The effect of estrogen signaling modulation on female-specific microglial heterogeneity in the mouse hippocampus

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ABSTRACT

Ovarian aging is characterized by an exhaustion of follicular reserve and subsequent cessation of estrogen production. Decreased estrogen induces systemic inflammation with wide impacts across body systems, including in the brain. We hypothesize that estrogen signaling through microglial estrogen receptor-alpha (ERα) suppresses proinflammatory signaling pathways. However, the pathways responsible for estrogens' anti-inflammatory effects in microglia remains unresolved. We believe that ablation of endogenous estrogens during menopause induces neuroinflammation and promotes the transition of microglia to activated phenotypes (i.e., disease-associated microglia (DAM)) in rodents, contributing to various brain disease such as Alzheimer's disease (AD). Here, we tested the effect of estrogen signaling modulation on murine microglial heterogeneity using: 1) estrogen receptor knockout and 2) endogenous estrogen depletion with 4-vinylcyclohexene diepoxide (VCD), in combination with single cell transcriptomic endpoints to analyze microglial phenotypes. Upon VCD treatment in wild-type, alterations in the estrogen signaling pathways led to upregulation of senescent transcriptional programming in TREM2-independent DAMs. Overall, these data will elucidate the estrogen response pathways associated with female-biased microglial heterogeneity.

METHODS

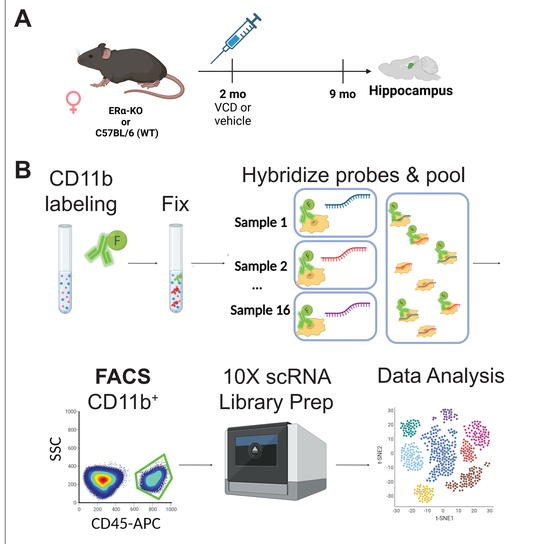


Figure 1. Schematic of experimental methods. **(A)** To test the effect of estrogen depletion on microglial heterogeneity, chemical ovariectomy (with 4-vinylcyclohexene [VCD]) or estrogen receptor alpha knockout were compared to wildtype female C57BL/6 controls. **(B)** Microglial heterogeneity was assessed by 10X scRNA-Seq, using the Flex kit.

RESULTS

TRANSCRIPTOMIC ANALYSIS OF HIPPOCAMPAL MICROGLIA

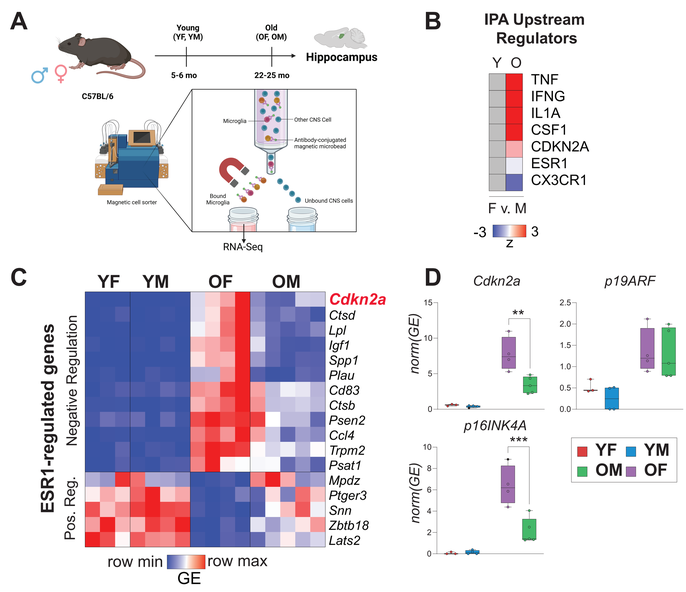


Figure 2. Sex differences in the hippocampal microglial transcriptome. **(A)** Hippocampus was collected from young (5-6 mo) and old (22-25 mo) mice of both sexes. A single cell suspension was created using enzymatic and mechanical dissociation with transcription and translation inhibitors to prevent *ex vivo* activational confounds. Microglia were labelled with CD11b magnetic beads and sorted using magnetic-activated cell sorting (MACS). Microglial RNA was isolated and processed for RNA-Seq analysis. **(B)** Heatmap visualizing IPA upstream regulators using genes differentially expressed by sex in the young and old groups. **(C)** Heatmap visualizing enrichment of ESR1-regulated genes, including *Cdkn2a*, a gene involved in cellular senescence. (D) *Cdkn2a* transcript analysis for *p19ARF and p16INK4A* variants. Boxplots are represented as normalized gene expression levels (n=3-5/group; Two-Way ANOVA, Tukey's post-hoc *p<0.05, **p<0.01, ***p<0.001)

