescence in single cells plotted as boxplot across each time frame for Dicer WT (black, $n = 695$ cells) and Dicer KO (red, $n = 226$ cells). Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range.

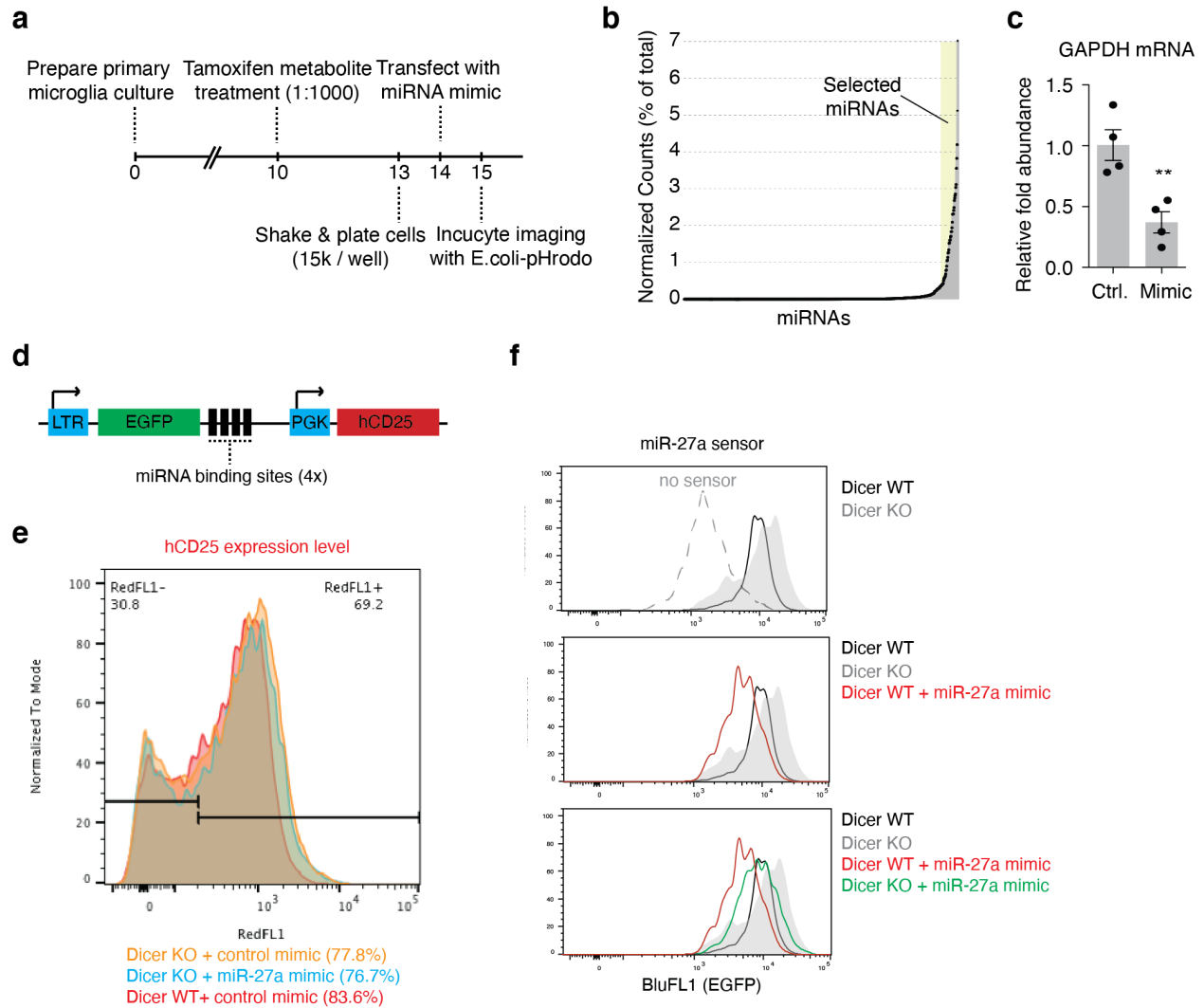


Figure 2.5. Microglial miRNA screen set up. (a) Schematic of timeline for miRNA mimic transfection of primary microglia cultures. (b) Line graph of the average percent of total counts contributed by individual miRNAs (x-axis) determined through miRNA-sequencing of microglia isolated from adult mice (Figure 2.1). (c) qPCR quantification of *Gapdh* mRNA after 24-hour transfection of a positive-control mimic targeting *Gapdh*. Normalized by *Actb* and relative fold change calculated using the $2^{-\Delta\Delta C_t}$ method. (d) Diagram of the miRNA sensory construct generated by the lab of K.M. Ansel and previously published¹³⁸. Insert in the MSCV retroviral backbone. LTR = long terminal repeat promoter driving EGFP expression; PGK = phosphoglycerate kinase promoter driving expression of human CD25 as a transduction control. In the presence of the miRNA of interest, miRNAs will bind the 4 identical and consecutive miRNA binding sites to suppress EGFP expression. (e) hCD25 expression level determined by APC fluorescence. Cells were gated for APC fluorescence before analyzing EGFP expression in (f). (f) EGFP expression measuring miRNA sensory activity for miR-27a in Dicer WT and Dicer KO microglia with and without transfection of the miR-27a miRNA mimic.

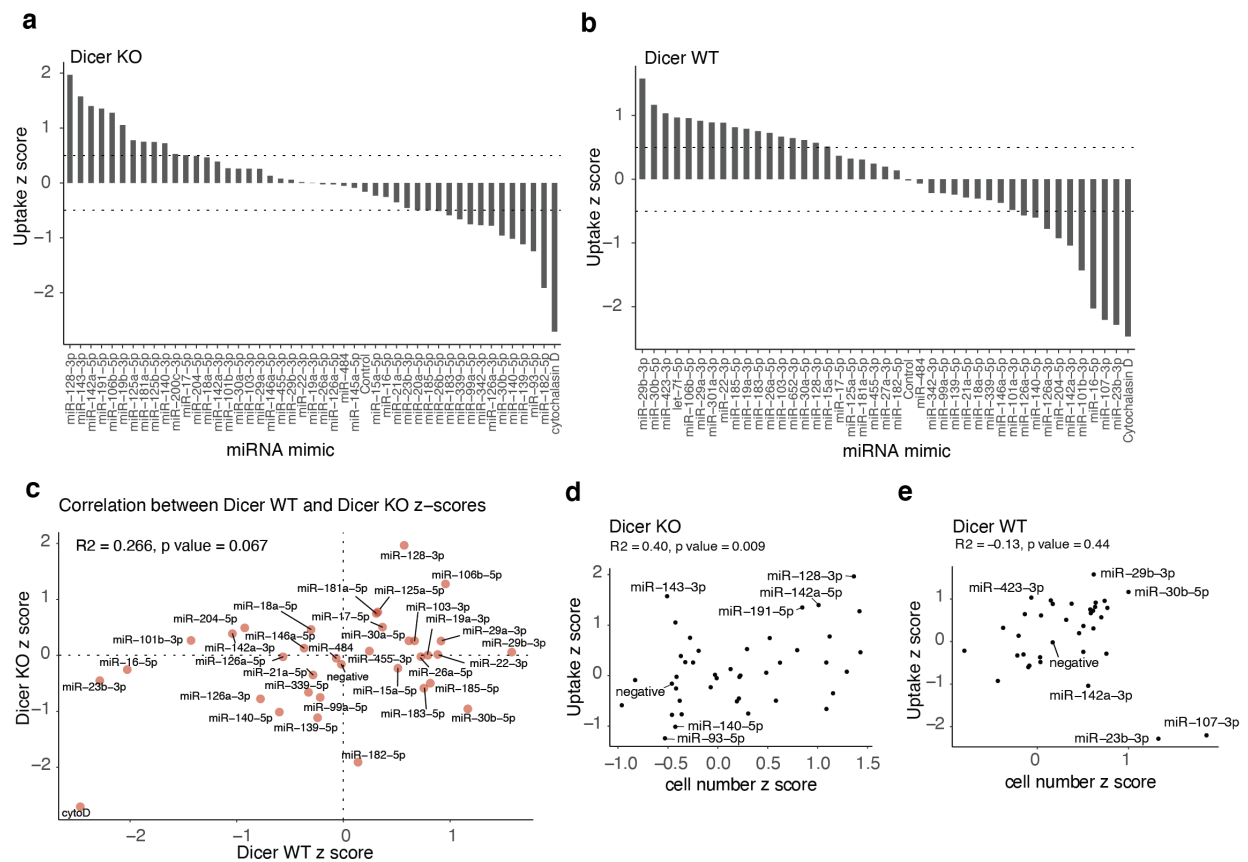


Figure 2.6. Individual miRNAs are able to robustly alter phagocytosis in Dicer KO and Dicer WT microglia. (a,b) Bar graph of z scores calculated by using the peak height of the pHrodo-*E. coli* fluorescence level for each miRNA mimic in Dicer KO (a) and Dicer WT (b) microglia. Dotted lines denote z score of ± 0.5 . (c) Scatterplot showing correlation between Dicer KO (y-axis) and Dicer WT (x-axis) uptake z scores for each miRNA mimic. $R^2 = 0.266$, $P = 0.067$, Pearson's correlation. (d,e) Scatterplot showing correlation between uptake z score (y-axis) and cell number z score (x-axis) for Dicer KO (d) and Dicer WT (e). $R^2 = 0.4$, $P = 0.009$ (d), $R^2 = -0.13$, $P = 0.44$ (e), Pearson's correlation.

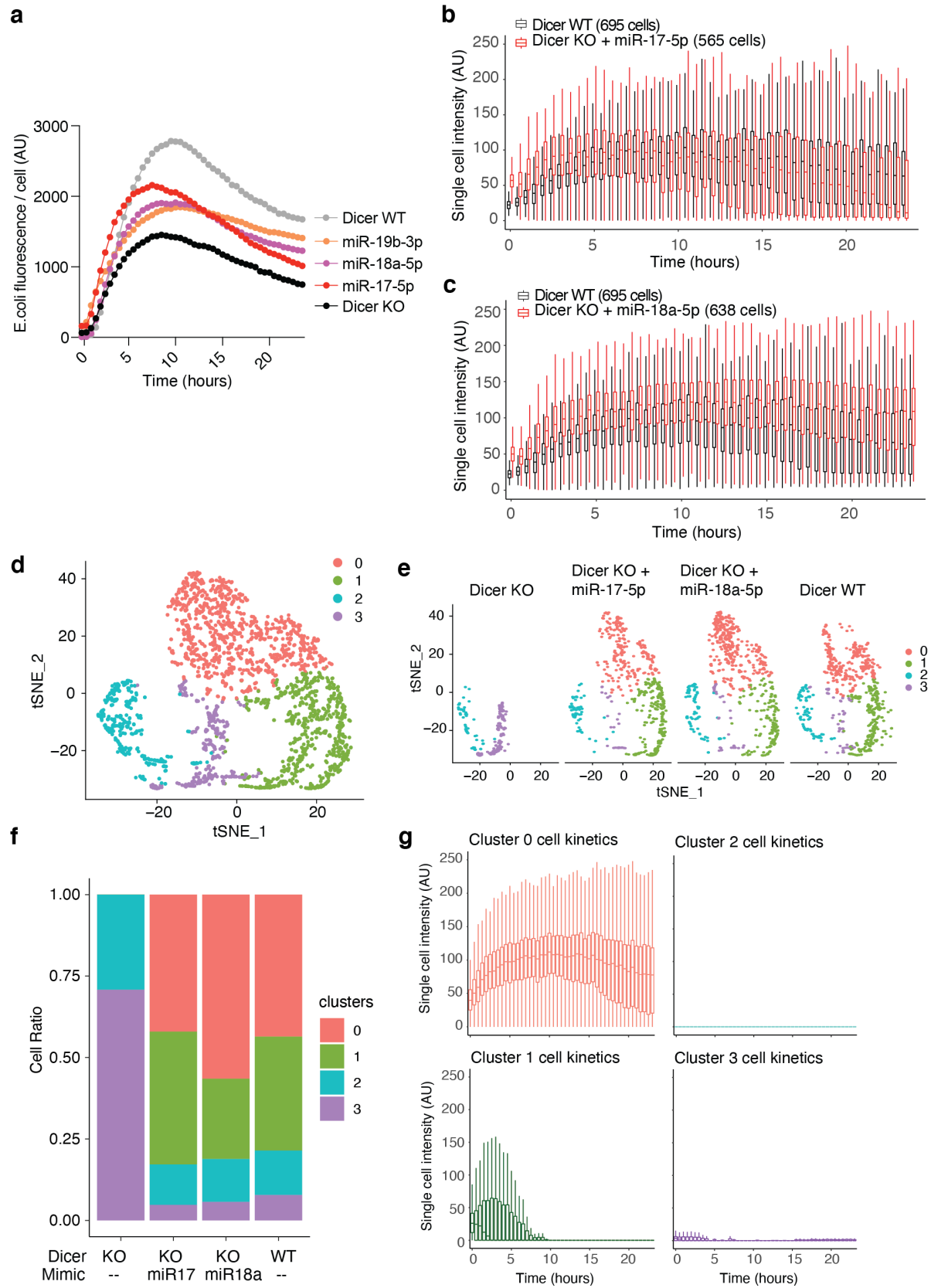


Figure 2.7. MiR-17~92 miRNA cluster partially rescues the Dicer KO phagocytosis deficit.

(a) Quantification of the average fluorescence across the entire field of view normalized by the number of cells for Dicer WT (grey), Dicer KO (black), and selected miRNAs from the miR-17~92 miRNA cluster transfected into Dicer KO cells. (b,c) Quantification of the fluorescence in single cells plotted as boxplots across each time frame for Dicer WT (black, $n = 695$ cells) and Dicer KO transfected with miR-17-5p miRNA mimic (red, $n = 565$ cells) (b) and miR-18a-5p (red, $n = 638$ cells) (c). (d,e) tSNE of single-cell quantification of fluorescence intensity of all cells analyzed (d) and split by condition (e). (f) Proportion of cells in each microglial cluster for each condition. (g) Fluorescence intensity change over time plotted as boxplots for each time point for each cellular cluster. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; dots, individual experimental values. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range.

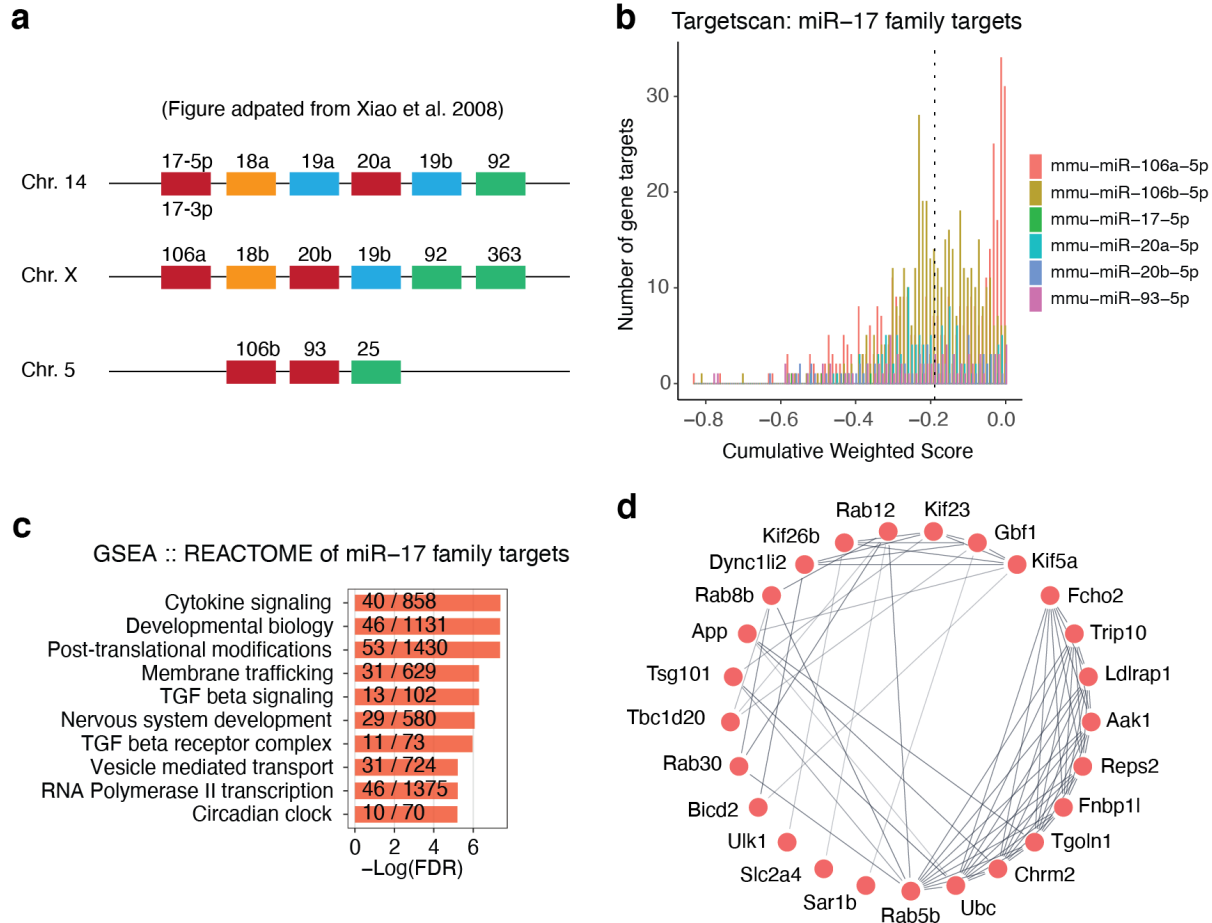


Figure 2.8. MiR-17 miRNA family predicted targets involved in innate immune system and phagocytosis-related pathways. (a) Schematic of miRNAs encoded by the miR-17~92 cluster. Figure adapted from Xiao et al. 2008⁴⁸. Each color denotes a different miRNA family based on seed sequence. (b) Histogram of the number of predicted gene targets of miR-17 family miRNAs for each binned cumulative weighted score determined by Targetscan^{5,176}. Dotted line at weighted score -0.2. (c) Bar plot of gene set enrichment analysis using the Reactome database for miR-17 family targets with weighted scores less than -0.2 determined in (b). (d) Cytoscape network visualization using the STRING database of genes involved in membrane trafficking determined in (c).

2.3.8 Supplementary Figure

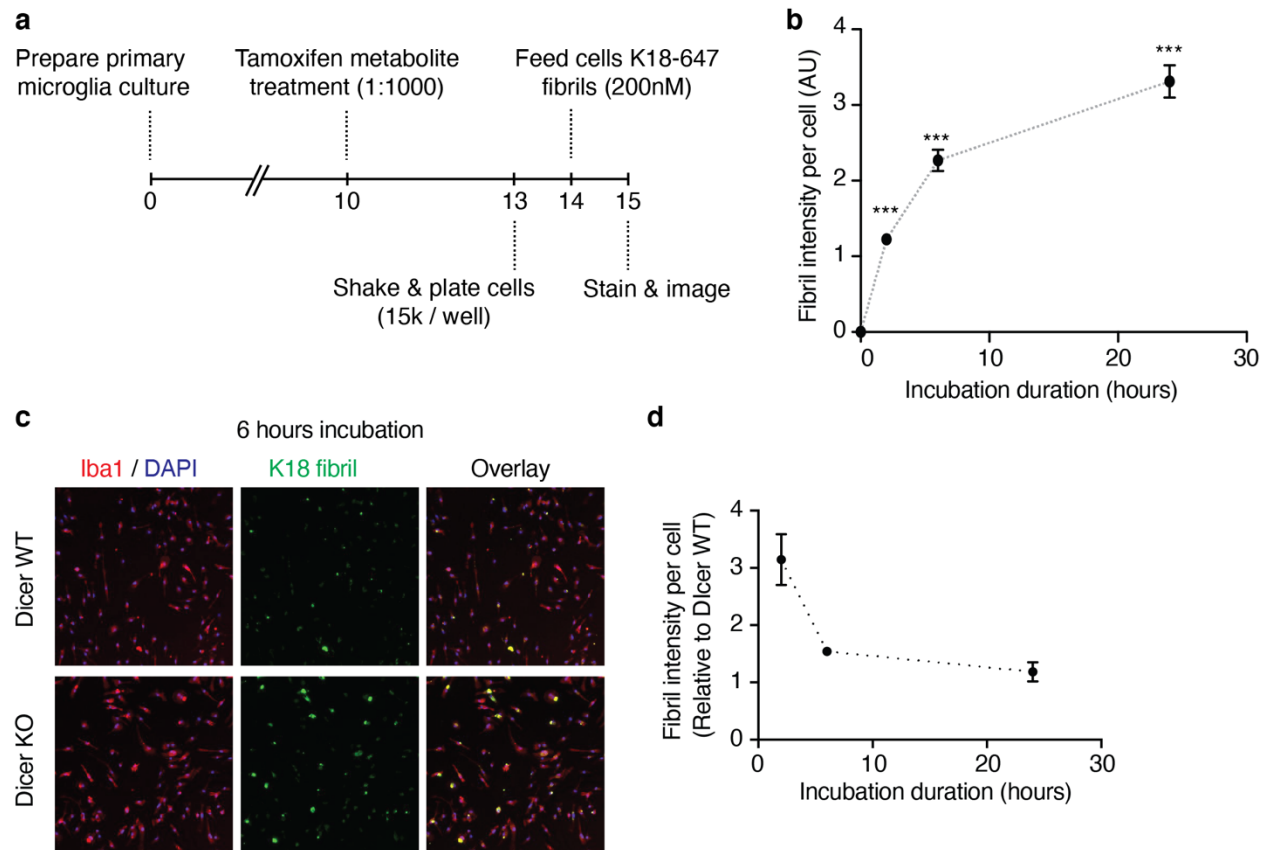


Figure S2.4. Dicer KO microglia have increased tau fibril accumulation. (a) Schematic of primary microglia culture tau fibril phagocytosis assay. (b) Quantification of fluorescence intensity per Dicer WT cell of tau fibrils conjugated with Alexa Fluor 647 at 2, 6, and 24 hours of incubation with tau fibrils. (c) Representative images taken by the Cellomics Arrayscan XTI of Dicer WT (top) and Dicer KO (bottom) stained for Iba1 (red). (d) Quantification fluorescence intensity per Dicer KO cell relative to Dicer WT levels at 2, 6, and 24 hours of incubation with tau fibrils.

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Chapter 3: AD-linked R47H-*TREM2* mutation induces sex-specific microglial states in mice and humans

3.1 Abstract

The hemizygous R47H variant of *TREM2*, a microglia-specific gene in the brain, increases risk for late-onset Alzheimer's disease (AD). In this study, we identified a subpopulation of microglia with disease-enhancing proinflammatory signatures associated with the R47H mutation in human AD brains and tauopathy mouse brains. Using transcriptomic analysis at the single-nuclei level from AD patients with the R47H or the common variant (CV)-*TREM2* with matched sex, pathology and *APOE* status, we found that the R47H mutation was associated with cell type- and sex-specific transcriptional changes in human AD brains, with microglia exhibiting the most robust alterations. Further characterization revealed that R47H-associated microglial subpopulations had enhanced inflammatory signatures including hyperactivation of Akt, one of the signaling pathways downstream of *TREM2*. In a newly-generated tauopathy knock-in mouse model expressing one allele of human *TREM2* (*hTREM2*) with either the R47H mutation or CV, R47H induced and exacerbated tau-mediated spatial memory deficits in female mice. Single-cell transcriptomic analysis of microglia from these mice also revealed transcriptomic changes induced by R47H that had significant overlaps with R47H microglia in human AD brains, including robust increases in proinflammatory cytokines, activation of Syk-Akt-signaling, and elevation of a subset of disease-associated microglial signatures. Strikingly, pharmacological Akt inhibition largely reversed the enhanced inflammatory signatures induced by R47H in primary microglia treated with tau fibrils. By unraveling the disease-enhancing properties of the R47H mutation in mouse and human, our findings shed light on an immune-linked AD subtype and provide new directions for modulating microglial immune responses to treat AD.

3.2 Introduction

Alzheimer's disease (AD) is the most common form of late-onset dementia. In addition to the pathological hallmarks of amyloid plaques and neurofibrillary tangles composed of hyperphosphorylated tau, AD is also characterized by increased microglial activation and an upregulation of cytokines in the brain. These aberrant microglial phenotypes in AD have been largely considered responses to the toxic buildup of plaques and tangles. However, genome-wide association studies have identified many risk alleles for late-onset sporadic AD that are highly expressed in microglia¹⁻⁴, providing compelling genetic evidence for important roles of microglia in AD pathogenesis. Among these risk genes, *TREM2* is the strongest immune-specific risk factor identified to date, with the heterozygous R47H point mutation significantly increasing the odds ratio of developing late-onset AD^{1,2}.

TREM2 is a single transmembrane receptor expressed exclusively in cells of the myeloid lineage, especially microglia^{5,6}. Upon ligand engagement, TREM2, together with its adaptor DAP12/TYROBP, recruits SYK and triggers several signaling cascades such as PI3K-AKT and MAPK pathways^{7,8}. These TREM2-dependent pathways in turn regulate many microglial functions, including inflammatory cytokine secretion, proliferation, phagocytosis, cell survival through mTOR signaling, and synapse elimination⁹⁻²⁰. While the exact ligands in the brain remain elusive and are likely to be context-dependent, TREM2 binds *in vitro* to apoptotic cells^{11,18}, anionic ligands^{11,21}, apolipoproteins²²⁻²⁴, and amyloid- β ²⁵⁻²⁷. Cleavage of TREM2 by metalloproteinases releases soluble TREM2²⁸⁻³⁰, which may regulate microglial cell survival and inflammation^{31,32}.

Accumulating studies suggest that TREM2 is involved in the stepwise activation of microglia in neurodegenerative processes. In the context of neurodegenerative mouse models, Trem2 is required for the conversion of microglia into disease-associated microglia (DAM) or a microglial neurodegenerative phenotype (MGnD)^{33,34}. This MGnD microglia-state can be activated by apoptotic cells and is partially

mediated through Trem2's interaction with Apoe³³. These microglia are characterized by downregulation of homeostatic genes, such as *P2ry12*, *Tmem119*, *Sall1*, and *Cx3cr1*, and upregulation of pro-inflammatory signatures such as *Apoe*, *Axl*, *Tlr2*, *Cd74*, and *Itgax*. Currently, it is unclear whether this DAM state is neuroprotective or neurotoxic for disease progression. Deletion of mouse Trem2 (*mTrem2*) prevents microglial conversion to this disease-state and protects against tau-induced atrophy^{35,36}. *mTrem2* deficiency in amyloid models, however, leads to increased amyloid toxicity, likely due to the role of Trem2 in plaque compaction³⁷⁻⁴⁰. Furthermore, human AD-microglia seem to be enriched in some of these DAM genes, such as *APOE*, *CD74*, *HLA-DRB1*, and show overlap in molecular pathways related to lipid and lysosomal biology. However, there is likely to be human-specific AD-microglia subpopulations since many gene signatures do not overlap between the mouse and human AD-associated microglia^{41,42}. These observations suggest the role of TREM2 and DAMs in neurodegenerative diseases is context- and disease state-specific.

Little is known about how the R47H mutation of *TREM2* contributes to AD. Previous studies reported that AD patients carrying the heterozygous R47H variant show higher neuritic plaque densities, reduced microglial coverage of amyloid plaques and more severe plaque-associated neuritic dystrophy, as well as increased accumulation of autophagosomes in microglia^{9,40,43}. Human AD patients carrying the R47H mutation also display higher levels of both total tau and phosphorylated tau in CSF compared to non-carriers^{44,45}. One transcriptomic study showed that at the bulk-tissue level, several immune-related genes are decreased in R47H carriers such as *IRF8*, *HLA-DRA* and *AIF1*, suggesting either a decrease in the number of microglia or decreased expression of these genes on a per-cell basis⁴⁶. However, comprehensive transcriptomic studies at either the single-cell level or with a large sample size of patient brain tissues have not been done. In mouse models, homozygous knock-in of R47H human *TREM2* (R47H-*hTREM2*) on a background lacking endogenous mTrem2 leads to deficits in microglial

amyloid plaque compaction and increases tau staining and dystrophic neurites bypassing plaques ^{40,47}. Similar to the *mTrem2* deficiency amyloid mouse model, protein expression of amyloid-plaque-induced microglial activation markers, such as *Clqa*, *Lyz2*, and *Spp1* is decreased ⁴⁶, suggesting that R47H may be neurotoxic due to its inability to activate microglia and compact amyloid plaques. In a recent study, male PS19 tauopathy mice expressing homozygous R47H-*hTREM2* exhibited reduced tau phosphorylation, brain atrophy, and synapse loss compared to mice expressing CV-*hTREM2* ⁴⁸, similar to the phenotype of *mTrem2*-deficient tauopathy mice. However, how the R47H mutation appears to protect against tauopathy in mice yet elevates AD risk in humans remains unanswered.

In the current study, we uncovered an R47H-enriched microglia subpopulation in human AD brains by performing single-nuclei RNA sequencing analysis of 16 AD patient brain tissues with and without the R47H mutation. To investigate the functional changes induced by R47H in AD, we used CRISPR to replace one allele of *mTrem2* in mice with CV- or R47H-*hTREM2*, generating a heterozygous R47H-*hTREM2* mouse model that was then crossed to a tauopathy model. Our heterozygous R47H-*hTREM2* tauopathy mice exhibited enhanced tau toxicity on spatial memory deficits. In addition, we showed that in response to tau pathology, R47H-associated microglia upregulated a subset of DAM signatures and increased expression of pro-inflammatory cytokines, which was reversed by inhibiting Akt. Together, our study uncovers novel disease-enhancing mechanisms of the R47H mutation and new directions for modulating brain immune responses to treat AD.

3.3 Results

R47H Mutation Induces Cell Type and Sex-Specific Transcriptional Changes in Human AD Brain Tissues

To dissect the pathogenic mechanisms associated with *TREM2*^{R47H} in human AD patients, we performed single-nuclei RNA-seq (snRNA-seq) of mid-frontal cortical tissues from 16 AD brains that harbored a single allele of the TREM2 common-variant (CV) or the R47H mutation (n=8 per group, Supplementary Table 1). The samples were matched in sex and *APOE* E3/E4 genotype (**Fig. 3.1 a**), as well as age, amyloid and tau burdens (**Fig. S3.1 a-c**). Following an established human snRNA-seq protocol^{49,50}, we sequenced 111,932 nuclei and used 83,051 nuclei for downstream analysis after removal of potential multiplets using DoubletFinder⁵¹ and filtering for low-quality nuclei determined by thresholding gene counts, UMI counts, and percent mitochondrial genes per nuclei (**Fig. 3.1a, Fig. S3.1 d-g**, Supplementary Table 2). Using reference gene sets for cluster annotations^{52,53}, we identified the major cell types of the brain and observed that cell types were similarly represented in all samples sequenced (**Fig. 3.1 b-c, Fig. S3.1 h-i**).

We first performed differential expression analysis to compare the effects of the R47H mutation in each cell type and sex. The mutation was associated with many transcriptional changes in all cell types in both sexes, with microglia having the largest total number of DEGs (**Fig. 3.1 d**, Supplementary Table 3). Interestingly, *TREM2*^{R47H} microglia induced sex-specific transcriptomic changes, with a significantly higher number of DEGs in male compared to female microglia and astrocytes, and far fewer changes in male compared to female excitatory neurons. We then examined the overlaps between the DEG signatures in different cell types and found that few R47H-induced DEGs were shared between male and female brains (purple, **Fig. 3.1 e**). There was also little overlap of the DEGs among different cell types

(rows, **Fig. 3.1 e**). Indeed, pathway analysis revealed that the top molecular pathways represented by the DEGs were cell-type and sex-specific (**Fig. 3.1 f**).

Human R47H AD-Microglia Exhibit Unique Microglial Disease Signatures Characterized by Hyperactivation of Syk-Akt Signaling

To further dissect the transcriptomic changes in microglia induced by *TREM2*^{R47H}, we subclustered the 4350 microglia cells from all samples and identified 9 different transcriptional states (**Fig. 3.2 a**, Supplementary Table 4). We focused on clusters 1-5, as clusters 6-9 had fewer than 30 cells each. When split by *TREM2* genotype, we found differential distributions of microglial subclusters between *TREM2*^{R47H} and *TREM2*^{CV} samples, with clusters 4 and 5 being more enriched in *TREM2*^{R47H} samples (**Fig. 3.2 b-c**), as well as differentially distributed by sex (**Fig. S3.2**). Cluster 4 and 5 cells had distinct transcriptomic signatures when compared with homeostatic microglial cluster 1 cells (**Fig. 3.2 d,e**). We then compared the gene signatures of these five microglial clusters identified in our AD *TREM2*^{R47H} transcriptomic dataset with previously-identified microglial signatures associated with high-pathology from AD *TREM2*^{CV} brains⁴¹ (Mathys-AD, **Fig. 3.2 f**). Clusters over-represented in the R47H carriers (clusters 4 and 5) had the most overlap, suggesting that *TREM2*^{R47H} led to further enrichment of the human AD-associated microglia subpopulations (**Fig. 3.3 a**). One of the upregulated markers in R47H-associated clusters was MS4A6A, an important AD risk gene that regulates soluble TREM2 in human AD⁵⁴. Other interesting components of the R47H-associated signature were acyl-coA synthase (ACSL1), which is involved in lipid metabolism and inflammation, serglycin (SRGN), an intracellular proteoglycan interacting with inflammatory mediators, and SLC11A1, a divalent transition metal (iron and manganese) transporter with antimicrobial function (**Fig. 3.2 d,e**, **Fig. S3.3 a**).

We then compared human R47H microglia in AD brains (cluster 4 or 5) with previously-identified mouse DAM microglia³⁴, and identified some overlap as well as significant differences (DAM, **Fig. 3.2 f**, **Fig. S3.3 b**). Notably, genes involved in microglial homeostasis (*CX3CR1*, *TMEM119*, *TGFBRI*, *CSF1R*), complement signaling (*CIQA*, *CIQB*, *CIQC*), and purinergic signaling (*P2RY12*, *P2RY13*, *GPR34*, and *ENTPDI*) were downregulated in human *TREM2*^{R47H} microglia and mouse DAMs. Upregulated genes overlapping with DAM signatures included *NAMPT*, *ATF3*, *SRGN*, *ATP1B3*, *ENPPI*, *CD83*, and *LPL*. However, many genes were differentially-regulated in human *TREM2*^{R47H} microglia and mouse DAM. Several genes upregulated in *TREM2*^{R47H} microglia were downregulated in mouse DAMs (*ABCA9*, *KLF2*, *STAB*, *MTSSI*, *GLUL*, *TNFRSF1B*, *ABCC3*), while a small subset of genes downregulated in *TREM2*^{R47H} microglia, including *SPPI*, involved in injury repair, and *SOCS6*, a negative regulator of the Jak-Stat pathway, were upregulated in DAMs (**Fig. S3.3 b**).

The R47H-unique signatures not overlapping with previously-identified human AD-microglial signatures or mouse DAMs were further analyzed using pathway enrichment analysis (**Fig. 3.2 f**). The top pathway in both cluster 4 and 5 was TNF- α signaling via NF- κ B, suggesting an elevated proinflammatory state (**Fig. 3.2 g**). R47H-induced changes were also associated with metabolic pathways, such as hypoxia, glycolysis and mTORC1 signaling, and immune pathways, such as interleukin (IL2 and IL6) and interferon response (**Fig. 3.2 g**), with many of these pathway genes being interconnected (**Fig. 3.2 h,i**). Interestingly, upstream and downstream mediators of TREM2 signaling, including AKT and SYK, were predicted to be activated in these human R47H-enriched microglia (**Fig. 3.2 j,k**). Together, in human AD patients, the R47H mutation expanded a unique microglial subpopulation characterized by elevated pro-inflammatory transcripts and reduced homeostatic microglial genes, and hyperactivation of TREM2-associated signaling molecules.

R47H-*hTREM2* Exacerbates Spatial Learning and Memory Deficits in Female Tauopathy Mice

To further dissect the molecular pathways induced by the R47H mutation, we generated knock-in mouse lines expressing one copy of CV- (*hTREM2*^{CV/+}) or R47H-*hTREM2* (*hTREM2*^{R47H/+}) cDNA at the *mTrem2* locus using CRISPR (**Fig. 3.3 a**). PCR and Sanger sequencing confirmed the correct recombination and insertion of human TREM2-CV and TREM2-R47H cDNA at the *mTrem2* locus (**Fig. S3.4 a,c-e**). We did not detect any non-specific integration in the *hTREM2*^{R47H/+} mouse line. However, a non-specific integration event occurred in *hTREM2*^{CV/+} mice at an unknown mouse genomic region (**Fig. S3.4 b,f-g**). Nevertheless, *hTREM2*^{CV/+} and *hTREM2*^{R47H/+} mice had equivalent expression levels of *hTREM2* mRNA (**Fig. 3.3 b**) and protein (**Fig. 3.3 c,d**), and *hTREM2*^{R47H} mice showed a dose-dependent reduction of *mTrem2* mRNA (**Fig. 3.3 e**), indicating successful replacement of *mTrem2* by *hTREM2*. The matched expression levels of CV- and R47H-*hTREM2* mRNA and protein allowed us to assess the consequences of heterozygous R47H variant *in vivo*.

Tau pathology strongly correlates with cognitive deficits in AD^{55,56}. To assess the effect of R47H-*hTREM2* on tau-induced cognitive deficits, we crossed *hTREM2*^{CV/+} and *hTREM2*^{R47H/+} mice with P301S mice, which express a human tau gene with the P301S mutation and develop hallmarks of tauopathy, including gliosis, tau inclusions, and cognitive deficits, such as deficits in hippocampal-dependent memory and spatial learning, mimicking human AD⁵⁷. The effects of a single allele of R47H-*hTREM2* on tau-induced spatial learning and memory were investigated using the Morris Water Maze. As expected, 7 to 9 month-old, non-transgenic mice preferred the target quadrant over the non-target quadrants while mice with P301S expression did not have a significant preference, indicative of spatial memory impairment (**Fig. 3.3 f,g**). Interestingly, R47H-*hTREM2* significantly worsened spatial learning in female P301S mice, but had no statistically significant effect on males (**Fig. 3.3 h,i**). P301S *hTREM2*^{R47H/+} mice also made significantly more search errors during the 72-hour probe trial than

controls (**Fig. 3.3 j**), indicative of worsened spatial memory. In contrast, male P301S *hTREM2*^{R47H/+} mice trended toward fewer search errors compared with male P301S mice during the probe trial (**Fig. 3.3 k**). The spatial learning and memory curves of P301S *hTREM2*^{CV/+} mice resembled that of P301S *mTrem2*^{+/+} (wild-type) mice regardless of sex (**Fig. 3.5 a-d**), suggesting the effects were R47H-mutation dependent. No differences were observed between genotypes in activity levels in the open field (**Fig. S3.5 e-h**) nor in the percentage of time spent in the open arms of the elevated plus maze (**Fig. S3.5 i-l**), ruling out hyperactivity and anxiety, which could confound the spatial memory test results.

Notably, the R47H mutation did not significantly impact the accumulation of insoluble tau aggregates detected using a conformation-specific antibody, MC1⁵⁸, suggesting that the disease-enhancing effects of R47H-*hTREM2* in female P301S mice were not mediated by elevating toxic tau load (**Fig. S3.6**). Indeed, even in the absence of tau pathology, the R47H mutation led to spatial learning deficits in females, without affecting baseline activity and anxiety levels (**Fig. S3.7**). Taken together, our findings suggest that the R47H mutation renders hippocampal circuitry particularly vulnerable in both the absence and presence of tau pathology in a sex-dependent manner.

R47H-*hTREM2* Increases Inflammation in Female Tauopathy Mice

To dissect how R47H-*hTREM2* microglia affects tau-mediated learning and memory, we next performed bulk-tissue RNA-seq of the hippocampus from 7- to 9-month-old male and female P301S *hTREM2*^{R47H/+} and P301S *hTREM2*^{CV/+} mice with matching *mTrem2* expression (**Fig. 3.4 a**), and their littermate P301S *mTrem2*^{+/+} controls. Differential expression analysis revealed that R47H-*hTREM2* induced significant transcriptomic changes in females. 94 genes were upregulated by the mutation, including several DAM genes (e.g., *Ccl6*, *Clec7a*, *Siglec5*, *Cd9*, *Cd63*)³⁴ and other inflammatory genes (e.g., *Cxcl5*, *Ccl9*), while 28 genes were downregulated, including neuron-associated genes (e.g., *Adora2a*, *Syt6*, *Serpina9*, *Penk*) (**Fig. 3.4 b**, Supplementary Table 5). In contrast, in male mice, R47H-*hTREM2* only significantly altered

3 genes in P301S mice (**Fig. 3.4 c**), consistent with the lack of a significant effect of R47H-*hTREM2* on cognitive deficits in male P301S mice at this age (**Fig. 3.3**). The effects were specific to the R47H mutation, as CV-*hTREM2* had no significant transcriptional effect in female P301S mice and only altered 18 transcripts in male mice (**Fig. S3.8**).

Using weighted gene-correlation network analysis (WGCNA), we examined the transcriptomes of female P301S *hTREM2*^{CV/+} and P301S *hTREM2*^{R47H/+} mice and their P301S *mTrem2*^{+/+} littermate controls, and identified four statistically significant correlational modules related to P301S *hTREM2*^{R47H/+} mice (**Fig. 3.4 d**). Pathway analysis showed that the brown module, which exhibited the most significant positive correlation with the P301S *hTREM2*^{R47H/+} genotype, was enriched with transcripts encoding cytokines/chemokines and cytokine receptors (e.g., *Ccr5*, *Ccl5*, *Ccl3*, *Cxcl5*) (**Fig. 3.4 e-f**, Supplementary Table 6). The cyan module, which negatively correlated with the P301S *hTREM2*^{R47H/+} genotype, was significantly enriched in transcripts encoding axon guidance molecules (e.g., *Sema6b*, *Sema3f*, *Epha8*, *Ephb6*) (**Fig. 3.4 e**, Supplementary Table 6). Together, these data suggest an upregulation of pro-inflammatory transcripts and a concomitant decrease in neuronal signaling genes in female P301S *hTREM2*^{R47H/+} mice compared to control mice.

R47H-*hTREM2* Enhances Expression of Disease-associated Microglial Signature and Akt Signaling in Female Tauopathy Mice

We next performed single-cell RNA-seq using the Smart-Seq2 platform to specifically probe the effects of the R47H mutation on the microglial transcriptome⁵⁹. Microglia were isolated from the hippocampal tissue of 8-month-old female *mTrem2*^{+/+}, *hTREM2*^{R47H/+}, P301S *mTrem2*^{+/+}, and P301S *hTREM2*^{R47H/+} mice, gating on CD45^{low}CD11b⁺ cells (**Fig. S3.9 a,b**). Out of the 1,480 cells that were sorted, 1,424 passed quality control (**Fig. S3.9 c-g**). *mTrem2* expression level was higher in *mTrem2*^{+/+} microglia compared to the *hTREM2*^{R47H/+} microglia, confirming the replacement of one allele of *mTrem2* (**Fig.**

S3.9 h). Two distinct clusters were identified by unsupervised clustering of these 1,424 cells (**Fig. S3.5 a**). While cluster 1 microglia were found in all 4 genotypes, cluster 2 microglia were mainly associated with the expression of P301S tau (**Fig. 3.5 b**). Strikingly, *hTREM2*^{R47H/+} expression significantly increased the proportion of cluster 2 microglia in P301S tau mice (**Fig. 3.5 b-c**). Compared to cluster 1 cells, cluster 2 cells have significant upregulation of transcripts including several DAM transcripts, such as *Clec7a*, *Ctsb*, *Axl*, *Cst7*, *Apoe*, and *Cd63* (**Fig. 3.5 d,e**, Supplementary Table 7), consistent with the increased inflammation observed in the bulk-tissue RNA-seq data (**Fig. 3.4 b**). While classical microglial genes, such as *Hexb*, were present in both clusters, the homeostatic microglial gene *P2ry12* was downregulated in cluster 2 cells (**Fig. 3.5 e**). A direct comparison of cluster 2 marker genes versus DAM signature genes showed a statistically-significant positive correlation (**Fig. 3.5 f**). Thus, in the presence of tau pathology, *hTREM2*^{R47H/+} enhances the DAM-like subpopulation and increases expression of *Trem2*-dependent microglial transcripts associated with neurodegeneration (MGnD), such as *Apoe*, *Itgax*, *Lpl*, *Axl*, and *Cst7*^{33,34} (red, **Fig. 3.5 f**). We further examined the microglial *Apoe* expression in brain sections of P301S *hTREM2*^{R47H/+} mice compared to P301S *hTREM2*^{+/+} mice by RNAscope and confirmed that the proportion of microglia expressing *Apoe* was significantly increased in P301S *hTREM2*^{R47H/+} mice (~90%) compared to P301S *mTrem2*^{+/+} (~60%) in the dentate gyrus of the hippocampus (**Fig. 3.5 g-i**).