DISTRIBUTION OF HORMONE RECEPTORS

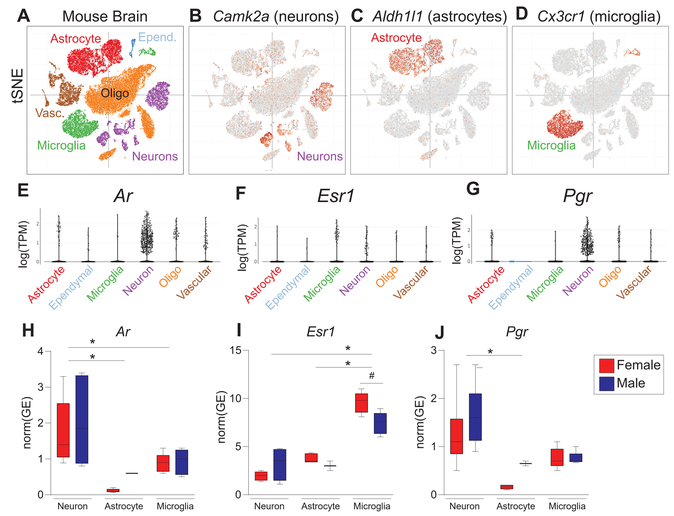


Figure 3. Distribution of sex steroid receptors across cell types in the mouse brain. Single cell data (A-G) obtained from the Broad Institute Single Cell Portal "Atlas of the Aging Mouse Brain" (Ximerakis et al., 2019) **(A)** tSNE plot of single cell RNA-seq data from C57BL6/J male mouse brain. **(B)** Expression of *Camk2a* is localized to the neuronal clusters. **(C)** Expression of *Aldh1l1* is localized to the astrocytic cluster. **(D)** Expression of *Cx3cr1* is localized to the microglial cluster. **(E-G)** Violin plots of sex steroid receptor genes: Androgen receptor (Ar), Estrogen receptor alpha (Esr1), and Progesterone receptor (Pgr). Expression in single cells is plotted as log(TPM). **(H-J)** Hippocampus from Camk2a-NuTRAP (neuron), Aldh1l1-NuTRAP (astrocyte), and Cx3cr1-NuTRAP (microglia) mice of both sexes were isolated and processed for TRAP-RNA-Seq (n=2-6/group). Boxplots of Ar, Esr1, and Pgr are represented as normalized gene expression. Two-way ANOVA was performed and main/simple effects were assessed (*p<0.05, main effect cell type, #p<0.05, simple effect sex).

CLUSTERING ANALYSIS OF HIPPOCAMPAL MICROGLIA

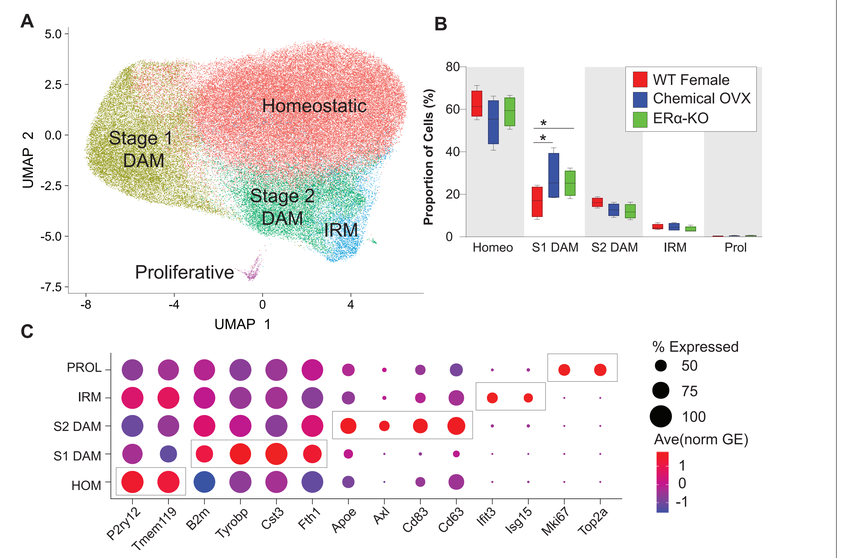
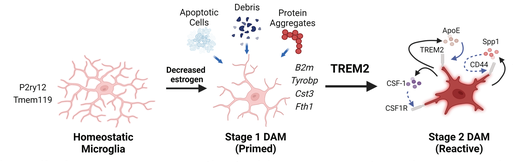


Figure 4. scRNA-seq clustering analysis of hippocampal microglia. **(A)** tsNE plot visualizing microglial single cells from the female mice hippocampus. **(B)** Representation of the expression of various microglial phenotypes. Proportion of cells within each cluster for the three experimental groups: WT, Chemical OVX, ERα-KO. Boxplots represent the proportion of cells in each cluster (%) (n=4/group) (Two-way ANOVA, BHMTC, FDR<0.2). **(C)** Dot plot visualizing the expression of known microglial cell marker genes.

CONCLUSIONS & FUTURE DIRECTIONS



-Hippocampal microglia become more activated in response to aging, especially in females. Pro-inflammatory regulators and senescent marker genes were upregulated in aged females.

-Ablation of endogenous estrogen production or ERα led to increased expression of stage 1 DAM, which may be associated with female-biased neuroinflammation. ERα-mediated estrogen signaling pathway in hippocampal microglia might be an appropriate therapeutic target for improving sex- and age-related, systemic inflammation.

-Future studies will test the interactive effects of sex chromosomes and sex hormones on sex-biased, systemic aging phenotypes, such as Alzheimer's disease.

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