Upstream regulator analysis further predicted activation of *Trem2*-associated downstream signaling molecules, such as *Tyrobp*, *Akt*, *Syk* and *Src*, as well as activation of upstream regulators of *Trem2* such as *Csf1* and *Csf2*⁷ (**Fig. 3.5 j,k**). To directly assess microglial Akt activation, we performed immunostaining of phospho-Akt-Ser473, the major phosphorylation site required for Akt activation. While most of the phospho-Akt signal in P301S *mTrem2*^{+/+} was localized to non-Iba1+ cells, strong phospho-Akt signaling was localized to Iba1+ microglia in P301S *hTREM2*^{R47H/+} brains, confirming

enhanced microglial Akt signaling induced by the R47H mutation (**Fig. 3.5 l,m**). In sum, *hTREM2^{R47H/+}* expression in female tauopathy mice induced similar features observed in AD *TREM2^{R47H}* human microglia (**Fig. 3.2 j,k**), including an expanded DAM-like subpopulation with enhanced Syk-Akt signaling and increased expression of a subset of transcripts previously found to be *Trem2*-dependent.

Aside from modulating the microglial inflammatory response, Trem2 is also involved in other key microglial functions. To test these, we assessed the microglial response to injury and phagocytosis^{12,16,36,60}. The R47H mutation, however, did not alter the microglial response to laser-induced injury compared to *hTREM2^{CV/+}* or *mTrem2^{+/+}* controls (**Fig. S3.10 a-c**, Supplementary Movie 1). The effects of R47H on phagocytosis were assessed by acquiring time-course images of primary microglia incubated with pH-rodo-conjugated *E. coli* substrates. Consistent with a previous study in HEK293 cells²⁹, we detected no difference in the dynamics of fluorescence intensity over time among these three genotypes (**Fig. S3.10 d-e**), suggesting no change of phagocytic activity of *E. coli* induced by R47H. Due to heterogeneity in the phagocytosis kinetics between cells, we further performed a more detailed single-cell image tracking analysis by clustering cells based on different fluorescence dynamics, which correlated well with the bulk fluorescence analysis (**Fig. S3.10 f**). Again, *hTREM2^{R47H/R47H}* microglia exhibited similar patterns of phagocytic behaviors at the single-cell level as *hTREM2^{CV/CV}* and *mTrem2^{+/+}* microglia (**Fig. S3.10 g**).

Akt Activation Underlies Tau-mediated Proinflammatory Signatures in R47H-*hTREM2* Microglia

Our results so far showed that the R47H mutation enhances proinflammatory microglial responses in human AD and in female mouse tauopathy brains. We next investigated how the R47H mutation affects microglial response to tau specifically *in vitro*. To maximize the R47H effects, we treated *hTREM2^{R47H/R47H}* microglia with tau fibrils and used *mTrem2^{+/+}* microglia as controls. Compared to *mTrem2^{+/+}* microglia, tau fibril stimulation significantly upregulated genes enriched in several signaling

pathways in *hTREM2*^{R47H/R47H} microglia (**Fig. 3.6 a,b**). The cytokine–cytokine receptor interaction pathway was one of the top pathways altered by *hTREM2*^{R47H/R47H} (**Fig. 3.6 b**), consistent with our observation in female P301S *hTREM2*^{R47H/+} mice (**Fig. 3.4 e**). Moreover, Akt and Syk involved in Trem2-signaling pathways were again predicted to be activated in these microglia (**Fig. 3.6 c-e**), similar to observations in our tauopathy mouse model and human AD tissues.

Next, we directly tested the extent to which Akt signaling contributes to exaggerated inflammatory responses in tau-treated *hTREM2*^{R47H/R47H} microglia. We acutely inhibited Akt in *hTREM2*^{R47H/R47H} microglia cultures with MK-2206, an allosteric Akt-specific inhibitor⁶¹ before treating with tau fibrils. Transcriptomic analysis showed that MK-2206 specifically inhibited the Akt pathway (**Fig. 3.6 f**) without altering the Syk pathway (**Fig. 3.6 g**). Strikingly, transcriptomic analysis demonstrated that, out of 838 DEGs (adjusted p value < 0.05, log2FC > 1 or < -1) between *hTREM2*^{R47H/R47H} and *mTrem2*^{+/+} microglia treated with tau fibrils, 477 of them were reversed towards *mTrem2*^{+/+} control levels upon Akt-inhibition (green columns, **Fig. 3.6 h**, Supplementary Tables 8-9). These genes were enriched in pathways related to cytokine–cytokine receptor interaction (**Fig. 3.6 i-k**). These transcriptional changes were further confirmed by measuring secreted cytokines in response to tau fibrils with a multiplex immunoassay. Out of the 26 cytokines altered by the R47H mutation, 11 of them were significantly rescued by MK-2206 (**Fig. 3.6 l**). These results suggest that at both the RNA and protein levels, hyperactivation of Akt signaling may mediate some of the R47H-induced pro-inflammatory signatures in response to tau pathology. Remarkably, the predicted activation of TREM2-associated signaling pathways was consistent in all three R47H models – human AD carriers, tauopathy mice, and primary mouse microglia treated with tau fibrils (**Fig. 3.6 m**), supporting hyperactive Akt as one of the consistent mechanisms underlying the disease-enhancing property of the R47H mutation in mouse and human.

3.4 Discussion

Compelling human genetic studies strongly suggest that maladaptive innate immune responses are associated with elevated risk of developing late-onset AD. Recent single-cell transcriptomic findings suggest that a subpopulation of microglia is enriched in response to AD-related pathologies (DAM or MGnD) ^{33,34}. Still, among the DAM signature genes, the identity of those that are disease-enhancing microglial genes (DEMs), disease-mitigating microglial genes (DMMs), or mere bystanders remains elusive. As the strongest immune-specific risk gene, the R47H-*TREM2* variant provides a unique model to help define immune-mediated DEMs in human AD. Through single-nuclei transcriptomic analysis of mid-frontal cortical tissues from 16 AD human patients carrying the R47H or CV variant of *TREM2*, we uncovered a microglia subpopulation enriched in AD R47H-*TREM2* carriers. We further established the disease-enhancing property of R47H-enriched microglia in a newly-developed R47H-*hTREM2* knock-in tauopathy mouse model by combining functional behavior studies and single-cell microglial transcriptomic analysis. We showed that in the context of tauopathy, R47H-*TREM2* microglia are characterized by a heightened inflammatory state, partially mediated by Akt hyperactivation.

In previous studies in amyloid mouse models, the homozygotic R47H mutation was found to dampen the microglial response to amyloid pathology, and correlated with increased neurotoxicity ^{40,47}. Paradoxically, in a tauopathy mouse model, the homozygotic R47H mutation was shown to be neuroprotective against neurodegeneration ⁴⁸. Our current study provides the first line of evidence that the heterozygotic R47H mutation is disease-enhancing in the presence of tau pathology. While the R47H mutation did not impact general microglial functions such as phagocytosis and response to acute injury, the mutation exacerbated tauopathy-induced spatial learning and memory deficits in female tauopathy mice without affecting other cognitive domains, such as activity or anxiety levels. Importantly, this enhanced toxicity in our tauopathy model was not due to differences in tau-pathology load. Instead,

transcriptomic profiling revealed that the toxic effects of R47H-*hTREM2* on tau-mediated cognitive deficits in female mice were associated with significant and profound transcriptional changes, particularly increased expression of pro-inflammatory genes. Meanwhile, the lack of toxic cognitive effects of R47H in male tauopathy mice was associated with few transcriptional changes. These findings support the notion that R47H-induced cognitive-deficits are driven by disease-enhancing microglial responses to stimuli including pathogenic tau, but not by directly influencing tau load itself.

Our study is the first to characterize the R47H-enriched microglial subpopulation in human AD brains and tauopathy mouse brains using single-cell or single-nuclei transcriptomic analyses. To identify specific microglial signatures induced by the R47H mutation in human brains, we ensured that samples were matched in AD pathology levels, *APOE* genotype, and sex. We discovered that, at the single-nuclei level, there was an increase in a subpopulation of microglia in R47H-AD brains characterized by heightened TNF- α signaling via NF- κ B and interferon responses, altered metabolic pathways, and enhanced AKT signaling. Similar to a previous observation of reduced microglial activation signatures in AD R47H-*TREM2* carriers using bulk-tissue RNA-seq⁴⁶, we found decreased expression of *AIF1* and *IRF8* in the R47H-associated microglial clusters. However, we also found overwhelming increases in expression of other immune-related genes, including interferon and interleukin-related genes (*IL7*, *CD44*, *IFI44L*, *TNFRSF1B*) and DAM-related genes (*NAMPT*, *ATF3*, *SRGN*, *ATP1B3*, *ENPP1*, *CD83*, *LPL*)³⁴, as well as increases in AD-associated genes defined previously such as (*SRGN*, *HIF1A*, *MS4A6A*, *SAT1*, *CTSB*)⁶², highlighting the complexity of microglial states and a need to use a diverse set of marker genes to define the activation state of microglia in the human AD brain.

We showed that R47H-*TREM2* in human AD and in tauopathy mice exhibited heightened proinflammatory states, diminished homeostatic signature, and enrichment of DAM signature genes. These findings were in contrast to findings in *Trem2*-deficient microglia in amyloid and tau mouse

models, which exhibit microglia in homeostatic states with blocked induction of DAM/MGnD signatures^{33,34}. Our findings are also distinct from studies using homozygous R47H-*hTREM2* in 5XFAD mice⁴⁷ and in P301S mice⁴⁸, and provide compelling evidence that the heterozygotic R47H mutation does not phenocopy *TREM2* deficiency in humans, which results in Nasu-Hakola disease, and in mouse tauopathy. This distinction is important, since the vast majority of R47H carriers in AD are heterozygotes, and we previously demonstrated that *Trem2* haploinsufficiency can have opposing effects on tau pathology and microglial activation compared with *mTrem2*^{-/-}³⁶. How exactly the homozygous and heterozygous R47H mutation differ in their downstream signaling is unclear. It is conceivable that the interaction of R47H- and CV-TREM2 could alter ligand-binding properties, including conformation of the hybrid receptor and ligand-selectivity, leading to heightened pro-inflammatory signaling. Nevertheless, the R47H-microglia from our tauopathy mice shared similar features to the R47H-associated human microglia, with enhanced TREM2-Akt-cytokine signaling and pro-inflammatory signatures as well as sex-specific transcriptomic differences, illustrating the strong relevance of our R47H knock-in mouse model to the human condition.

AD is strongly modified by sex, with many studies showing sex-differences in the incidence, prevalence, pathological findings, and disease progression rates⁶³⁻⁶⁶. The sex-specific effects of R47H-*hTREM2* uncovered in our behavioral tests and transcriptomic studies in both mouse and human may be mediated by the differences between male and female microglial transcriptomes⁶⁷⁻⁶⁹. Indeed, the R47H mutation led to disease-enhancing effects only in female, not male mice of the same age group. The lack of detrimental effects of R47H on male tauopathy mice is consistent with a recent study in which only male mice were used⁴⁸. In human tissues, we also observed sex-specific microglial changes induced by the R47H mutation, though there were greater transcriptomic changes in male compared to female microglia. The distinct sex-specific microglial response induced by R47H in mice and humans could

potentially be explained by differences in their cognitive status. In the human studies, male and female patients were matched in their Cognitive Dementia Ratings, with both sexes having robust transcriptional changes induced by R47H-*TREM2* microglia, while in mice, the lack of transcriptional changes in microglia was correlated with male R47H tauopathy mice that exhibited little tau-mediated cognitive impairment.

In a previous study, induction of the MGnD-state by Trem2, including upregulation of *ApoE*, has been shown to be sex-specific³³. Interestingly, ApoE4 increases risk for late-onset sporadic AD to a greater extent in women^{70,71}, and female ApoE4 knock-in mice have spatial memory deficits not seen in males⁷². Microglia-derived ApoE is a major source of plaque-associated ApoE and is thought to be the driver of neurodegeneration in tauopathy mouse models^{73,74}, suggesting that sex-specific differences in microglia may impact the sex-dependent effect of ApoE4 in AD pathogenesis⁷⁵. However, how R47H-*TREM2* and different *APOE* genotypes might interact to affect microglial function is unknown. We focused our current assessment of R47H effects in the same *APOE* background (*APOE3/4*) to control for potential influences of different *APOE* isoforms. Further studies are needed to confirm and extend our observations and to investigate the effects of different *APOE* isoforms.

Since DAMs exhibit responses that could be either protective or detrimental, blocking or enhancing all DAMs is unlikely to result in neuroprotection in AD patients. By identify disease-enhancing properties associated with the R47H mutation, strategies could be developed to suppress DEMs specifically and reprogram microglial responses more precisely. In our current study, *hTREM2*^{R47H/R47H} microglia treated with tau fibrils also exhibited enhanced pro-inflammatory gene expression and hyperactive Akt-signaling, as observed in R47H microglia in human AD patients. Pharmacological inhibition of Akt corrected more than half of the genes altered by R47H, including cytokines, suggesting that Akt inhibitors could be explored as therapeutic strategies to reprogram disease-enhancing microglial

states. While further *in vivo* studies are required to validate these findings, our study opens new avenues for developing microglia-targeted therapies for AD.

3.5 Methods

Mice

CRISPR/Cas9-mediated knock-in of the common variant (CV) or R47H human *TREM2* cDNA in place of *mTrem2* was done by injecting embryos with Cas9, short-guide RNA (sgRNA), and donor vectors (generated by PNA Bio). The human *TREM2* cDNA sequence was flanked on each side by 1-kb homology arms for the *mTrem2*. The sequences are as follows: *Trem2* targeted region 5'CCTGCTGCTGATCACAGGTGGGA and sgRNA sequence (antisense) 5'TCCCACCTGTGATCAGCAGCAGG. Potential off-target genes were identified with CRISPR off-target prediction software (<http://www.crispor.tefor.net>). There were no predicted off-targets for 1- or 2-basepair mismatches. CV hTREM2 and R47H hTREM2 lines were maintained independently and backcrossed to nontransgenic C57BL/6 mice for two to three generations, then crossed to *Cx3cr1*^{GFP/GFP} or P301S mice. *Cx3cr1*^{GFP/GFP} (<https://www.jax.org/strain/005582>) were crossed with CV or R47H hTREM2 knock-in lines to obtain *Cx3cr1*^{GFP/+}*hTREM2*^{R47H/+}, *Cx3cr1*^{GFP/+}*hTREM2*^{CV/+}, and *Cx3cr1*^{GFP/+}*mTrem2*^{+/+} littermates for both lines. P301S transgenic mice (<https://www.jax.org/strain/008169>) were crossed with CV or R47H hTREM2 knock-in mice to generate P301S *hTREM2*^{R47H/+} and littermate P301S *mTrem2*^{+/+} mice, as well as P301S *hTREM2*^{CV/+} and littermate P301S *mTrem2*^{+/+} mice. Mice of both sexes were used, and analyses based on sex are included in the main and supplementary figures. Mice underwent behavioral testing at 7 to 9 months of age and had not been used for any other experiments. At 8 to 9 months of age, the same mice were used for pathology and RNA-seq studies after completion of behavioral tests. *Cx3cr1*^{GFP/+} mice for in vivo imaging were studied at 12 to 17 months of age. All mouse protocols were approved by the Institutional Animal Care and Use Committee, University of California, San Francisco and Weill Cornell Medicine.

Human Postmortem Samples

Tissues from the mid-frontal cortices from brains of AD donors carrying the R47H mutation (n=8, 5 females and 3 males) and or the common variant (CV) (n = 8; 5 females and 3 males) were used for single-nuclei RNA-sequencing, for a total of 16 samples. Samples were matched in age, tau and amyloid pathology burden, *APOE* genotype, and Clinical Dementia Ratings. Samples were obtained from the Mayo Clinic brain bank and derived from several different studies: State of Florida Alzheimer's Disease Initiative (ADI), Alzheimer's cases derived from the Mayo Clinic (ADC), and cases obtained from outside sources, usually because of atypical clinical syndromes (such as corticobasal syndrome, frontal lobe dementia or progressive aphasia), but AD as the underlying pathology (Consult). Mayo Clinic brain bank operates under procedures approved by the Mayo Clinic institutional review board (IRB# is 15-009452). All brains were donated after consent from the next-of-kin or an individual with legal authority to grant such permission. The institutional review board has determined that clinicopathologic studies on de-identified postmortem tissue samples are exempt from human subject research according to Exemption 45 CFR 46.104(d)(2).

Additional information about the donors can be found in Supplementary Table 1.

Isolation of Nuclei from Frozen Postmortem Human Brain Tissue

The protocol for isolating nuclei from frozen postmortem human brain tissue was adapted from a previous study with modifications^{49,50}. All procedures were done on ice or at 4°C. In brief, postmortem brain tissue was placed in 1500 µl of Sigma nuclei PURE lysis buffer (Sigma, NUC201-1KT) and homogenized with a Dounce tissue grinder (Sigma, D8938-1SET) with 20 strokes with pestle A and 15 strokes with pestle B. The homogenized tissue was filtered through a 35-µm cell strainer, and were centrifuged at 600 g for 5 min at 4°C and washed three times with 1 ml of PBS containing 1% BSA, 20 mM DTT and 0.2 U µl⁻¹ recombinant RNase inhibitor. Then the nuclei were centrifuged at 600 g for 5

min at 4°C and re-suspended in 800 µl of PBS containing 0.04% BSA and 1x DAPI, followed by FACS sorting to remove cell debris. The FACS-sorted suspension of DAPI-stained nuclei were counted and diluted to a concentration of 1000 nuclei per microliter in PBS containing 0.04% BSA.

Droplet-based Single-nuclei RNA-seq of Human Brain Tissue

For droplet-based snRNA-seq, libraries were prepared with Chromium Single Cell 3' Reagent Kits v3 (10x Genomics, PN-1000075) according to the manufacturer's protocol. The snRNA-seq libraries were sequenced on the NovaSeq 6000 sequencer (Illumina) with 100 cycles.

Analysis of Droplet-Based Single-nuclei RNA-seq Data from Human Brain Tissue

Gene counts were obtained by aligning reads to the hg38 genome with Cell Ranger software (v.3.1.0) (10x Genomics). To account for unspliced nuclear transcripts, reads mapping to pre-mRNA were counted. Cell Ranger 3.1.0 default parameters were used to call cell barcodes. We further removed genes expressed in no more than 2 cells, cells with unique gene counts over 4,000 or less than 200, and cells with high fraction of mitochondrial reads (> 5%). Potential doublet cells were predicted using DoubletFinder⁵¹ for each sample separately with high confidence doublets removed. Normalization and clustering were done with the Seurat package v3.0.1⁷⁶. In brief, counts for all nuclei were scaled by the total library size multiplied by a scale factor (10,000), and transformed to log space. A set of 2000 highly variable genes were identified with *SCTransform* from *sctransform* R package in the variable stabilization mode. This returned a corrected unique molecular identifiers (UMI) count matrix, a log-transformed data matrix, and Pearson residuals from the regularized negative binomial regression model. Principal component analysis (PCA) was done on all genes, and *t*-SNE was run on the top 20 PCs. Cell clusters were identified with the Seurat functions FindNeighbors (using the top 20 PCs) and FindClusters (resolution = 0.02). In this analysis, the neighborhood size parameter pK was estimated using the mean-variance normalized bimodality coefficient (BCmvn) approach, with 20 PCs used and pN set as 0.25 by

default. For each cluster, we assigned a cell-type label using statistical enrichment for sets of marker genes^{52,53} and manual evaluation of gene expression for small sets of known marker genes. Differential gene expression analysis was done using the FindMarkers function and MAST⁷⁷. To identify gene ontology and pathways enriched in the differentially expressed genes (DEGs), we tested enrichment of gene annotation collections from the Molecular Signatures Database (MSigDB) gene annotation database v6.1^{78,79}. For convenience, the MSigDB gene set collections have been assembled into an R package called “msigdb” which is publicly available from <https://github.com/mw201608/msigdb>. Enrichment analysis P values were calculated using the hypergeometric test (equivalent to the Fisher’s exact test, FET) in R language. To control for multiple testing, we employed the Benjamini-Hochberg (BH) approach⁸⁰ to constrain the false discovery rate (FDR).

Microglia Subclustering. The subset() function from Seurat was used to subset microglia-only cells. Seurat object was converted to Monocle3⁸¹⁻⁸³ cds for subclustering analysis as we found the integration between samples was most optimal using Monocle3. Cluster marker genes were determined using fit_models(model_formula_str = “~cluster”).

Quantitative Reverse-Transcription PCR

Snap-frozen cortices were thawed and homogenized with a 21G needle in RLT buffer with 1% β -mercaptoethanol. RNA was isolated with the RNeasy Mini-Kit (Qiagen), and the remaining DNA was removed by incubation with RNase-free DNase. Purified mRNA was then converted to cDNA with the iScript cDNA Synthesis Kit (Bio Rad). Quantitative RT-PCR was performed on the ABI 7900 HT sequence detector (Applied Biosystems) with PowerUp SYBR Green master mix (ThermoFisher Scientific). The average value of three replicates for each sample was expressed as a threshold cycle (C_t), the point at which the fluorescence signal starts to increase rapidly. Then, the difference (ΔC_t) between

C_t values for the transcript of interest and for mouse *Gapdh* was calculated for each sample. The gene expression for each sample was calculated using the $2^{-\Delta\Delta C_t}$ method and reported as fold change relative to the average of the *hTREM2*^{CV/+} sample. The following primers were used:

Primer: Human TREM2 Fwd: CCGGCTGCTCATCTTACTCT

Primer: Human TREM2 Rev: GGAGTCATAGGGGCAAGACA

Primer: Mouse GAPDH Fwd: TGGCCTTCCGTGTTCTTAC

Primer: Mouse GAPDH Rev: GAGTTGCTGTTGAAGTCGCA

Western Blot

Mouse brains were homogenized in RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA (ThermoFisher Scientific), 1 mM phenylmethyl sulfonyl fluoride, protease inhibitor cocktail (Millipore Sigma) and phosphatase inhibitor cocktail (Millipore Sigma). After sonication, brain lysates were centrifuged at 18,000 g at 4°C for 30 min. Supernatants were collected and protein concentrations were measured with the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). The same amount of protein was loaded onto a 4–12% SDS-PAGE gel (Invitrogen), transferred to nitrocellulose membranes (GE Healthcare), blocked with 5% milk, and immunoblotted in 2% milk. Bands in immunoblots were visualized by enhanced chemiluminescence (ThermoFisher Scientific) and quantified by densitometry with ImageJ (NIH). The antibody used for western blot was anti-TREM2 (1:500, Cell Signaling, #91068S). Immunoreactivity was detected with goat anti-rabbit HRP (1:2000, Millipore Sigma).

Isolation of Adult Microglia

Adult microglia were isolated from 3- to 4-month-old *mTrem2*^{+/+}, *hTREM2*^{R47H/+}, and *hTREM2*^{R47H/R47H} mice as described³⁶. Briefly, after perfusion, brains were chopped with a razor blade, incubated with 3% collagenase type 3 (Worthington), 3 U/ml dispase (Worthington) and DNase (Millipore Sigma) at 37°C,

inactivated with 2.5 mM EDTA (ThermoFisher Scientific) and 1% fetal bovine serum (FBS) (Invitrogen), filtered through a 70- μ m filter, centrifuged at 300 g for 5 min at 18°C and resuspended in fluorescence-activated cell sorting (FACS) buffer. Samples were incubated with myelin-removal beads (Miltenyi Biotec) for 15 min at 4°C, passed through a magnetic LD column (Miltenyi Biotec), centrifuged at 300 g for 10 min, and resuspended in FACS buffer. Cells were magnetically separated and sorted with CD11b beads (Miltenyi Biotec) and a magnetic MS column (Miltenyi Biotec). CD11b-positive cells were centrifuged at 300 g for 10 min, and RNA was extracted for RNA-seq.

Behavioral Tests

In all behavioral tests, *hTREM2^{CV/+}* and *hTREM2^{R47H/+}* mice were compared to their respective nontransgenic or P301S littermates. Experimenters were blinded to mouse genotypes throughout the experiments. Male and female mice were tested on separated days.

Morris Water Maze. The water maze consists of a pool (122 cm in diameter) containing opaque water ($20 \pm 1^\circ\text{C}$) and a platform (10 cm in diameter) 1.5 cm below the surface. Three different images were posted on the walls of the room as spatial cues. Hidden platform training (days 1–7) consisted of 14 sessions (two per day, 2 hrs apart), each with two trials. The mouse was placed into the pool at alternating quadrants for each trial. A trial ended when the mouse located the platform or after 60 sec had elapsed. At 24 and 72 hrs after training, the mice were tested in probe trials, in which the hidden platform was removed and mice were allowed to swim for 60 sec. Mice received 7 days of hidden platform training before the 24-hr and 72-hr probe trials. Visible platform testing was done 24 hrs after the last probe trial. Performance was measured with an EthoVision video tracking system (Noldus Information Technology).

Elevated Plus Maze. The maze consists of two 15 x 2-inch open arms without walls and two closed arms with walls 6.5 inches tall and is 30.5 inches above the ground. Mice were moved to the testing

room 1 hr before testing to acclimate to the dim lighting. Mice were individually placed in the maze at the intersection of the open and closed arms and allowed to explore the maze for 10 min.

Open Field. Mice were individually placed into brightly lit automated activity chambers equipped with rows of infrared photocells connected to a computer (San Diego Instruments). Open field activity was recorded for 5 min. Recorded beam breaks were used to calculate total time of activity.

Immunohistochemistry and Image Analysis

Mice were transcardially perfused with phosphate-buffered saline (PBS). The brains were cut vertically into hemibrains. Half of each brain was flash frozen at -80°C for RNA-seq analyses; the other half was placed first in 4% paraformaldehyde for 48 hr and then in a 30% sucrose solution for 48 hr at 4°C and cut into 30- μm -thick sections with a freezing microtome (Leica). For Fig. S3.6, free-floating sections (8–10 per mouse) were washed in PBS, placed in sodium citrate buffer for 30 min at 90°C for antigen retrieval, permeabilized with PBS containing 0.5% Triton X-100 for 10 min, and blocked in PBS containing 10% normal goat serum for 1 hr. Sections were then placed in PBS with 5% normal goat serum and primary antibodies overnight at 4°C . The next day, sections were washed in PBS containing 0.1% Triton X-100, incubated with Alexa-conjugated secondary antibodies in PBS with 5% normal goat serum and washed in PBS containing 0.1% Triton X-100. Images were acquired with a Keyence BZ-X700 microscope and a 10x objective. Immunoreactivity was quantified with ImageJ software (NIH). Antibodies used for staining were anti-MC1 (1:500, kind gift from Dr. Peter Davies) and anti-Iba1 (1:500, Wako). Secondary antibodies used for staining were donkey anti-mouse 488 and donkey anti-rabbit 546 (both 1:500, ThermoFisher Scientific).

For Fig. 3.5 l and Fig. 3.5 m, free floating sections were washed in Tris-buffered saline (TBS) buffer followed by incubation in Antigen decloaker reagent (Biocare Medical, Cat#RV1000M) in TBS at 95°C three times for 5 min each time, shaking at 600rpm. They there then washed with TBS containing 0.5%

Triton x-100 (TBST), brain sections were incubated with 10% BSA in TBST for 1 hour at room temperature followed by incubation at 4°C overnight with anti-Iba1 antibody (goat, 1:500, Abcam #Ab5076) and anti-phospho-Akt-Ser473 antibody (rabbit, 1:100, Cell signaling #9271) in TBST containing 0.5% BSA. Next day, brain sections were washed with TBST three times and incubated for 1 hour at room temperature with donkey anti-goat secondary antibody conjugated with Alexa Fluor 488 (1:500, Invitrogen, #A11055) and donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 568 (1:500, Invitrogen, #10042) in TBST containing 0.5% BSA. Brain sections were washed three times with TBST, mounted on slides in Vectashield mounting reagent containing DAPI, air dried, and sealed with nail polish. Stained sections were imaged by Keyence BZ-X710 All-in-One Fluorescence Microscope using the 40x objective.

RNA-Seq and Analysis of Adult Microglia

RNA from adult microglia was extracted from *mTrem2^{+/+}* (n = 7), *hTREM2^{R47H/+}* (n = 6), and *hTREM2^{R47H/R47H}* (n = 7) mice with the Qiagen RNeasy Mini Kit. RNA concentration was determined with a NanoDrop, and RNA quality was measured with a Bioanalyzer and Agilent RNA Pico Chip. Samples with an RNA integrity number >7 were considered of good quality and used for subsequent steps. Libraries were then prepared with the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina. Library quality was assessed with a Bioanalyzer and the Agilent High Sensitive DNA Chip. Individual library concentrations were measured with the Qubit dsDNA HS Assay Kit and submitted for SE50 sequencing on an Illumina HiSeq 4000. Quality control was done on base qualities and nucleotide composition of sequences. Alignment to the GRCm38.84 *Mus musculus* (mm10) refSeq (refFlat) reference gene annotation was done with the STAR spliced read aligner and default parameters. Counts were normalized with the median of ratios method.

RNA-Seq and Analysis of Bulk Hippocampal Tissue

Hippocampal RNA was isolated with the Qiagen RNeasy Mini Kit. After quality control analysis with a Bioanalyzer, the RNA was sent to Novogene for library preparation and PE150 sequencing with an Illumina HiSeq 4000 instrument. 48 samples were sent in for sequencing: 6 samples each from male and female P301S CV and R47H hTREM2 knock-in mice, and 3 samples each for male and female P301S *mTrem2*^{+/+} littermates of the *hTREM2*^{CV/+} line and male and female P301S *mTrem2*^{+/+} littermates of the *hTREM2*^{R47H/+} line.

Quality Control. We used multiple clustering methods to examine the quality of replicates and to identify possible outlier samples for exclusion if necessary. For the hTREM2 R47H line (LG72), three samples were excluded from further analysis based on clustering. Clustering techniques were applied to variance stabilizing transformed expression values, fragments per kilobase of transcript per million mapped reads, and values of log counts per million. The Pearson correlation coefficient was first used as a distance metric between samples. Hierarchical cluster analysis was then applied to measure similarity between the Pearson correlations. The hierarchical clustering algorithm was an iterative process. Each iteration joined the two most similar clusters (based on Pearson correlation) and computed the distance between remaining clusters, continuing until there is just a single cluster. The distances between clusters were computed at each stage using the complete linkage clustering method (see manual for R hclust function).

Differential Expression Analysis. Differential gene expression was calculated with the R package DESeq2⁸⁴. Counts were normalized with the median of ratios [Anders & Huber 2010]. Genes with <15 counts across all samples were excluded from analysis. The false discovery rate (FDR) was calculated with the Benjamini-Hochberg method⁸⁰.

Weighted Gene Co-expression Network Analysis. Weighted gene co-expression network analysis (WGCNA) was done on normalized expression data with the R package WGCNA v1.51⁸⁵. The top 5000 most variable genes were used to create modules, and the soft-thresholding power parameter was set to 14. The minimum module size was 30 genes, and modules with a module eigengene dissimilarity below 0.2 were merged, creating 11 modules of 43–1361 genes each after removal of the module (gray) that contain genes that do not belong to any other module. KEGG and GO biological processes were used for pathway analysis as described above.

Pathway Enrichment Analysis. Gene network analyses of RNA-seq data were done with gene set enrichment analysis (GSEA)⁷⁸; cell types were defined by the top 100 genes expressed by each CNS cell type⁸⁶. Pathway analysis was done with Gene Ontology (GO) biological processes^{87,88} and the Kyoto Encyclopedia of Genes and Genomes (KEGG)⁸⁹.

Network Visualization. Networks were visualized with Cytoscape (version 3.6.1)⁹⁰, the STRING database⁹¹, and perfuse force directed layout.

Mouse Hippocampus Single-cell RNA-seq

Preparation of samples. Brain tissue was prepared using a previously published protocol⁵⁹. Briefly, 8-month-old female mice were anesthetized with avertin and transcardially perfused with phosphate-buffered saline (PBS). The brain without the cerebellum was harvested and collected into cold media with 15mM HEPES, 0.5% glucose in 1X Hanks' Balanced Salt Solution without phenol red (ThermoFisher Scientific 13185-052) on ice. The entire procedure was done on ice. The hippocampus was extracted and chopped into pieces using a razor blade then further homogenized using a 2ml douncer containing 2ml medium A with 80uL DNase (12500 units/mL) and 5ul recombinant RNase inhibitor (Takara Bio 2313B). Homogenized tissue was filtered through a 70um strainers to obtain a single cell suspension. Cells were washed with medium A and resuspended in 850uL MACS buffer with 1.8uL

RNase inhibitor (sterile-filtered 0.5% BSA, 2mM EDTA in 1 X PBS). Cells were incubated with 100uL myelin removal beads (MACS Miltenyl Biotec) for 10 min, and loaded onto LD columns (Miltenyl Biotec). Cells were collected and washed for FACS staining.

Single Cell Sorting for Single-cell RNA-seq. Cells were blocked in 5uL mouse Fc block for 5 min on ice then incubated with primary antibodies for 10 min then washed with FACS buffer (sterile-filtered 1% FCS, 2mM EDTA, 25mM HEPES in 1XPBS). Cells were incubated with secondary antibodies for 10min then washed with FACS buffer. Cells were resuspended in 500uL FACS buffer with RNase inhibitor (Takara Bio 2313B, 1:500) and 0.5ul Propidium Iodide (ThermoFisher Scientific P3566, 1:1000) for single cell index sorting. Cell sorting/flow cytometry analysis was done on the cell sorter (BD InFlux) at the Stanford FACS Facility. The following gates were used for sorting microglia: (1) forward scatter-area (FSC-A)/side scatter-area (SSC-A) (2) Trigger Pulse Width/ FSC (3) Live- Dead negative using PI (4) CD45^{low}CD11b⁺ and CD45^{hi}CD11b⁺. Single cells were sorted into 96-well plates containing 4uL lysis buffer containing 4U Recombinant RNase Inhibitor (Takara Bio 2313B), 0.05% Triton X-100, 2.5mM dNTP mix (ThermoFisher Scientific R0192), 2.5uM Oligo-dT30VN (50-AAGCAGTGGTATCAACGCAGAGTACT30VN-30), and ERCC Spike-ins (ThermoFisher Scientific 4456740) diluted at 1:2.4x10⁷. Plates were vortexed, spun down and frozen on dry ice, and plates were stored at -80°C freezer. Antibodies used for FACS: rabbit anti-mouse Tmem119 (Abcam ab210405, ~200ug/ul, 1:400 dilution), CD45-PE-Cy7 (ThermoFisher Scientific 25-0451-82, 1:300), CD11b-BV421 (BioLegend 101236, 1:300), goat anti-rabbit Alexa 488 (ThermoFisher Scientific 11034, 1:300).

Single-cell RNA-seq library preparation. Sequencing libraries were prepared following the Smart-seq2 published protocol⁹². Briefly, plates were thawed and incubated at 72°C for 3 min in order to anneal RNAs to the Oligo-dT30VN primer. The 6uL of the reverse transcription mixture was added to each well: 95U SMARTScribe Reverse Transcriptase (100U/μl, Clontech 639538), 10U RNase inhibitor

(40U/μl), 1XFirst-Strand buffer, 5mM DTT (Promega P11871), 1M Betaine (Sigma-Aldrich B0300-5VL), 6mM MgCl₂, 1μM TSO (Exiqon, Rnase free HPLC purified). RT was performed at 42°C for 90 min, followed by 70°C, 5 min. 15ul of PCR amplification mix containing the following reagents was added to each well: 1X KAPA HIFI Hotstart Master Mix (Kapa Biosciences KK2602), 0.1uM ISPCR Oligo (AAGCAGTGGTAT CAACGCAGAGT), 0.56U Lambda Exonuclease (5U/ul, New England BioLabs M0262S). cDNA was amplified using the following PCR program: (1) 37°C 30 min; (2) 95°C 3 min; (3) 23 cycles of 98°C 20 s, 67°C 15 s, 72°C 4 min; (4) 72°C 5 min. cDNA samples were purified using PCRClean DX beads (0.7:1 ratio, Aline C-1003-50), and resuspended in 20ul EB buffer. cDNA quality was examined with a Fragment Analyzer (AATI, High Sensitivity NGS Fragment Analysis Kit: 1 bp - 6000 bp, Advanced Analytical DNF-474-1000). To make libraries, all samples were diluted down to 0.15ng/ul in 384-well plates using Mantis Liquid Handler (Formulatrix) and Mosquito X1 (TTP Labtech) with customized scripts. Nextera XT DNA Sample Prep Kit (Illumina FC-131-1096) was used at 1/10 of recommendation volume, with the help of a Mosquito HTS robot for liquid transfer. Tagmentation was done in 1.6ul (1.2ul Tagment enzyme mix, 0.4ul diluted cDNA) at 55°C, 10 min. 0.4ul Neutralization buffer was added to each well and incubated at room temperature for 5 min. 0.8ul Illumina Nextera XT 384 Indexes (0.4ul each, 5uM from 4 sets of 96 indexes) and 1.2ul PCR master mix were added to amplify whole transcriptomes using the following PCR program: (1) 72°C 3 min; (2) 95°C 30 s; (3) 10 cycles of 95°C 10 s, 55°C 30 s, 72°C 1 min; (4) 72°C 5 min. Libraries from one 384 plate were pooled into an Eppendorf tube and purified twice using PCRClean DX beads. Quality and concentrations of the final libraries were measured with Bioanalyzer and Qubit, respectively. Libraries were sequenced on the Illumina HiSeq 4000 at the Weill Cornell Medicine Genomics and Epigenomics Core Facility.

Processing of Single-cell RNA-seq Raw Data. Prinseq ⁹³ v0.20.4 was first used to filter sequencing reads shorter than 30 bp (`-min_len 30`), to trim the first 10 bp at the 5' end (`-trim_left 10`) of the reads, to trim reads with low quality from the 3' end (`-trim_qual_right 25`) and to remove low complexity reads (`-lc_method entropy, -lc_threshold 65`). Then, Trim Galore v0.4.3 (<https://github.com/FelixKrueger/TrimGalore>) was applied to trim the Nextera adapters (`-stringency 1`). The remaining reads were aligned to the mm10 genome by calling STAR ⁹⁴ v2.5.3a with the following options: `-outFilterType BySJout,-outFilterMultimapNmax 20,-alignSJoverhangMin 8,-alignSJDBoverhangMin 1,-outFilterMismatchNmax 999,-outFilterMismatchNoverLmax 0.04,-alignIntronMin 20,-alignIntronMax 1000000,-alignMatesGapMax 1000000,-outSAMstrandField intronMotif`. Picard was then used to remove the duplicate reads (`VALIDATION_STRINGENCY = LENIENT, REMOVE_DUPLICATES = true`). Finally, the aligned reads were converted to counts for each gene by using HTSeq (`-m intersection-nonempty, -s no`) ⁹⁵.

Quality Control for Single-cell RNA-seq Data. We used the following criteria to filter out cells with low sequencing quality. The distribution of total reads (in logarithmic scale) was fitted by a truncated Cauchy distribution, and data points in two tails of the estimated distribution were considered as outliers and eliminated. Fitting and elimination were then applied to the remaining data. This process was run iteratively until the estimated distribution became stable. The threshold was set to the value where the cumulative distribution function of the estimated distribution reaches 0.05. Cells with small numbers of detected genes and poor correlation coefficients for ERCC (low sequencing accuracy) were dropped. 1424 cells were retained for downstream analysis after filtering from 1480 cells.

Clustering Analysis of Single-cell RNA-seq Data. The Seurat R package v3.0.1 was used to perform unsupervised clustering analysis on the filtered scRNA-seq data ^{76,96}. Gene counts were normalized to the total expression and log-transformed. Principal component analysis was performed on the scaled data

using highly variable genes as input. The JackStrawPlot function was used to determine the statistically significant principal components. These principal components were used to compute the distance metric and generate cell clusters. Non-linear dimensional reduction (t-SNE) was used to visualize clustering results. Differentially expressed genes were found using the FindAllMarkers function that ran Wilcoxon rank sum tests.

Ingenuity Pathway Analysis. DEGs and their log₂-fold change expression values were inputted into IPA (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>). For Trem2-signaling pathway predictions, previously published molecules involved in Trem2-signaling were manually curated and highlighted through network construction.

RNAscope

Mouse brains were harvested and snap-frozen on dry ice followed by OCT embedment. Frozen brains were cut into 20 µm sections using a cryostat and mounted onto superfrost plus slides. The RNAscope assay was carried out with RNAscope Fluorescent Multiplex Assay (ACD, 320513). Briefly, after dehydration with ethanol, sections were permeabilized with Protease IV. Then samples were hybridized with RNA probes C1qa (ACD, 441221-C2) for activated microglia and Apoe (ACD, 313271-C3) at 40°C for 2 h. Signal was amplified according to the instructions. After counterstain with DAPI, sections were mounted by Prolong Gold (Thermo Fisher, P36930). Line-scanning confocal images of hippocampal dentate gyrus region were obtained with a confocal microscope (LSM880, Carl Zeiss Microscopy, Thornwood, NY) and a 40x objective (1-µm focal plane intervals, one field of view per brain section) within a day post-mounting of sections. Images were examined by maximum intensity Z-projection and analysis was done using ImageJ v2.0.0 (NIH). All analysis and imaging were done blinded to the genotype of the samples.

Isolating Primary Microglia: *in vitro* Tau Fibril Stimulation Assay and MK-2206 Treatment

Cortices was harvested from postnatal day 3 pups. The meninges were removed, and the cortical tissue was finely chopped with a razor blade and digested in 0.25% trypsin with DNase (Millipore Sigma) at 37°C for 25 min. Digestion was stopped with DMEM containing 10% FBS. The tissue was then triturated and spun at 200 g for 5 min, and the pellet was resuspended in DMEM and 10% FBS and plated in T75 flasks that had been pre-coated with 5 µg/ml poly-D-lysine (Millipore Sigma) and rinsed with water. Mixed cultures were maintained in flasks for 10–11 days. The flasks were then shaken for 2 hr, spun at 200 g for 15 min, and the cells were plated at a density of 150,000/well in a 24-well plate in DMEM and 10% FBS. After 24 hr, cells were treated with or without 1 µg of 0N4R tau fibril per well (from Dr. Jason Gestwicki) for approximately 16 hr before lysis for RNA isolation. For drug treatment, cells were treated with either 1 µM MK-2206 (MedChem Express) suspended in DMSO or no drug with DMSO as a control for 6 hours of incubation. Then, cells were treated with 1 µg of 0N4R tau fibril per well for 24 hours. The conditioned media were collected and debris was removed by spinning at 4°C for 10 minutes at 3,000 g. The levels of 32 cytokine/chemokine in the conditioned media were measured by a Milliplex mouse cytokine/chemokine 32-plex kit (Millipore #MCYTMAg-70K-PX32) with the Luminex® MAGPIX® CCD Imager.

RNA-Seq and Analysis of Primary Microglia

RNA was isolated using the *Quick*-RNA MiniPrep kit (Zymo Research). For experiments with MK-2206 drug treatment, RNA was sent to Novogene for library preparation and sequencing. For experiments without drug treatment, RNA concentration was determined with a NanoDrop, and RNA quality was measured with a Bioanalyzer and Agilent RNA Pico Chip. Samples with an RNA integrity number >7 were considered of good quality and used for subsequent steps. Libraries were then prepared with the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina. Library quality was assessed with a

Bioanalyzer and the Agilent High Sensitive DNA Chip. Individual library concentrations were measured with the Qubit dsDNA HS Assay Kit and submitted for SE50 sequencing on an Illumina HiSeq 4000. Quality control was done on base qualities and nucleotide composition of sequences. Alignment to the GRCm38.84 *Mus musculus* (mm10) refSeq (refFlat) reference gene annotation was done with the STAR spliced read aligner and default parameters. Differential gene expression analysis was done with the R package DESeq2. Counts were normalized with the median of ratios method. Genes with <15 counts across all samples were excluded from analysis. The FDR was calculated with the Benjamini-Hochberg method. Pathway analysis was done using Gene Set Enrichment Analysis referencing Gene Ontology Biological Processes dataset. Predicted upstream activators of the transcriptome were determined using Ingenuity Pathway Analysis software (Qiagen).

In Vivo Imaging

For intravital imaging with two-photon microscopy, thinned-skull windows were made in 12–17-month-old *Cx3cr1*^{GFP/+} *hTREM*^{R47H/+}, *Cx3cr1*^{GFP/+} *hTREM2*^{CV/+}, and *Cx3cr1*^{GFP/+} *mTrem2*^{+/+} as described previously ³⁶. Briefly, mice were anesthetized, the skull was exposed, and a small area over the cortex was thinned manually and with a high-speed drill (K.1070 High Speed Rotary Micromotor drill; Foredom). Mice were fixed onto a custom-made head plate and imaged with an Ultima IV multiphoton microscope (Bruker) equipped with MaiTai DeepSee-eHP lasers (Spectra Physics) tuned to 920 nm for imaging and InSight X3 lasers (Spectra Physics) tuned to 880 nm for ablation. Z-stacks of images were acquired every 3 min in 1-μm steps with a 25x water-immersion objective at 2.4 optical zoom. Extensions and retractions of processes during baseline recordings were manually traced with the mTrackJ plugin. The movement of microglial cells toward a laser ablation site was analyzed by normalizing the number of processes near the injury site at each time point to the overall microglial density at that time point.

Primary Microglia Phagocytosis Experiment

Experimental and Imaging Set-up: Primary microglia were prepared as described above. Microglia were seeded at 30,000 cells / well in a 96-well plate. After 24 hours, media was replaced with FluoroBrite DMEM media (ThermoFisher) with 10% FBS and 0.015 mg pH-rodo *E. coli* (ThermoFisher; P35366) was added to each well. 4 fields of view were taken for each well every 30 minutes using Incucyte (Essen BioScience). Background fluorescence was subtracted and population-level fluorescence information was determined through the Incucyte analysis software using Top-Hat and threshold based on negative control well with no pH-rodo *E. coli*.

Single-cell image tracking. Model Training: The single-cell analysis pipeline was adapted from previous work, using the Usiigaci framework⁹⁷. A custom FIJI/ImageJ macro was written to manually annotate phase contrast images, such that a unique RGB value corresponded to an individual cell mask⁹⁸. A preprocessing step converted the 8-bit RGB image into a 16-bit grayscale mask image (8-bit would yield max of 255 unique cells per image), such that each unique RGB cell mask was a unique grayscale value. A Mask R-CNN model implemented in Tensorflow and Keras (open-source Matterport Inc. – MIT License), was trained to segment cells in phase contrast microscopy images⁹⁹⁻¹⁰². The model had a learning rate of 1×10^{-3} for training and used a Resnet-101 backbone^{97,101}. The network heads were trained first for 50 epochs and then the full network was trained for another 100 epochs. All model training was performed using a GPU on Google Colab. The imgaug package was used for data augmentation during training, incorporating image rotations and flips, along with brightness augmentations and gaussian blur augmentations stochastically. Transfer learning was performed based on final weights from Usiigaci model. The average of two different models (epochs 130 and 134) were used for instance segmentation during this study. These models were chosen based on lowest validation

loss during training. The model was trained on 20 images with ~50 cells per image to assess accuracy of the model on data that were not used during model training.

Analysis of Phase Contrast Microscopy Images: Phase contrast and fluorescent image were exported as TIFF images from the Incucyte system. Top-hat background subtraction was performed on the fluorescence image to remove autofluorescence. Time-lapse images were registered using a manual drift correction FIJI plug-in ¹⁰³. The output from the Mask R-CNN model was fed into a K-D tree-based cell tracking pipeline based on trackpy, from the Usiigaci pipeline. Once cells from each frame were annotated, a python script was used to measure fluorescence of each cell over the entire time-lapse. A threshold was manually set for each individual experiment. Average intensity measurements above the threshold were measured for each individual cell by taking the total intensity within a cell above the threshold divided by the area of the cell above the set threshold. Cells with more than 30 missing time points and cells with more than 5 missing time points within the first 10 frames were excluded from the analysis and a linear regression was used to impute missing data from the time-lapse. Cells that contained enough datapoints to be included in the cut-off but missing datapoints at the beginning and end of the time-lapse were filled with zeros.

Clustering of Single-Cell Fluorescence Data: Clustering algorithms were adapted from the Seurat R package⁷⁶ for single-cell RNA sequencing analysis. Briefly, the fluorescence measurement for each cell at each time point was scaled such that the mean expression across cells is 0 and all time points were used to generate a PCA. For clustering and generation of the tSNE, FindNeighbors, FindClusters (resolution 0.15), and RunTSNE functions were used.

Quantification and Statistical Analysis

Data were analyzed with GraphPad Prism v.7 (GraphPad Software, San Diego, California USA, www.graphpad.com), STATA12 (StataCorp. 2011. Stata Statistical Software: Release 12. College

Station, TX: StataCorp LP), or R¹⁰⁴. A multilevel mixed-effects linear regression model fitted with STATA12 was used to analyze latency in the Morris water maze. R was used to calculate the area under the curve for cumulative search errors in the Morris water maze. Outliers were removed with Prism's outlier analysis algorithm. All statistical details can be found in the figure and figure legends. $P < 0.05$ and $FDR < 0.05$ was considered statistically significant, unless otherwise noted. All values are expressed as mean $\pm$ SEM, unless otherwise noted. A subset of mice from the behavior cohort was randomly selected for single-nuclei RNA-seq and bulk RNA-seq studies. Data and visualizations were done using ggplot2^{105,106}.

3.6 Data Availability

Full western blots (Fig. 3.3) are available as a source data figure and RNA-seq gene lists with statistics are available in the respective supplementary tables accompanying this article. All RNA-seq data was deposited in the Gene Expression Omnibus (GEO) under the following series accession numbers: bulk RNA-seq of mouse hippocampus and primary microglia; GSE136389, human single-nuclei; GSE154578, mouse single-cell; GSE140670. If you are a reviewer, please consult the journal editor for the access token. Any code used for data analysis can be made available upon request.

3.7 Acknowledgements

We thank Dr. Junli Zhang from the Gladstone Institutes Transgenic Core for microinjections into embryos to generate knock-in human TREM2 mice; Allan Villanueva and Elizabeth Fan for assistance with genotyping the mice; Dr. Li-Huei Tsai and Dr. Yueming Li for their time discussing the data and feedback on the manuscript; Dr. Jason Gestwicki and his lab for synthesizing the 0N4R tau fibrils; Dr. Rosa Rademakers and Dr. Nilufer Taner for genotyping of the human brain tissues and Christopher Fernandez De Castro for assistance with the tissues; Rose Horowitz for help with immunostaining; and Stephen Ordway and Kathryn Claiborn for manuscript editing. All *in vivo* imaging was done by L.K., with helpful advice from Dr. Mario Merlini and Dr. Katerina Akassoglou, at the Gladstone Center for In Vivo Imaging Research. MC1 immunohistochemistry imaging was done by F.A.S. using the Gladstone Histology Core microscopes. Behavioral assays were done by F.A.S. and D.L. using equipment from the Gladstone Behavioral Core. RNA-sequencing was done by the UCSF Center for Advanced Technology (bulk-seq), and Weill Cornell Medicine Genomics and Epigenomics Core Facility (single-cell and single-nuclei RNA-seq). The authors are also grateful to the Flow Cytometry Core and the Integrated Genomics Operation at MSKCC for their assistance. This work was supported by the National Institute of Health Grants R01AG051390, U54NS100717, R01AG054214, and Rainwater Foundation (to L.G.), the National Institute of Aging Grant F31 AG058505 (to F.A.S.), the National Institute of Aging Grant F30 AG062043-02 and National Institute of Health Grant T32GM007618 (to L.K.), the Early Postdoctoral Mobility Fellowship from the Swiss National Science Foundation P2BSP3 151885 (to H.M.), National Institute of Aging Grant K01 AG057862 (to T.E.T), National Institute of Mental Health Grant R01 MH110504 (to G.Y.), National Institute of Aging Grant R01 AG057555 (to L.X.), UK Dementia Research Institute, which receives its funding from DRI Ltd, funded by the UK Medical Research Council, Alzheimer's Society and Alzheimer's Research UK, Medical Research Council

MR/N026004/1, Wellcome Trust 202903/Z/16/Z, Dolby Family Fund, National Institute for Health Research University College London Hospitals Biomedical Research Centre, Research Centre at University College London Hospitals NHS Foundation Trust and University College London (to J.H.), the National Institute of Aging Grants U01AG046170, RF1AG054014, RF1AG057440 and R01AG057907 (to B.Z.), and the Alan and Sandra Gerry Foundation (to L.M.).

3.8 Main Figures

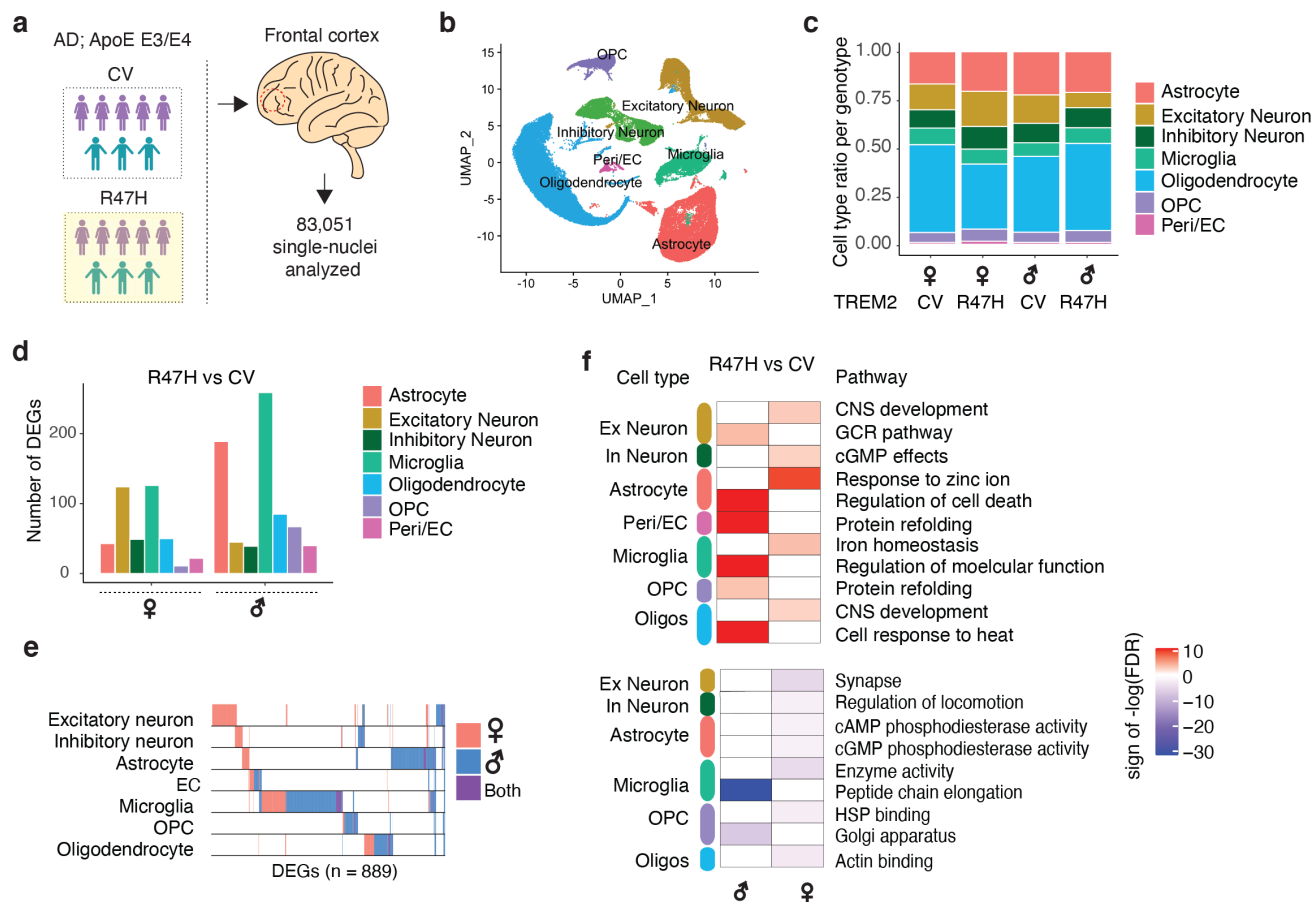


Figure 3.1. R47H Mutation Induces Cell Type- and Sex-Specific Transcriptional Changes in Human AD Patient Brains. (a) Schematic showing the sex and genotypes of age-matched human donors used for single-nuclei RNA-seq. $n = 83,051$ nuclei were isolated from the mid-frontal cortex of AD patients carrying the CV-TREM2 variant ($n=8$) and AD patients carrying the R47H-TREM2 variant. Purple and turquoise cartoons denote females and males, respectively. See also Supplementary Table 1. (b) UMAP plots of all single nuclei and their annotated cell types. Peri/EC = pericyte/endothelial cells, OPC = Oligodendrocyte precursor cells. (c) Proportion of cell types for each of the 4 genotypes. (d) Number of DEGs between R47H vs. CV samples for each cell type and each sex. $FDR < 0.05$. (e) Binary plot indicating whether a gene (column) is a DEG or not in a given cell type (rows) or in each sex (pink: female; blue: male; purple: overlapping in both sexes) ($n=889$ DEGs). (f) Heatmap of the Gene Ontology pathway enrichment analysis for DEGs determined in (d). Colors denote upregulated (+1, red, upper panel) or downregulated (-1, blue, lower panel) pathway multiplied by the $-\log_{10}(FDR)$. Ex Neuron = Excitatory neurons, In Neuron = Inhibitory neurons, Oligos = oligodendrocytes. See also Fig. S3.1 and Supplementary Tables 1-3.

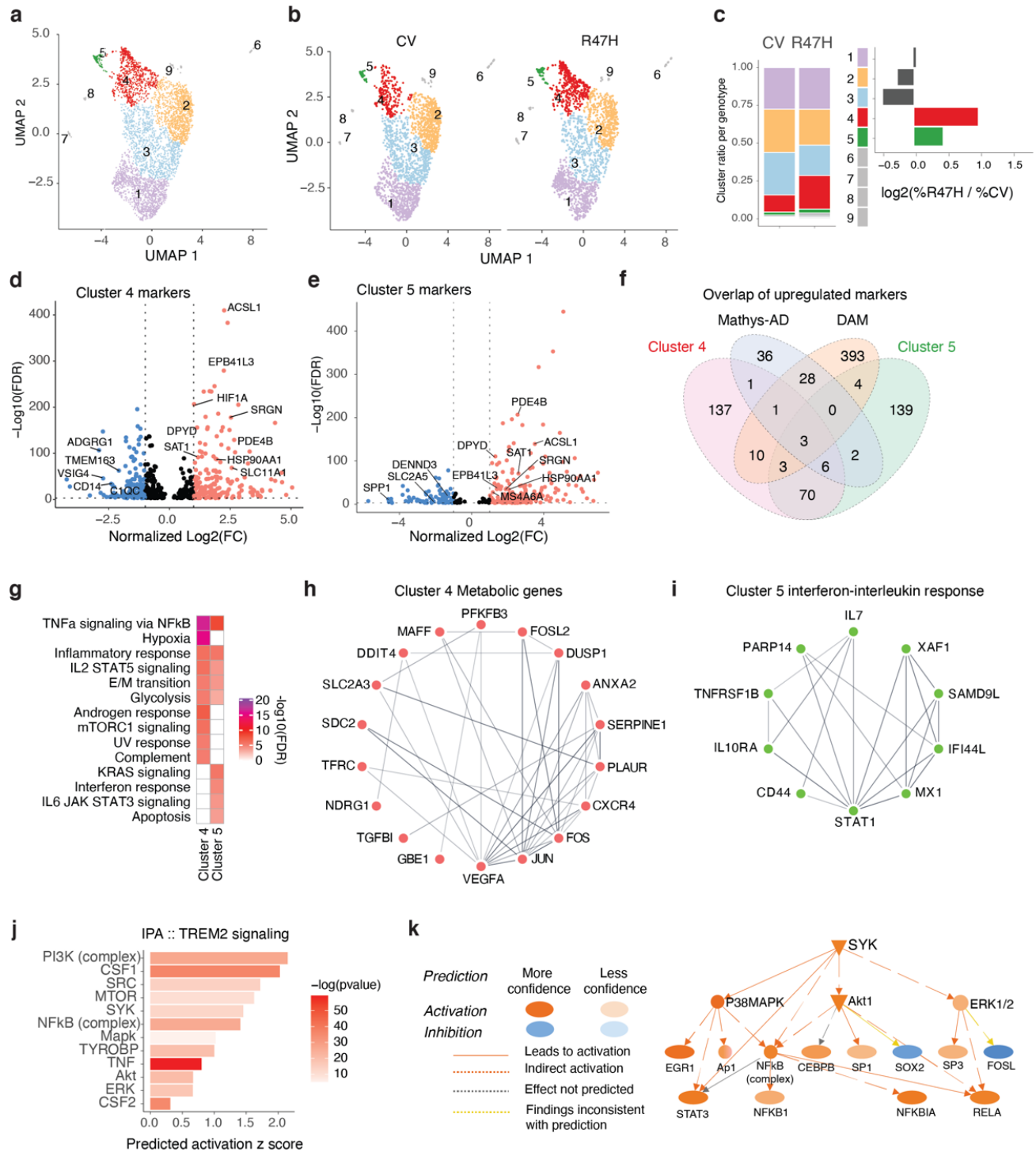


Figure 3.2. R47H Mutation Increases TREM2-Signaling in a Unique Microglia Cluster in Human AD Patient Brains. (a-b) UMAP of microglia subclusters for all nuclei (a) or split by TREM2 genotype (b). (c) Proportion of microglia subcluster per genotype (left) and log2 ratio of fraction in R47H versus CV (right). (d-e) Volcano plot of significant DEGs (FDR < 0.05) between homeostatic cluster 1 compared to cluster 4 (d) or cluster 5 (e). Genes overlapping with Mathys et al. highlighted ⁴¹. See also

Supplementary Table 4. (f) Venn diagram showing number of overlapping upregulated markers from (d) and (e), DAM signature ³⁴ and Mathys AD-microglia signature ⁴¹. (g) Heatmap of GSEA Hallmark pathways based on the unique, non-overlapping markers for clusters 4 and 5 identified in (f). Colors denote $-\log_{10}(\text{FDR})$. (h-i) Protein-protein interaction networks determined through the STRING database for genes overlapping in metabolic pathways: Hypoxia, mTORC1, and glycolysis for cluster 4 pathways (h) and interferon-interleukin pathways: Interferon response, IL2, and IL6 signaling for cluster 5 pathways (i) based on (g). (j) IPA of genes involved in TREM2 signaling based on DEGs identified in (d) and (e). (k) Diagram of SYK-AKT activation networks predicted by IPA upstream regulator analysis in (j). See also Fig. S3.2-S3.3 and Supplementary Table 4.

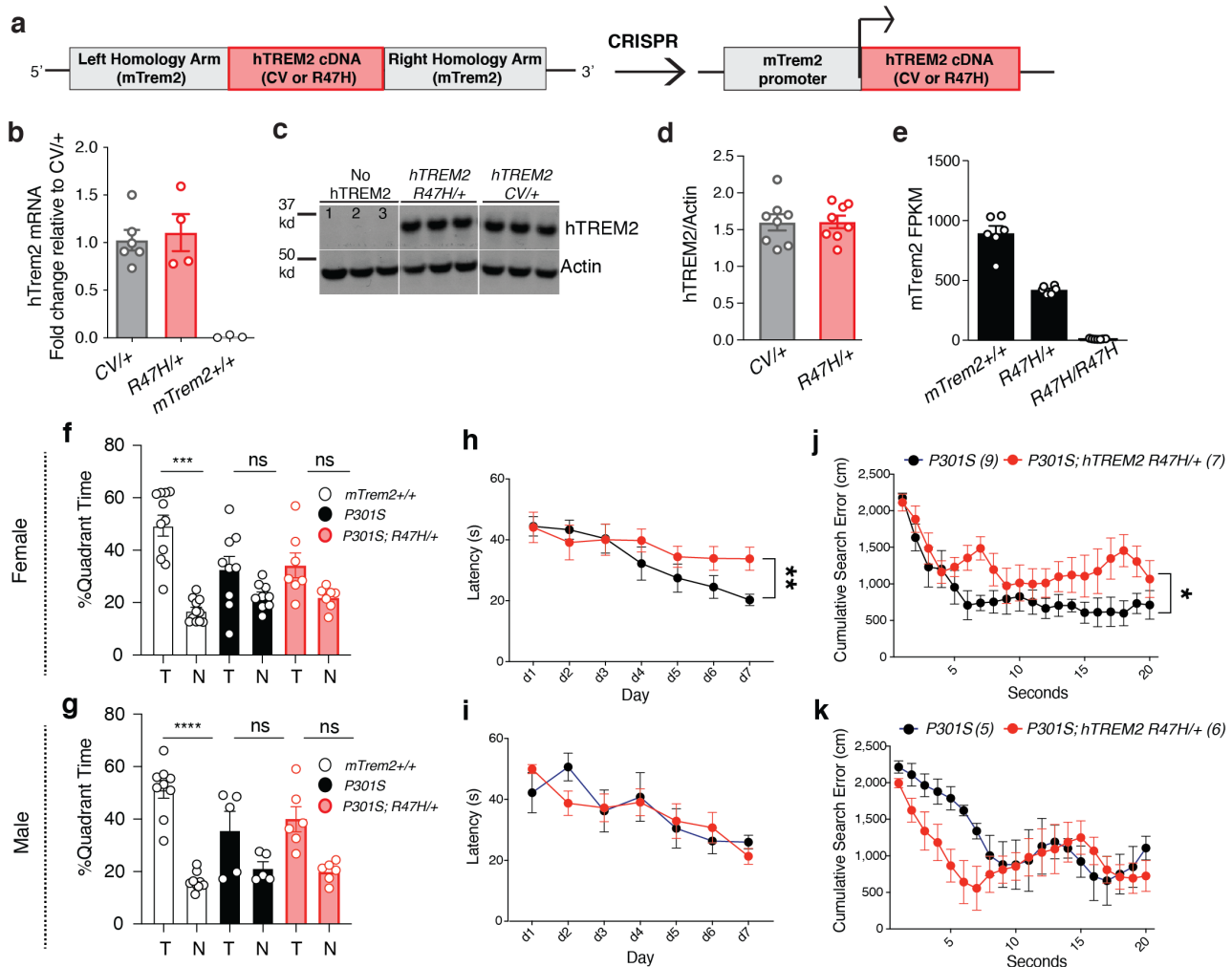


Figure 3.3. R47H-hTREM2 Exacerbates Spatial Learning and Memory Deficits in Female Tauopathy Mice. (a) The human *TREM2* donor vector was designed with two 1-kilobase long arms homologous to mouse *Trem2* (*mTrem2*) flanking the common variant (CV) or R47H human *TREM2* (*hTREM2*) cDNA sequence. When inserted into the genome, *hTREM2* cDNA is driven by the endogenous *mTrem2* promoter. (b) Quantitative real-time PCR analysis of cortical tissue from 3-4-month-old mice for *hTREM2* mRNA. Samples were run in triplicate, and averages of the three wells were used for quantification. $2^{-\Delta\Delta CT}$ calculation method used, normalized to *Gapdh* and relative to *hTREM2*^{CV/+}. Each dot represents the average of three wells from one mouse. Two-tailed Mann-Whitney U-test comparing CV/+ and R47H/+. (c) Representative western blot of RIPA-soluble cortical lysates from 8-9-month-old mice immunoblotted for hTREM2 and β -actin. Lane 1=*mTrem2*^{-/-}, Lanes 2-3=*mTrem2*^{+/+}. (d) Quantification of hTREM2 levels normalized by β -actin of the entire cohort by western blot. Student's two-tailed t-test. (e) *mTrem2* mRNA expression level from RNA-seq of adult microglia isolated from whole brain of 3-4-month-old mice. FPKM, fragments per kilobase of transcript per million mapped reads. Kruskal-Wallis statistic=16.01, $p=1.289e-007$, Kruskal-Wallis test with Dunn's post-hoc analysis. (f,g) Percentage of time spent in the target (T) or the average time spent in the nontarget (N) quadrants during the 24-hr probe for P301S *hTREM2*^{R47H/+} females (f) and males (g) and their sex-matched *mTrem2*^{+/+} and P301 *mTrem2*^{+/+} littermate controls. *** $t=6.088$, $p=0.0001$ (f), **** $t=7.834$, $p=5.077e-005$ (g), paired two-tailed Student's t-test for $n>8$, two-tailed Mann-Whitney U-

test for $n < 8$. (h,i) Latency to reach the platform during hidden trials (d1-d7) for female (h) and male (i) P301S *hTREM2*^{R47H/+} and their P301S *mTrem2*^{+/+} littermate control mice. ** $p = 0.003$, STATA mixed-effects modeling. (j,k) Cumulative search error for female (j) and male (k) P301S *hTREM2*^{R47H/+} and their P301S littermate control mice. * $U = 9$, $p = 0.0164$, two-tailed Mann-Whitney U-test of area under the curve. Behavioral data represent the combination of two behavioral cohorts that were run independently. Values are mean $\pm$ SEM. See also Fig. S3.4-S3.7.

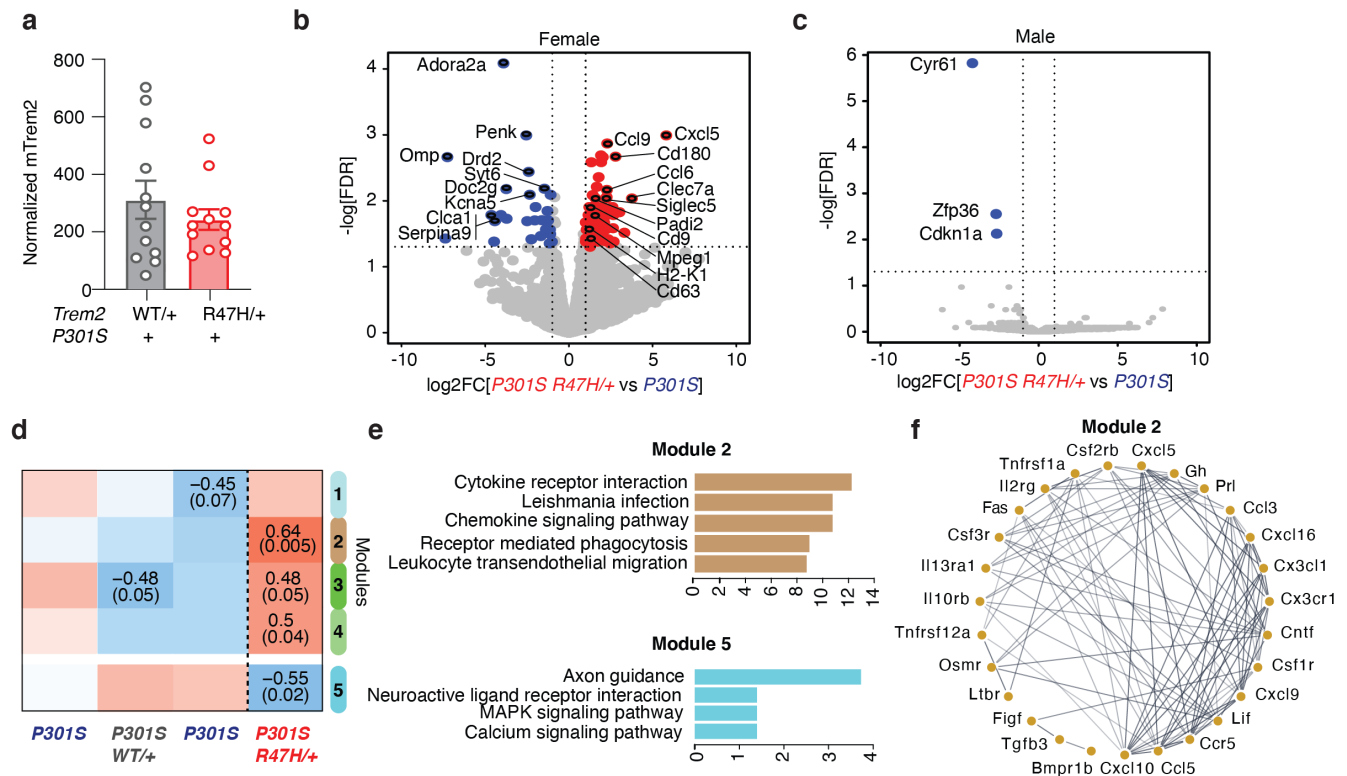


Figure 3.4. R47H-hTREM2 Increases Inflammatory Signatures in Female Tauopathy Mice. (a) Bar plot of normalized *mTrem2* RNA expression of bulk hippocampal tissue from 8–9-month-old P301S *hTREM2*^{R47H/+} and P301S *hTREM2*^{CV/+} mice. Student’s two-tailed t-test. (b) Volcano plot of bulk RNA-seq data of hippocampal tissue from 8–9-month-old female P301S *hTREM2*^{R47H/+} mice and line-specific female P301S littermate controls. Blue dots are genes with significantly higher normalized counts in P301S controls than in P301S *hTREM2*^{R47H/+} samples (28 mRNAs). Red dots are genes with significantly higher normalized counts in P301S *hTREM2*^{R47H/+} samples than P301S controls (94 mRNAs). Highlighted upregulated genes are disease-associated microglial (DAM) genes and genes involved in inflammation while highlighted downregulated genes are the most significantly downregulated genes. Vertical dashed lines indicate $\log_2FC \pm 1$. Horizontal dashed line indicates $-\log_{10}(0.05)$. Wald test used. (n = 3 mice for P301S; n = 5 mice for P301S *hTREM2*^{R47H/+}). See also Supplementary Table 5. (c) Volcano plot of bulk RNA-seq data of hippocampal tissue from male P301S *hTREM2*^{R47H/+} and line-specific male P301S littermate controls. Blue dots are genes with significantly higher normalized counts in P301S controls than P301S *hTREM2*^{R47H/+} samples (3 mRNAs). Vertical dashed lines indicate $\log_2FC \pm 1$. Horizontal dashed line indicates $-\log_{10}(0.05)$. Wald test used. (n = 2 mice for P301S; n = 5 mice for P301S *hTREM2*^{R47H/+}). (d) Heatmap of results from weighted gene co-expression network analysis (WGCNA) of bulk RNA-seq data from (b) and Fig. S3.8 a, with only the significant module associations shown (top number: Pearson correlation, bottom number: adjusted p-value). Brown and cyan modules were the most significant. ** $p = 0.005$, * $p = 0.02$. (e) Top 5 enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways of genes in brown and cyan modules from the WGCNA in (d). Colors of the bars represent the WGCNA module. See also Supplementary Table 6. (f) Network analysis using STRING database on genes from the KEGG “cytokine receptor interaction” gene set of the brown module. See also Fig. S3.8 and Supplementary Table 5-6.

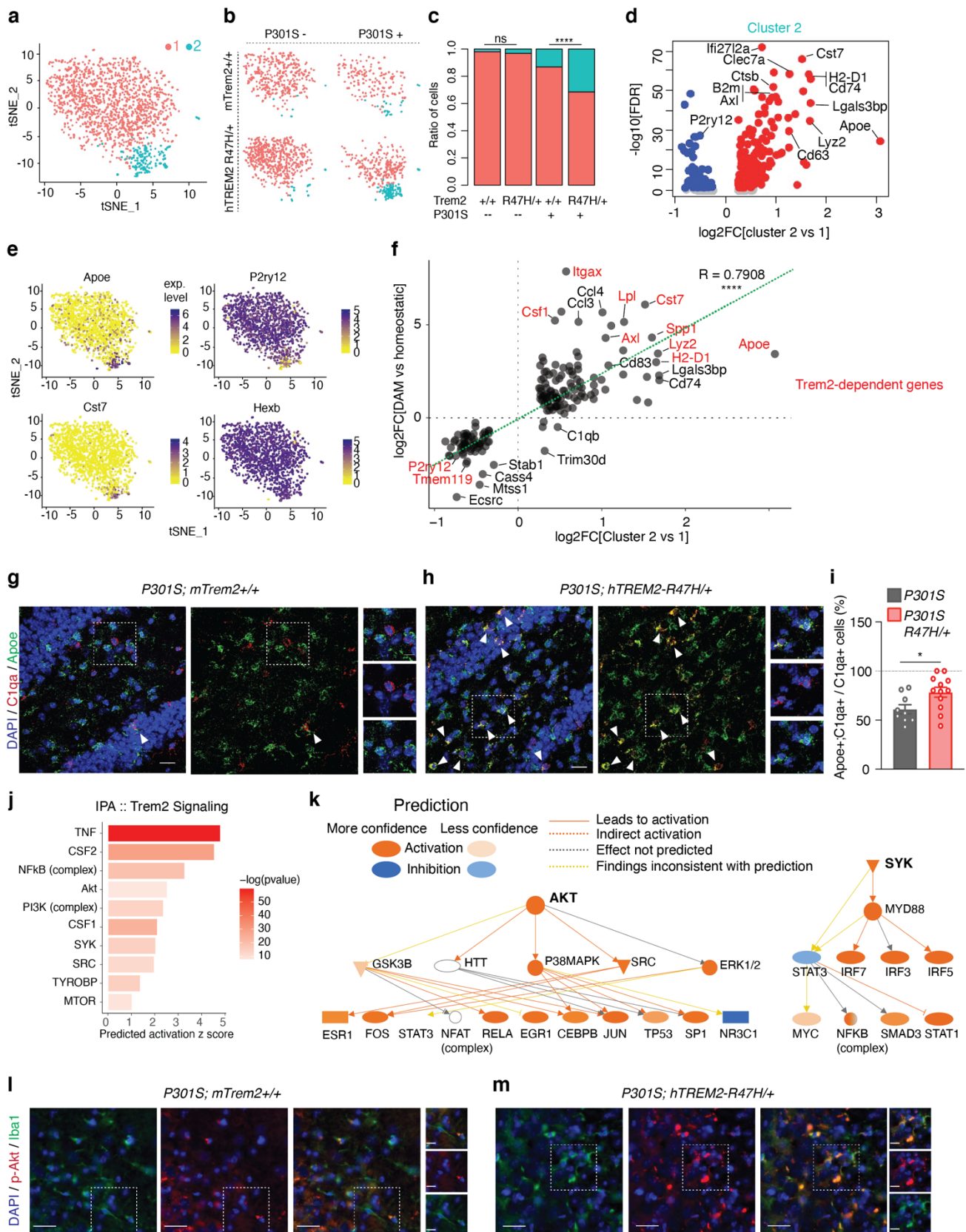


Figure 3.5. R47H-hTREM2 Enhances the Disease-Associated Microglia Population and Elevates Akt/Syk Signaling. (a) t-SNE plot of all 1,424 microglial cells analyzed and clustered. ($n = 3$ *mTrem2*^{+/+}, 2 *hTREM2*^{R47H/+}, 1 P301S, 2 P301S *hTREM2*^{R47H/+}, 8-month-old female mice). (b) t-SNE plots based on clustering from (a) split by genotype. (c) Ratio of cells in each cluster by genotype. **** $p < 0.0001$, two-sided Fisher's exact test. (d) Volcano plot of DEGs defining cluster 2 compared to cluster 1. See also Supplementary Table 7. (e) Feature plots of transcript expression overlaid onto t-SNE of all microglial cells. Colored scale bar denotes normalized expression level. (f) Correlation scatterplot of DEGs in the microglial cluster 2 vs cluster 1 comparison (x-axis) compared to disease-associated microglia (DAM/MGnD) versus homeostatic microglia (y-axis) previously published³⁴. Red genes are *Trem2*-dependent. $r = 0.7908$, **** $p < 2.2e-16$, Pearson's correlation. (g-h) Representative images of RNAscope using probes against *Clqa* (red) and *Apoe* (green) of P301S (g) and P301S *hTREM2*^{R47H/+} (h) dentate gyrus sections. White triangles highlight *Clqa*⁺; *Apoe*⁺ microglial cells. Dashed regions are zoomed in on the right side of the image. Scale bar = 20 μm , 10 μm for zoomed images. (i) Quantification of RNAscope images for percent of cells that are *Clqa*⁺; *Apoe*⁺ over total *Clqa*⁺ cells. $n = 9$ sections, 3 mice for P301S; 12 sections, 4 mice for P301S *hTREM2*^{R47H/+}. Student's t-test, * $p = 0.0254$, $t = 2.426$, $df = 19$. (j) Ingenuity Pathway Analysis (IPA) upstream regulator prediction for Trem2-signaling molecules based on cluster 2 markers from (d). Bar color denotes $-\log_{10}(p\text{value})$. (k) IPA example activated networks from (j) and their downstream predicted targets. (l-m) Representative images of immunostaining against phospho-Akt-Ser473 (red) and Iba1 (green) for P301S (l) and P301S *hTREM2*^{R47H/+} (m) CA3 sections. Dashed regions are zoomed in on the right side of the image. Scale bar = 25 μm , 12.5 μm for zoomed images. Values are mean $\pm$ SEM. Each sequencing dataset represents one independent sequencing experiment. See also Fig. S3.9-S3.10 and Supplementary Table 7.

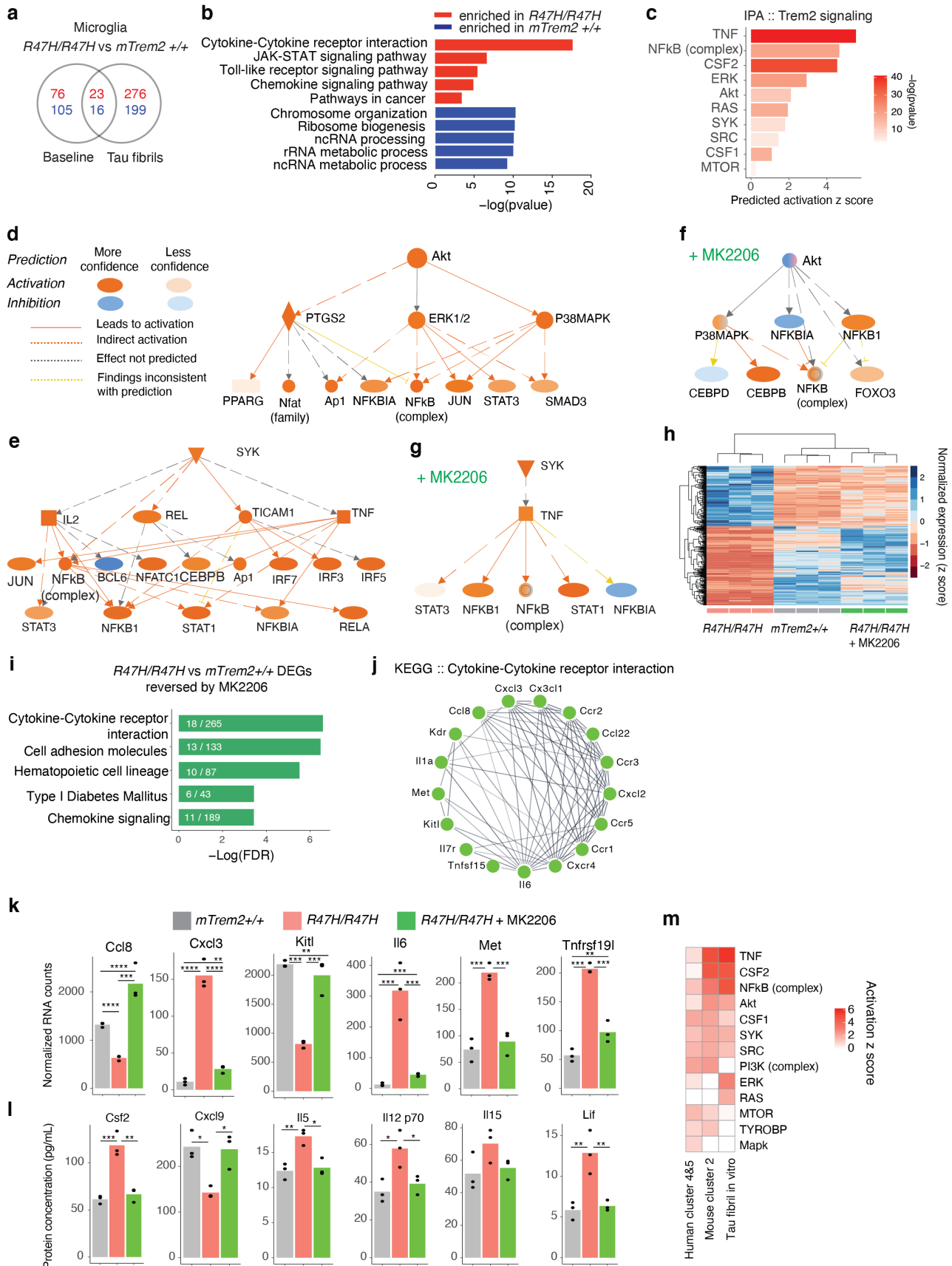


Figure 3.6. R47H-hTREM2 Primary Microglia Induce an Akt-dependent Pro-inflammatory Signature in Response to Tau Fibrils. (a) Venn diagram of differentially expressed genes between *mTrem2*^{+/+} and *hTREM2*^{R47H/R47H} primary microglia with or without tau fibril stimulation. Red and blue numbers denote upregulated and downregulated genes, respectively. (n = 3 biological replicates for all conditions). (b) KEGG pathway enrichment analysis of the genes from (a) that were uniquely changed in *hTREM2*^{R47H/R47H} microglia under tau fibril stimulation conditions. (c) IPA upstream regulator prediction for Trem2-signaling molecules based on genes that were uniquely changed in *hTREM2*^{R47H/R47H} microglia in response to tau fibril stimulation from (a). (d-e) IPA Akt (d) and Syk (e) activated networks from (c) and their downstream predicted targets. (f-g) IPA Akt (f) and Syk (g) networks and their predicted inhibition/activation states after MK2206 (Akt-inhibitor) treatment of *hTREM2*^{R47H/R47H} vs. *mTrem2*^{+/+} microglia with tau fibril stimulation. (h) Heatmap showing z scores of normalized expressions of 477 genes that are changed by *hTREM2*^{R47H/R47H} compared to *mTrem2*^{+/+} and are reversed towards control expression levels with MK2206 treatment. (i) KEGG pathway enrichment analysis of genes highlighted in (h). (j) STRING network representation of the genes in the “Cytokine-Cytokine receptor interaction” pathway from (i). (k) Barplots of example genes changed by *hTREM2*^{R47H/R47H} but reversed back to normal expression levels by MK2206. (l) Barplots of example cytokines measured by MAGPIX changed by *hTREM2*^{R47H/R47H} but reversed back to normal protein expression levels by MK2206. (m) Heatmap comparing the IPA predicted activation z score of TREM2 signaling molecules for all three models (Fig. 3.2, Fig. 3.5). See also Supplementary Tables 8-9.

3.9 Supplementary Figures

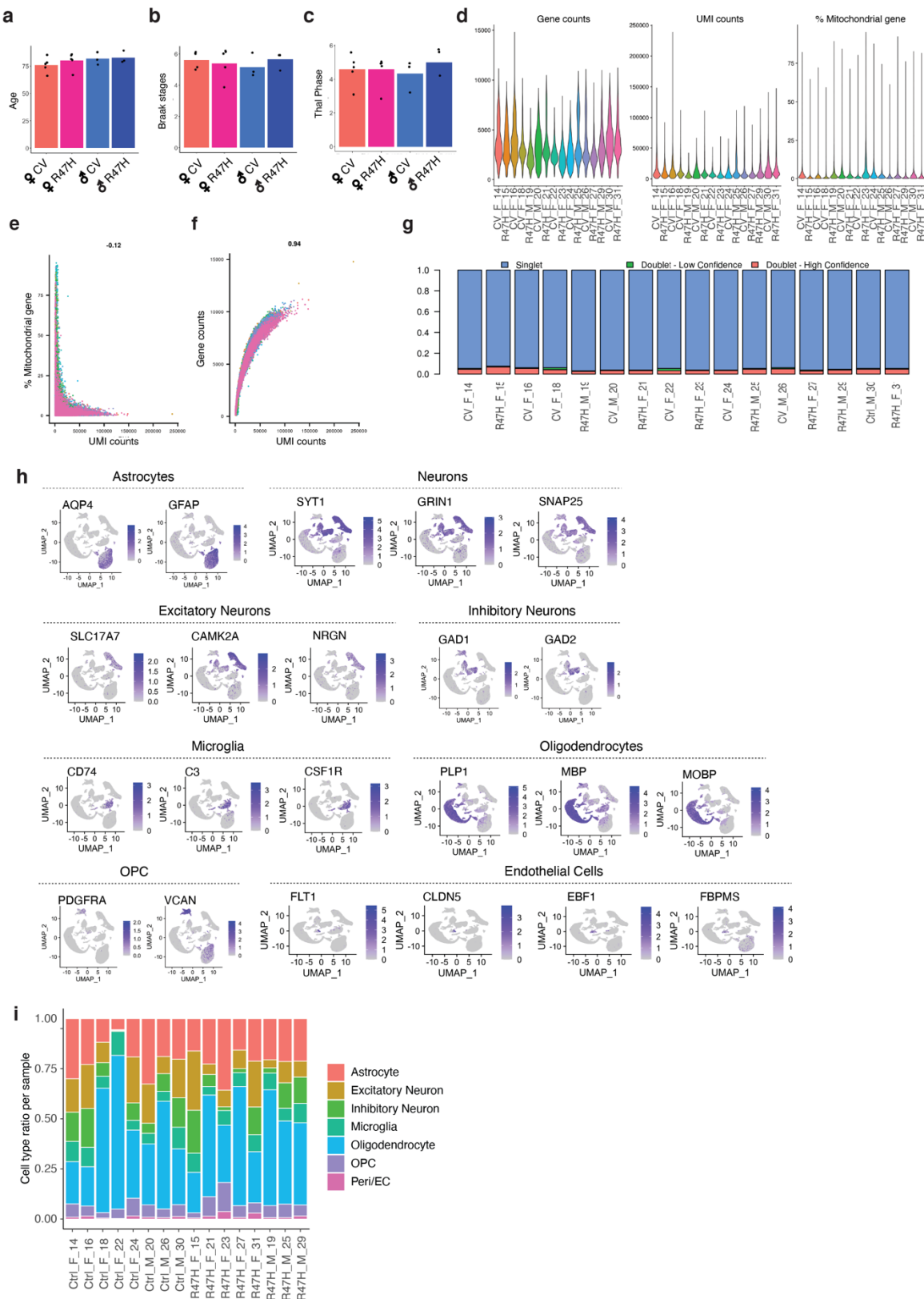


Figure S3.1. Quality Control Assessment of Single-Nuclei RNA-Seq of Human AD Patient Brain Tissues (Related to Fig. 3.1). (a-c) Bar plots of age (a), Braak stage (b), and Thal phase (c) of the patient tissues. (d) Violin plots showing spread of total genes, total UMIs, and percent of mitochondrial genes detected per nuclei for each individual sample. (e-f) Correlation between UMI counts and percentage of mitochondrial genes per nuclei (e) and total genes detected (f) for all samples. (g) Ratio of nuclei in each sample determined as singlets, doublets (low confidence), and doublets (high confidence). Only high-confidence doublets were removed for downstream analysis. (h) Feature plots showing expression of cell type marker genes for each cell type. (i) Proportion of different cell types captured per sample.

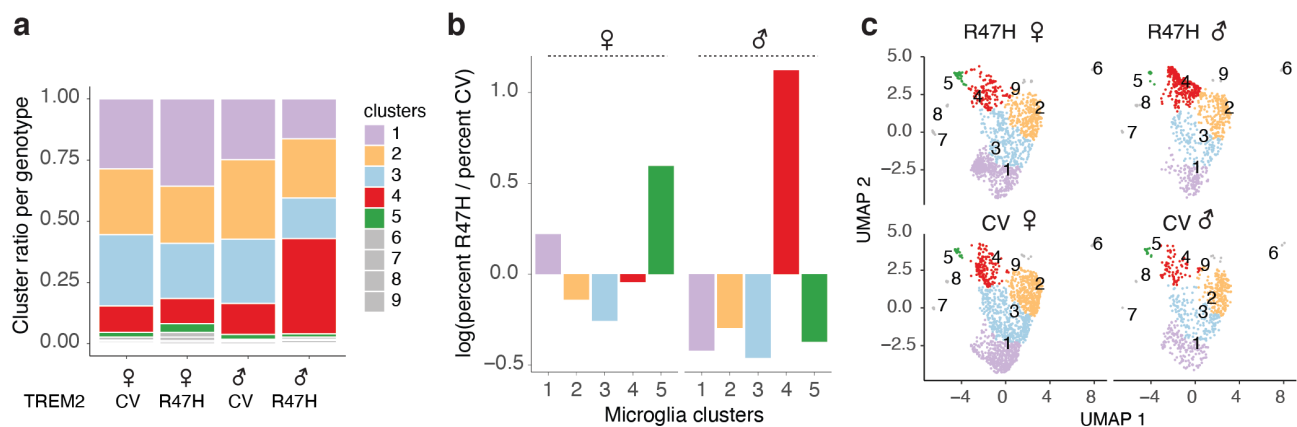


Figure S3.2. R47H-hTREM2 Induces a Sex-Specific Change in the Microglial Transcriptome of Human AD Patients (Related to Fig. 3.2). (a) Microglia subcluster ratio per genotype and sex. (b) Log2 ratio of microglia subcluster fraction in R47H versus CV for each sex. (c) UMAP split by genotype and sex. Colors denote subclusters from (a).

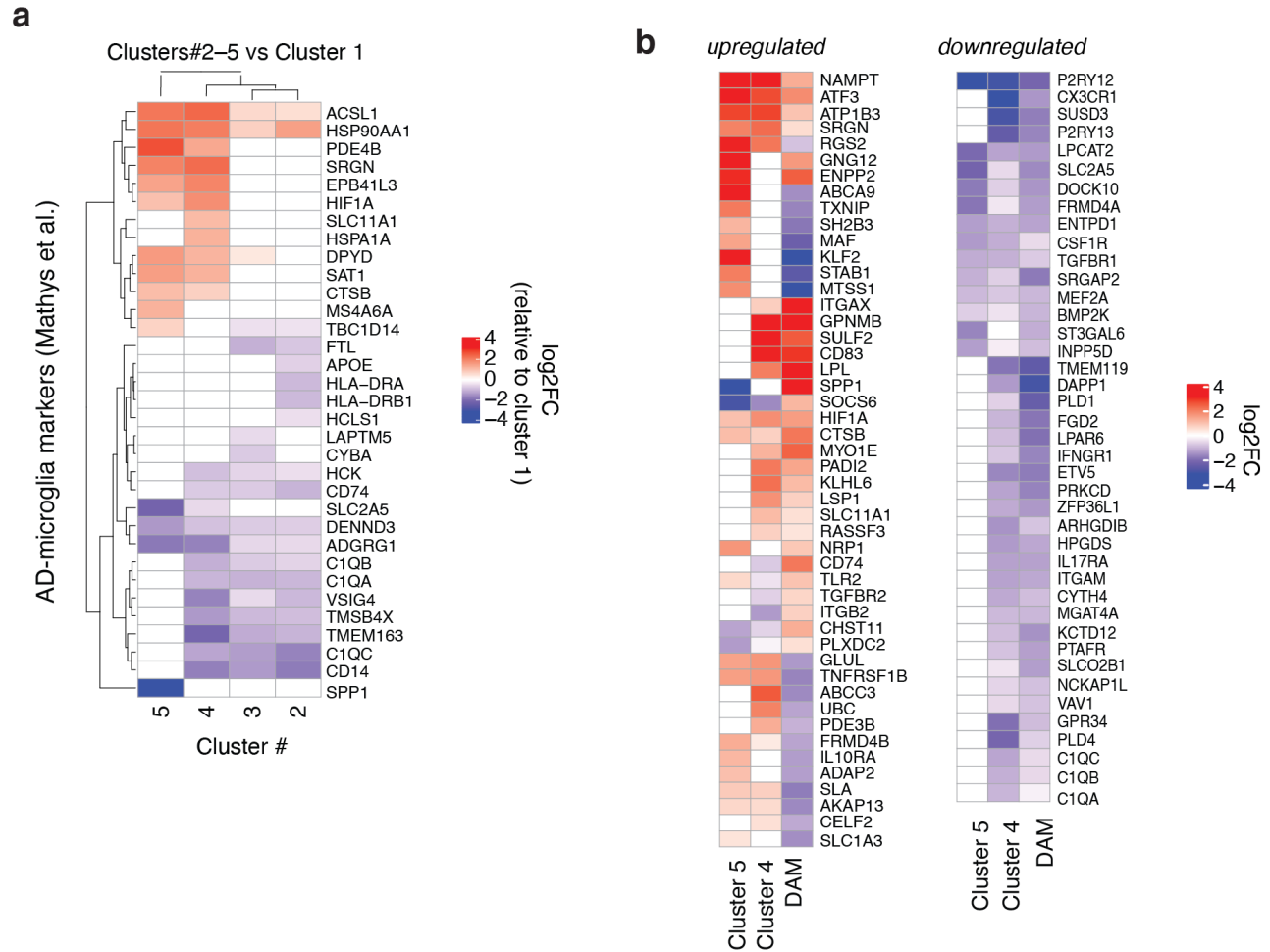


Figure S3.3. R47H-associated Microglia Signature Overlaps with Previously-identified AD-associated Signatures (Related to Fig. 3.2). (a) Heatmap showing Log2-fold change values of DEGs from (Fig. 3.2 d) and (Fig. 3.2 e) compared to AD-associated microglia signature identified in Mathys et al. (Mic1)⁴¹. (b) Heatmap showing Log2-fold change values of DEGs from (Fig. 3.2 d) and (Fig. 3.2 e) compared to the DAM signature³⁴.

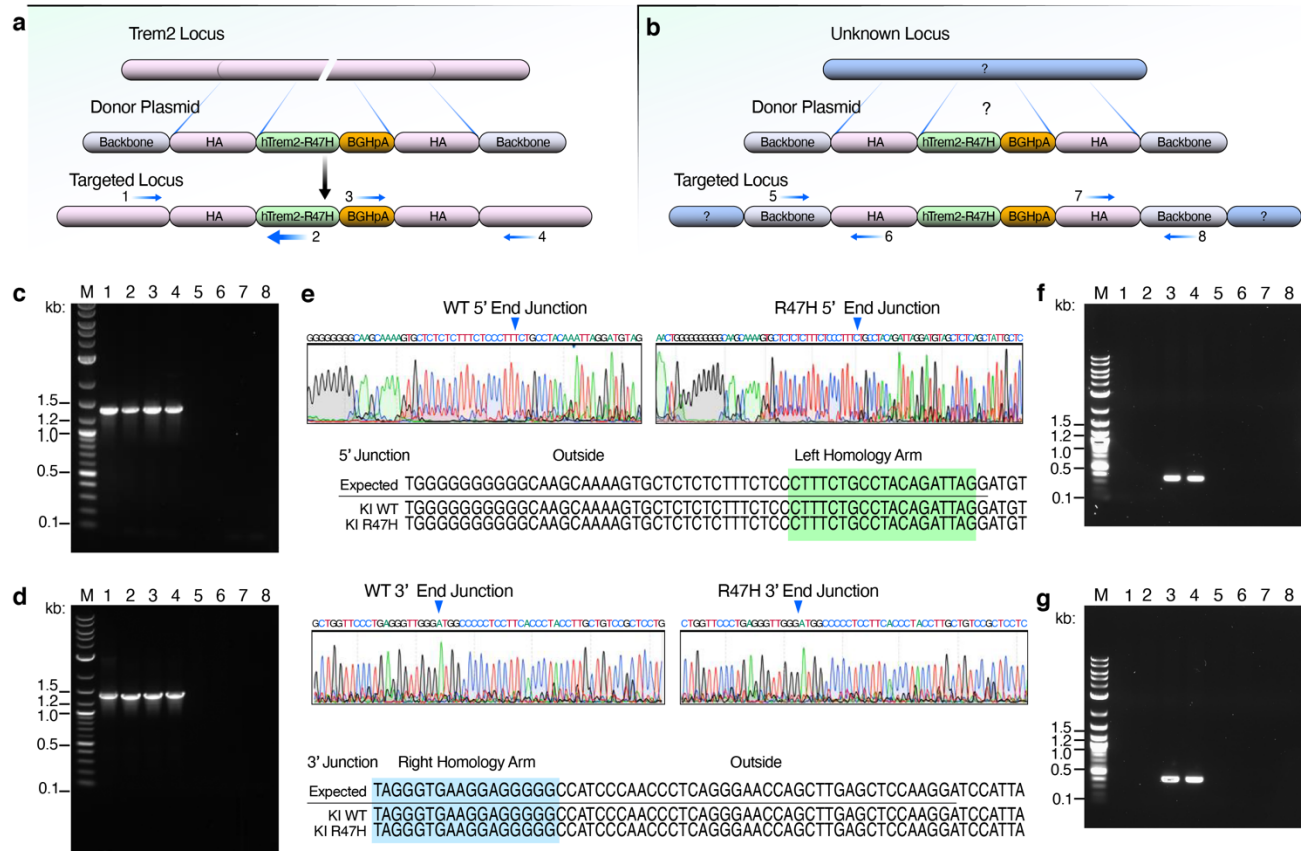


Figure S3.4. Characterization of CV-*hTREM2* and R47H-*hTREM2* Knock-in Mouse Lines (Related to Fig. 3.3). (a) Diagram of the strategy to detect the specific targeting of the R47H-*hTREM2* (or CV-*hTREM2*) into the mouse *Trem2* genomic locus and primer locations for detecting integration. PCR primers labeled as arrows and are located as follows: Primer 1 is located outside of the left homology arm, Primer 2 is on 5' end of *hTREM2* cDNA, Primer 3 is on BGHpA, Primer 4 is located outside of the right homology arm. (b) Diagram of the strategy to detect non-specific integration of the R47H-*hTREM2* (or CV-*hTREM2*) cDNA in the mouse genome. PCR primers labeled as arrows and are located as follows: Primer 5 is on the vector, Primer 6 is on the left arm, Primer 7 is on the right arm, Primer 8 is on the vector. (c) PCR screening for the 5' end specific homologous recombination of CV-*hTREM2* and R47H-*hTREM2* mice using Primers 1 and 2. Lane M = 1kb plus DNA ladder, Lanes 1-2 = R47H-*hTREM2*, Lanes 3-4 = CV-*hTREM2*, Lanes 5-6 = C57BL/6 control, Lanes 7-8 = H₂O control. The size of PCR product is about 1345 bp. (d) PCR screening for the 3' end specific homologous recombination of CV-*hTREM2* and R47H-*hTREM2* mice using Primers 3 and 4. Lane M = 1kb plus DNA ladder, Lanes 1-2 = R47H-*hTREM2*, Lanes 3-4 = CV-*hTREM2*, Lanes 5-6 = C57BL/6 control, Lanes 7-8 = H₂O control. The size of PCR product is about 1339 bp. (e) Sanger sequencing chromatograms for the junctions. Sequences of the intended and obtained knock-in junctions in the mouse lines show correct homologous recombination. 5' end of left homology arm is highlighted in green and the 3' end of right homology arm is highlighted in blue. (f) PCR detection of non-specific integration on 5' end of the CV-*hTREM2* and R47H-*hTREM2* cDNA using Primers 5 and 6. Lane M = 1kb plus DNA ladder, Lanes 1-2 = R47H-*hTREM2*, Lanes 3-4 = CV-*hTREM2*, Lanes 5-6 = C57BL/6 control, Lanes 7-8 = H₂O control. The PCR product size is 347 bp. (g) PCR detection of non-specific integration on 3' end of the CV-*hTREM2* and R47H-*hTREM2* cDNA using Primers 7 and 8. Lane M =

1kb plus DNA ladder, Lanes 1-2 = R47H-*hTREM2*, Lanes 3-4 = CV-*hTREM2*, Lanes 5-6 = C57BL/6 control, Lanes 7-8 = H₂O control. The PCR product size is 398 bp.

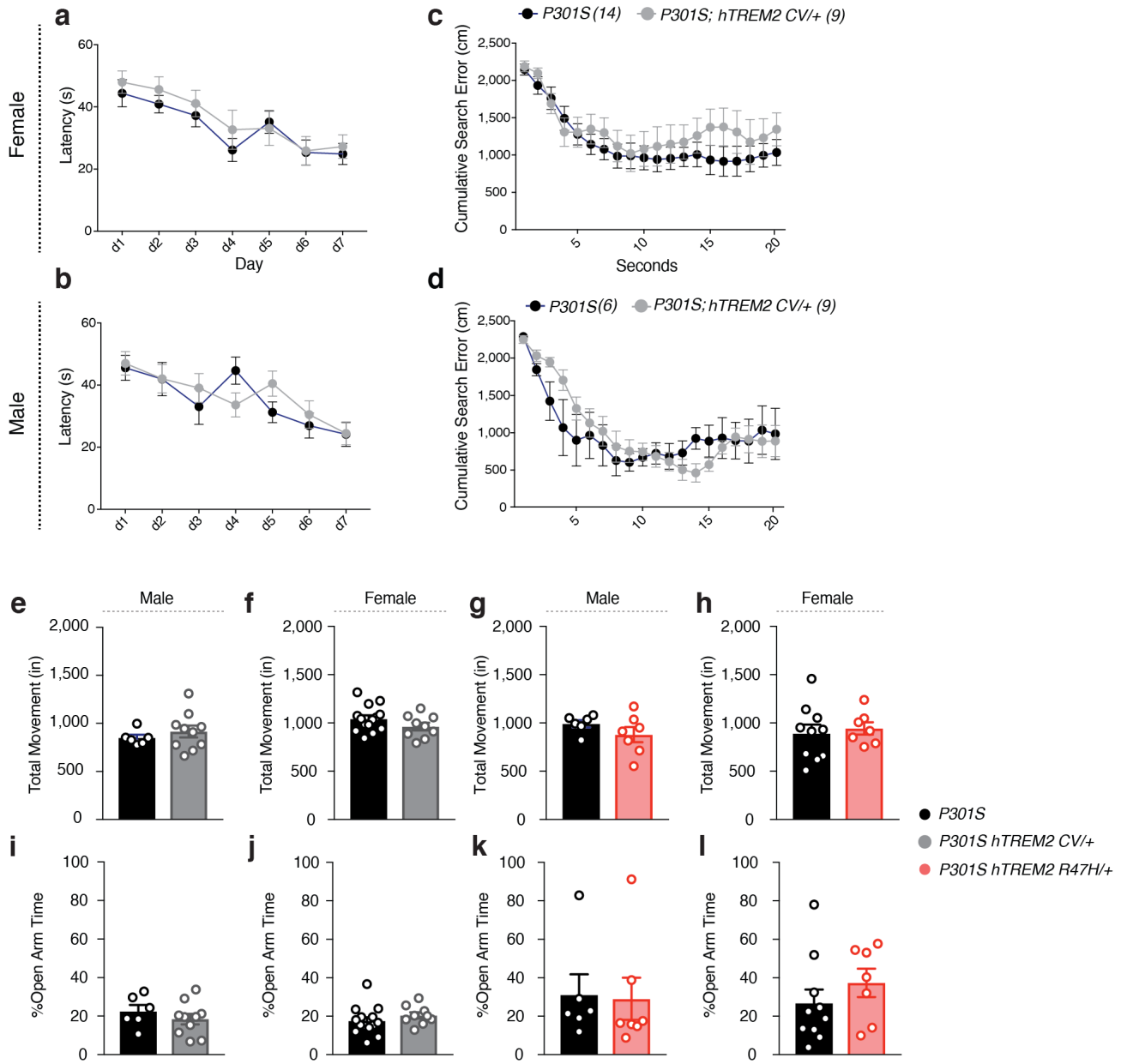


Figure S3.5. WT-*hTREM2* Tauopathy Mice Behave Similar to *mTrem2*^{+/+} Tauopathy Mice (Related to Fig. 3.3). (a-d) Latency to reach the platform during hidden trials (d1-d7) and cumulative search errors during the 72-hr probe trial for female (a,c) and male (b,d) P301S and P301S *hTREM2*^{CV/+} mice. STATA mixed-effects modeling for (a,b). Two-tailed Mann-Whitney U-test of area under the curve for (c,d). (e-h) Open-field assessment of overall activity of 7–9-month-old P301S *hTREM2*^{CV/+} male (e) and female (f) and P301S *hTREM2*^{R47H/+} male (g) and female (h) mice and littermate P301S controls. Two-tailed Student's t-test for $n > 8$, two-tailed Mann-Whitney U-test for $n < 8$. (i-l) Elevated plus maze assessment of anxiety levels in 7–9-month-old *hTREM2*^{CV/+} male (i) and female (j) mice and *hTREM2*^{R47H/+} male (k) and female (l) mice and littermate P301S controls. Two-tailed Student's t-test for $n > 8$, two-tailed Mann-Whitney U-test for $n < 8$.

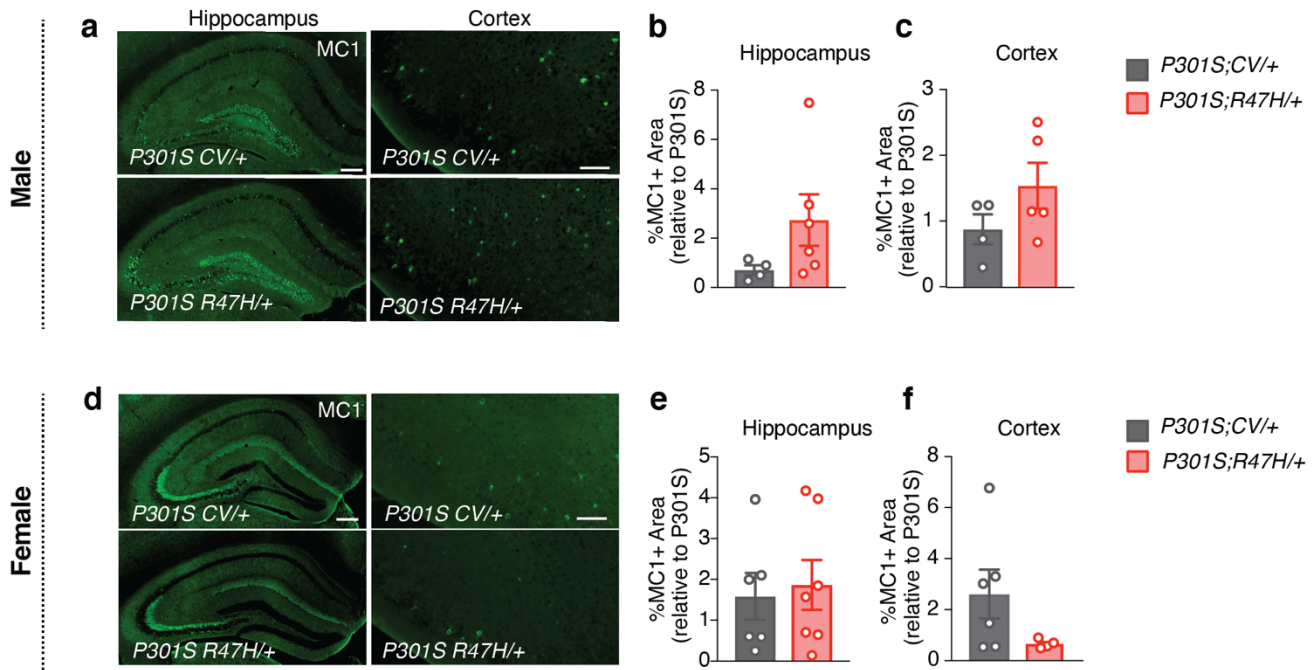


Figure S3.6. R47H-*hTREM2* Does not Alter Tau Pathology Load (Related to Fig. 3.3). Immunohistochemical analysis of 8–9-month-old mice. Each circle in bar graphs represents the average of 8–10 sections per mouse. Values are normalized to line-specific, sex-matched *P301S mTrem2*^{+/+} littermate controls. (a,d) Representative images of MC1 immunostaining in the hippocampus and entorhinal cortex of male (a) and female (d) mice. (b-c, e-f) Percentage of MC1+ area in male hippocampus (b) and cortex (c) and female hippocampus (e) and cortex (f) of *P301S hTREM2*^{CV/+} and *P301S hTREM2*^{R47H/+} mice. Entire hippocampus and entire cortex were quantified. Two-tailed Mann-Whitney U-test. Values are mean ± SEM.

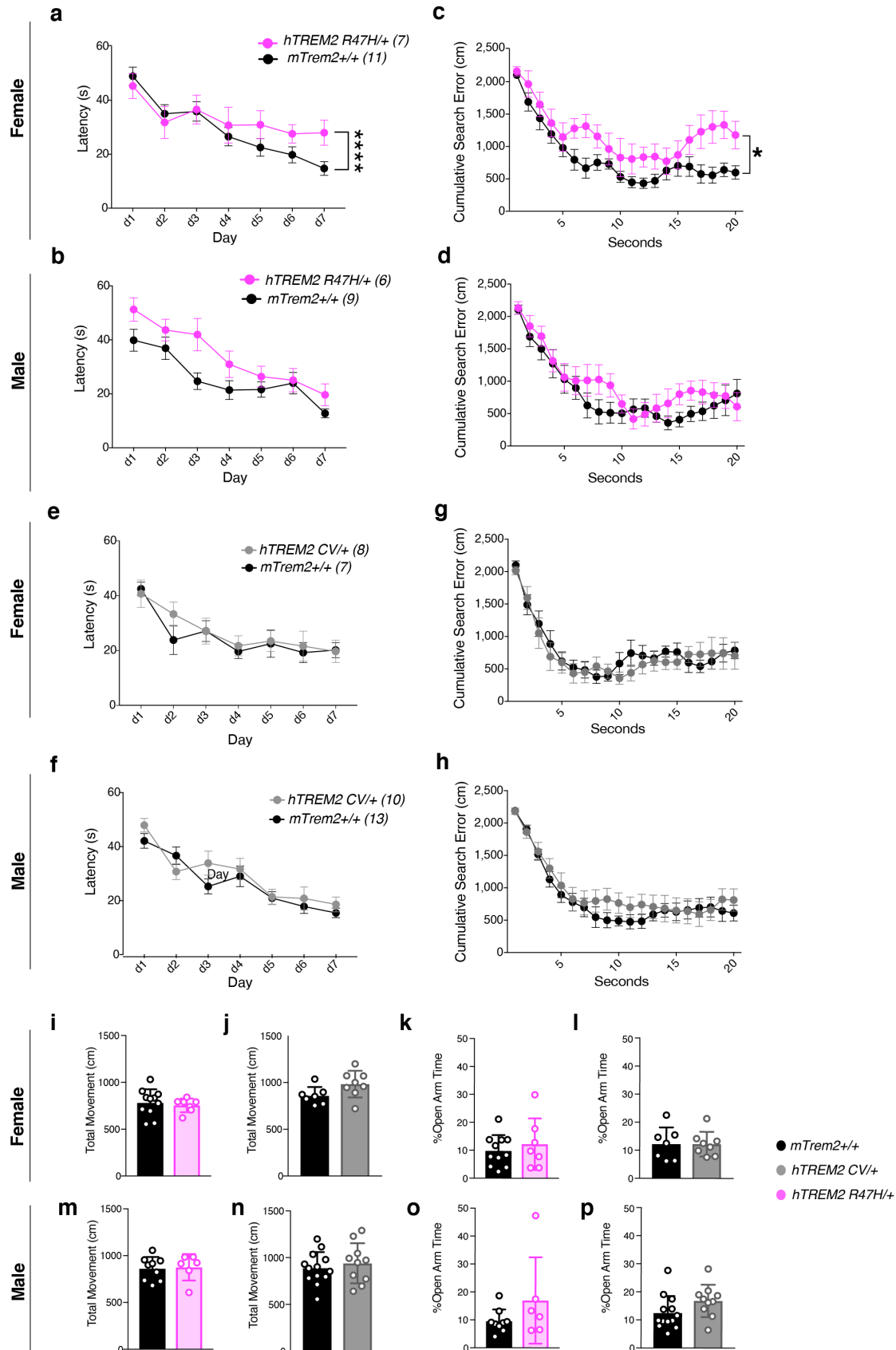


Figure S3.7. R47H-*hTREM2* Induces Spatial Learning and Memory Deficits in Female Mice (Related to Fig. 3.3). Morris water maze assessment of spatial learning and memory in 7–9-month-old *hTREM2*^{R47H/+} and *hTREM2*^{CV/+} mice and their littermate controls. (a,b) Latency to reach the platform during hidden trials (d1–d7) for *hTREM2*^{R47H/+} females (a) and males (b) and their littermate sex-matched *mTrem2*^{+/+} controls. ****p=0.0001, STATA mixed-effects modeling. (c,d) Cumulative search errors during the 24-hr probe trial for *hTREM2*^{R47H/+} females (c) and males (d) and their littermate sex-matched *mTrem2*^{+/+} controls. * U=13, p=0.0204, Two-tailed Mann-Whitney U-test of area under the curve. (e,f) Latency to reach the platform during hidden platform trials (d1–d7) for *hTREM2*^{CV/+} females (e) and males (f) and their littermate sex-matched *mTrem2*^{+/+} controls. STATA mixed-effects modeling. (g,h) Cumulative search errors during the 24-hr probe trial for *hTREM2*^{CV/+} females (g) and males (h) and their littermate sex-matched *mTrem2*^{+/+} controls. Two-tailed Mann-Whitney U-test of area under the curve. (i-p) Open-field assessment of overall activity (i,j,m,n) and elevated plus maze assessment of anxiety levels (k,l,o,p) of 7–9-month-old *mTrem2*^{+/+}, *hTREM2*^{CV/+}, and *hTREM2*^{R47H/+} mice. Values are mean ± SEM.

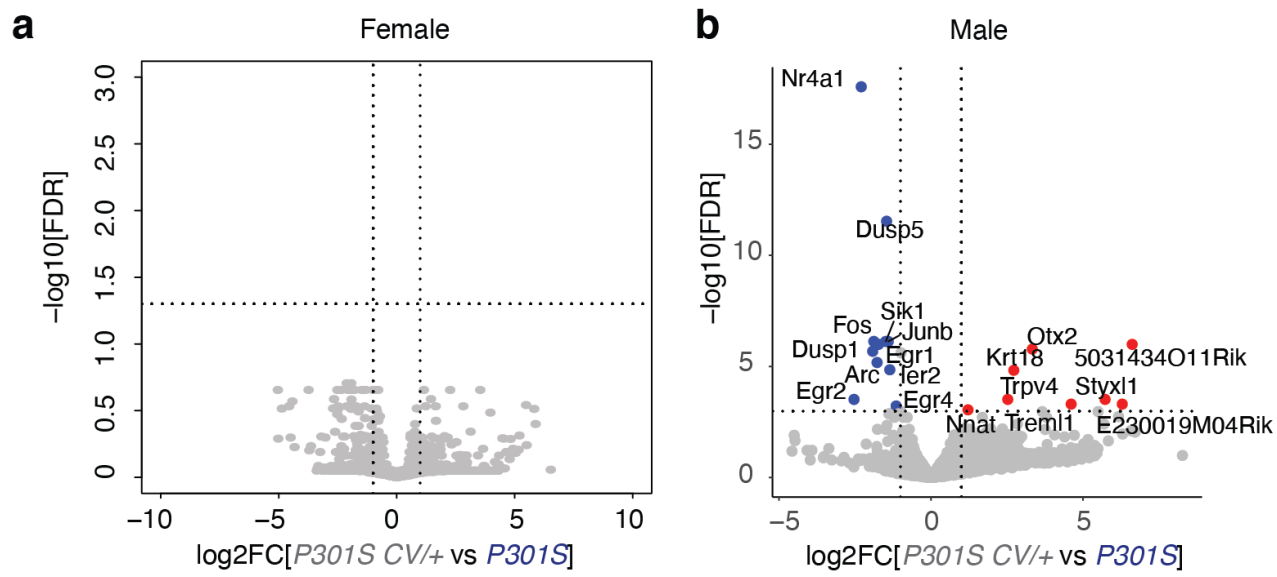


Figure S3.8. CV-*hTREM2* has Similar Transcriptomic Profiles to *mTrem2*^{+/+} (Related to Fig. 3.4). (a-b) Volcano plot of bulk RNA-seq data of hippocampal tissue from female (a) and male (b) P301S *hTREM2*^{CV/+} and line-specific female and male P301S controls, respectively. Blue dots are genes with significantly higher normalized counts in P301S controls than in P301S *hTREM2*^{CV/+} samples (11 mRNAs). Red dots are genes with significantly higher normalized counts in P301S *hTREM2*^{CV/+} samples than P301S controls (7 mRNAs). Vertical dashed lines indicate $\log_2FC \pm 1$. Horizontal dashed line indicates $-\log_{10}(0.05)$. Wald test used. (n = 3 mice for P301S; n = 6 mice for P301S *hTREM2*^{CV/+} for each sex).

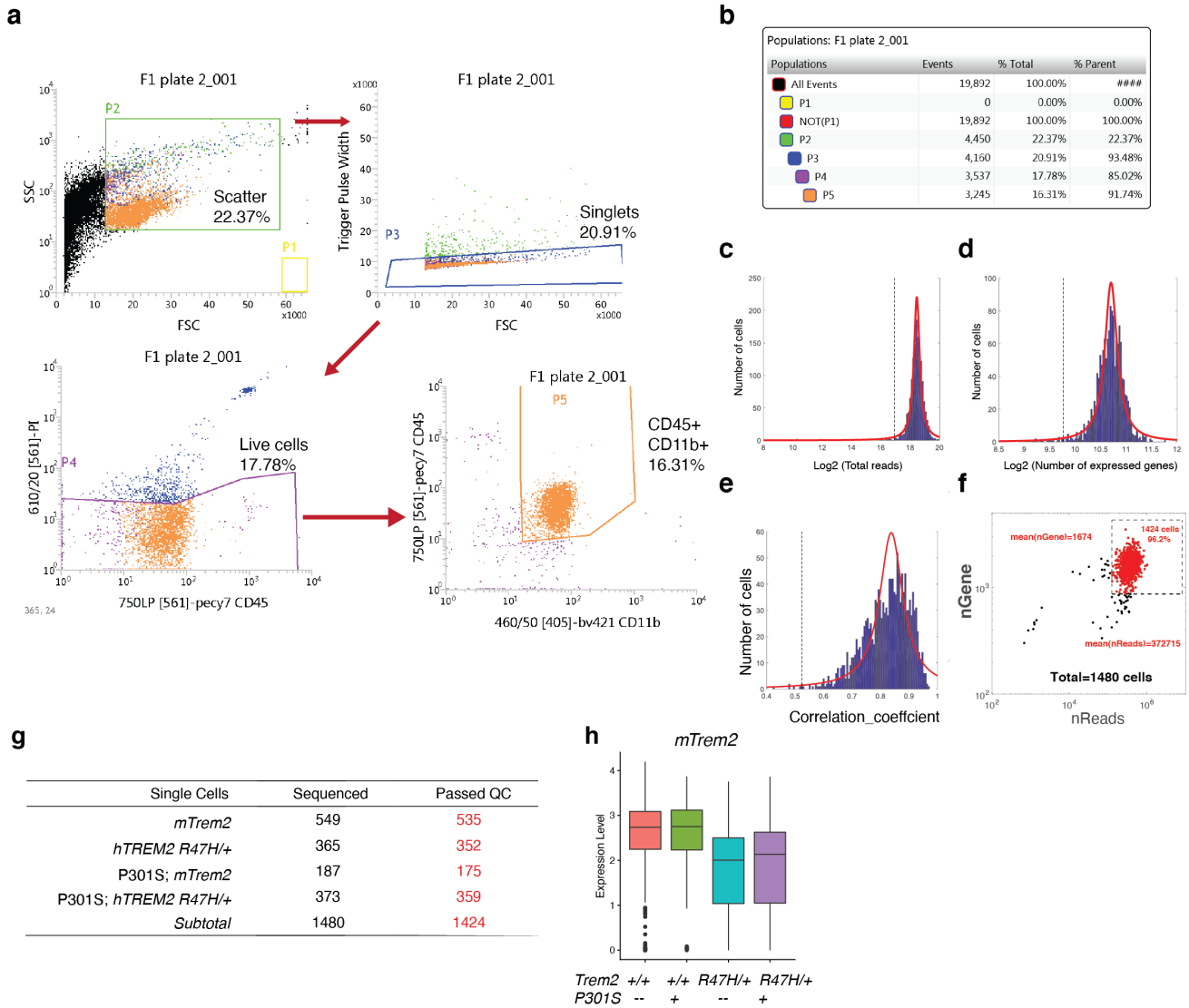


Figure S3.9. Single-Cell RNA-Seq of Brain CD45⁺;CD11b⁺ Cells (Related to Fig. 3.5). (a) Representative FACS plots showing gating strategies and cells sequenced. (b) Number of cells and proportions based on FACS from (a). (c-e) Quality control criteria for the single-cell sequencing data. Fitted curves for histograms of mapped reads (c), number of detected genes (d) and ERCC correlation coefficient (e) are labeled in red. Dashed lines are statistical cutoffs. Cells that passed all three criteria were retained for analysis. (f) Scatter plot highlighting cells that passed QC (red) among all cells sequenced. Each dot is a cell. (g) Summary table for the numbers of cells sequenced and cells that passed QC (red). 1424 cells were retained for analysis. (h) Normalized *mTrem2* expression level. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; dots, outliers.

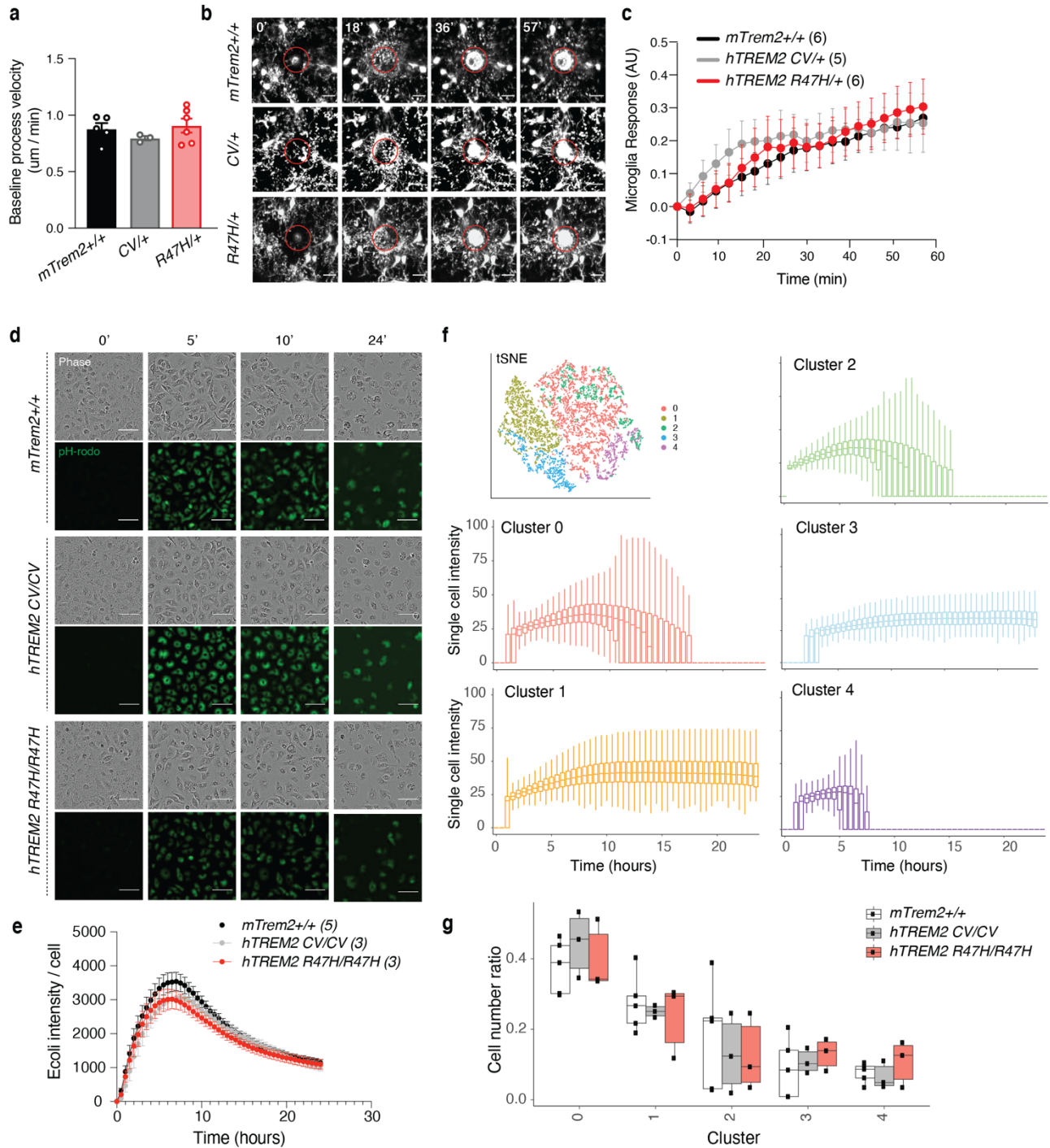


Figure S3.10. R47H-*hTREM2* Does Not Affect Microglial Injury Response or Phagocytosis (Related to Fig. 3.5). (a) Quantification of microglial process velocity in 12-17-month-old mice during 10 min of baseline recordings ($n = 5$ $mTrem2^{+/+}$, $n = 3$ $hTREM2^{CV/+}$, $n = 6$ $hTREM2^{R47H/+}$ mice, ~25 processes/mouse). One-way ANOVA. (b) Representative images at 0, 18, 36, and 57 minutes after laser-induced injury. Red circle indicates region of injury. (c) Quantification of the normalized microglial response to focal laser-induced tissue injury for 60 min after injury. ($n = 6$ $mTrem2^{+/+}$, $n = 5$ $hTREM2^{CV/+}$, $n = 6$ $hTREM2^{R47H/+}$ mice). STATA mixed-effects modeling. Scale bars: hippocampus, 300 μm ; cortex,

200 μm ; hippocampal microglia, 20 μm , ablation, 15 μm . Values are mean $\pm$ SEM. All immunohistochemistry imaging and data are from one independent experiment. For *in vivo* imaging, one mouse was imaged per imaging session. (d) Representative images of primary microglia at 0, 5, 10, and 24 hours after incubation with pH-rodo conjugated *E. coli* substrates. Scale bar: 100 μm . (e) Bulk fluorescence intensity quantification of field of view normalized by cell number. ($n = 5$ mTrem2 $^{+/+}$, $n = 3$ *hTREM2* $^{CV/+}$, $n = 3$ *hTREM2* $^{R47H/+}$ biologically independent experiments). (f) tSNE of single-cell quantification of fluorescence intensity of all cells analyzed and fluorescence intensity change over time plotted as boxplots for each time point for each cellular cluster. Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. (g) Boxplot of proportions of each genotype for each cell cluster determined in (f). Boxplot elements: center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; dots, individual experimental values. See also Supplementary Movie 1.

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Chapter 4: Conclusion

By leveraging sex-differences in microglia as a point of study, we have been able to uncover novel molecular pathways involved in AD pathogenesis. In Chapter 2.2, we focused on sex-dependent expression of miRNAs in adult microglia and, though at baseline they did not mediate sex-dependent phenotypes, they had dramatic sex-dependent outcomes when perturbed. This would suggest that male and female microglia utilize distinct mechanisms of adaptation that, when disrupted, can have sex-specific maladaptive consequences to disease. In Chapter 2.3, we explored how individual miRNAs modulate microglial phagocytosis, with a specific focus on a cluster of miRNAs that have paralogs on autosomal chromosomes as well as the X chromosome. Though preliminary, the study established a platform for further investigating these sex-specific miRNAs on microglial phenotypes. Finally, in Chapter 3, we found that the R47H mutation in a microglia-specific gene, *TREM2*, could also have sex-specific consequences in both humans and mice at the transcriptomic and functional levels. This finding suggests *TREM2*, like the *APOE4* AD risk-variant and perhaps *through* this APOE-pathway, can have sex-specific effects on disease.

One of the main questions that remains from these studies is the relevance of these sex-differences in the context of human disease and AD. Are differences in the transcriptomic profiles of male and female microglia simply a characterization of the differential response to disease pathology that ultimately has no influence on disease outcomes, or could these sex-differences be *drivers* of the sex-specific clinical outcomes of disease? More experimental studies to assess causality would need to be done in order to answer this question. For example, one could alter the profiles of female microglia to reflect the profile of male microglia through microglial implantation or hormonal manipulation early in development, and assess how such a manipulation changes

disease outcome. Another approach would be to dissect the specific pathways that are different between male and female microglia, target them through genetic or pharmacological studies and assess disease outcome. For example, one could manipulate the expression of an individual miRNA or a cluster of miRNAs in male versus female microglia in the context of tauopathy mouse models. Of note, these current studies were done at the end-stages of the pathology or disease. Further studies would need to be done to assess the longitudinal changes that occur in both mice and humans, and how sex-differences emerge or dynamically change over the course of disease. Clinically, sex-specific biomarkers could be developed to better track the clinical progression of AD in humans or use currently-available biomarkers, such as phospho-tau217¹, to do longitudinal studies to better understand the sex-differences observed in early stages of the disease.

Another overarching and persisting challenges of the field of sex biology is to parse out the contribution of hormonal and non-hormonal (including sex chromosomal) effects. This dissection is further complicated by aging. Hormonal fluctuations during the aging process (especially during menopause) can have profound effects on neurological disease outcomes². Mouse models can be used to study the effects of menopause, such as the use of 4-vinylcyclohexene diepoxide (VCD) to induce follicle depletion and ovary atrophy, leading to a gradual decline in hormones³. Mimicking these hormonal fluctuations in mice as well as using models to segregate hormonal from non-hormonal mechanisms of sex differentiation are crucial for understanding sexually dimorphic phenotypes. One way to segregate the hormonal from the sex chromosomal contribution to microglial sex differences is to use the “Four Core Genotype” (FCG) mouse model. In these mice, the testis-determining *Sry* gene is deleted from the Y chromosome and inserted into an autosome^{4,5}. Thus, XX and XY mice can develop ovaries or testis depending on whether they have the *Sry* gene. The effect of this model on microglia has yet to be

studied and could be used to better understand the hormonal versus the sex chromosome effects on microglial function. In addition, when crossed to mouse models of disease, the FCG model can be used as a powerful tool for understanding the mechanism of sex differential phenotypes in disease. For example, Smith-Bouvier et al. (2008) crossed FCG mice with experimental autoimmune encephalomyelitis (EAE) mice, a mouse model of MS, then gonadectomized them to remove sex hormones. They found that the disease was more severe in mice with the XX sex chromosomes than the XY group⁶, suggesting differential effects of the X and Y chromosome on disease progression outside the context of sex hormones. Such a model could have powerful implications in other models of neurodegeneration such as AD, where the sex differences in disease progression are not well-understood.

What is clear is that microglia from male and female rodents and humans exhibit sex-differences. As the field of neurodegenerative diseases has come to appreciate the role of microglia in a myriad of diseases, we should also appreciate how these cells may be contributing to sex differences in the diseased brain. Historically, clinical and basic science studies have largely focused on the male sex. Only in 1993 did the U.S. Food and Drug Administration and the National Institute of Health (NIH) require the inclusion of women in clinical trials⁷, and finally in 2014, NIH mandated balanced use of both sexes in preclinical, basic science studies⁸. By excluding one of the two sexes in our studies, we are potentially missing rich biological insights that can be uncovered through sex-differences and inherently biasing our results, ultimately doing a disservice to all patients. Studies need to be conducted to explore sex differences with the mindset that sex differences in cells, animals, and humans may be an adaptation to normalize the differences of the sexes that may become maladaptive in disease states and lead to differences in disease vulnerability and progression. Ultimately, studying sex-differences is not about focusing on one sex, but rather

about aiming to uncover novel disease mechanisms and develop potential therapies for patients of both sexes.

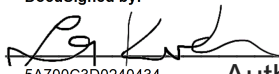
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