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Supplemental Information

Dynamic Neuroimmune Profile during Mid-life Aging in the Female Brain and Implications for Alzheimer Risk

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Supplemental Information

Supplemental Tables

Table S1. P-value of significant fold changes in gene expression, related to Figure 1

	Gene name	Reg 9m		Irreg 9m		Acyc 9m		Acyc 15m	
		p-value	padj	p-value	padj	p-value	padj	p-value	padj
Microglial reactivity	Aif1	5.71E-04							
	Apoe	1.98E-02							
	Cd68	2.39E-03		4.70E-02		2.33E-03		2.51E-03	
	Cd200					3.02E-02			
	Fscn1			1.64E-04	4.96E-03				
	Itgb2	1.74E-03		4.66E-02		1.45E-04	8.52E-03	5.31E-03	
	Nlrp3			1.53E-03	1.94E-02	2.51E-03			
	Sirpa			9.96E-05	3.64E-03				
	Tgfa	6.33E-04		3.90E-03	3.58E-02			7.04E-03	
	Trem2	2.71E-04							
	Tyrobp	2.55E-04							
Complement	C1qa	6.25E-04				3.47E-02		3.68E-04	1.34E-02
	C1qb	1.67E-02							
	C3							3.65E-02	
	C3ar1								
	C4a	1.09E-06	1.48E-02	3.29E-02		6.76E-06	9.83E-04	5.41E-05	3.63E-03
	C4b	1.19E-02		3.32E-03	3.20E-02	5.35E-04		7.02E-03	
Lipid metabolism	Vegfa			3.67E-02					
	Siglec1			1.02E-03	1.51E-02	4.33E-02			
	Abca1	2.13E-03		9.92E-05	3.64E-03	1.29E-02		6.35E-03	
	Abca3			1.99E-02					
	Abca7	3.23E-02		1.17E-02		8.36E-04	2.51E-02	5.06E-04	1.66E-02
	Ide			3.64E-03	3.40E-02	1.61E-02			
	Nos3			3.54E-02					
	Pla2g10			3.07E-02					
	Pla2g15			3.77E-02					
	Pla2g4b			6.54E-03		7.74E-04	2.42E-02	1.02E-04	5.76E-03
	Sox10	5.56E-03							
	Sox13	2.39E-03		4.26E-02					
TGF-beta signaling	Acvrl1			4.84E-02		4.12E-03		4.61E-02	
	Bmpr1b			3.80E-04	8.21E-03	2.39E-03	4.70E-02	1.69E-02	
	Chrd			1.89E-02		4.17E-04	1.63E-02	5.72E-05	3.76E-03
	Mapk3			2.61E-03	2.75E-02				
	Nog			2.17E-03	2.41E-02	1.38E-02		9.78E-03	
	Ppp2r1a			2.46E-04	6.40E-03	3.45E-02			
	Ppp2r2b			1.00E-02					
	Rbx1			3.60E-04	7.97E-03	3.74E-08	3.29E-05	3.36E-08	2.13E-05
	Rps6kb1			2.16E-03	2.40E-02				
	Rps6kb2			2.51E-02		3.85E-02			
	Skp1			6.88E-03		6.58E-06	9.83E-04	7.03E-06	8.91E-04
	Smad5			6.02E-03	4.79E-02	2.90E-02			
	Sp1			3.12E-04	7.31E-03				
	Tgfb3			5.74E-03	4.65E-02				
	RT1-A1	4.72E-02							

MHC-I	RT1-CE10	2.10E-02						2.87E-03	
	RT1-CE4							1.36E-02	
	RT1-CE7							1.11E-02	
	RT1-T24-2	2.20E-02		2.81E-02					
	RT1-T24-3	3.05E-04		1.38E-03	1.82E-02	6.15E-03			
	RT1-M3-1			3.45E-02				9.31E-04	2.46E-02
MHC-II	RT1-Ba								
	RT1-Bb					3.44E-02		4.60E-02	
	RT1-Da								
	RT1-Db1								
	RT1-Db2							3.95E-02	
	RT1-DMb	1.99E-02							
TNF Signaling	Tnfaip3								
	Apaf1								
	Fos								
	Jun			2.02E-02		4.60E-02			
	Casp9								
	Casp2								
	Cycs			8.83E-03		5.67E-05		4.54E-02	
	Fadd								
	Map4k2			5.56E-04	1.03E-02	3.04E-06	5.77E-04	8.93E-06	1.05E-03
	Tank								
	Mapk8								
	Madd					3.40E-02		4.67E-02	
	LOC100912399			6.67E-04	1.15E-02				
	Map2k4								
	Map3k14								
	Casp8								
	Ripk1								
	Bid								
	Tnf								
	Tnfrsf1a								
	Tradd								
	Traf2								
Type I & II Interferon Response	B2m	5.23E-03		1.39E-02					
	Casp4			6.76E-03					
	Cfb	3.20E-03		1.85E-03					
	Cfh			9.69E-03					
	Ddx60			3.20E-02					
	Elf1			7.10E-03					
	Gbp2			2.95E-02					
	Gbp4			4.14E-02					
	Herc6	1.26E-02		3.03E-05	1.83E-03	5.22E-03			
	Ifi44	1.10E-02		3.95E-03	3.61E-02				
	Ifih1	1.02E-02		8.06E-04	1.30E-02	3.84E-02		8.57E-03	
	Ifit2			1.68E-02					

	Ifitm3	4.13E-05	4.57E-02	4.45E-06	5.11E-04	4.05E-03			
	Ifnar2			1.19E-02				1.29E-02	
	Il15ra			3.51E-02				3.33E-02	
	IL18BP			1.87E-02				3.62E-02	
	Irf1	5.97E-03		2.02E-02				5.44E-03	
	Irf2								
	Irf4	7.85E-03		4.16E-02					
	Irf7	6.95E-03							
	Itgb7								
	Lap3	8.19E-03		5.46E-03	4.51E-02			1.95E-05	1.94E-03
	Lpar6			1.38E-02					
	Ly6e			2.92E-04	7.01E-03	4.72E-02			
	Mov10			1.59E-02		4.99E-02			
	Mvp	1.72E-02		2.99E-02					
	Nampt			1.54E-02					
	Ncoa3			1.31E-02		3.25E-04			
	Nfkb1	1.56E-02				4.92E-02		3.20E-02	
	Nlrc5					3.58E-02		1.99E-02	
	Nmi	3.25E-02		3.79E-02					
	Nup93			3.62E-05		6.45E-04	2.13E-02	7.04E-04	2.06E-02
	Ogfr			5.52E-04	1.03E-02				
	P2ry14			1.22E-02					
	Parp9			1.29E-02		1.78E-02			
	Parp14	2.85E-02		2.35E-02					
	Plscr1	6.99E-03		1.98E-04	5.54E-03			2.66E-03	
	Ripk2			3.71E-03	3.46E-02				
	Rnf213	3.90E-03		4.24E-06		1.38E-04	8.31E-03	4.98E-04	1.64E-02
	Rsad2	2.22E-02		3.53E-03	3.34E-02	3.61E-02			
	Smad9			1.06E-02				1.50E-02	
	Sp110	9.03E-03		6.24E-03	4.92E-02	1.81E-02			
	Sspn	3.58E-02							
	St3gal5			1.96E-03				4.39E-02	
	Tnfaip6	9.81E-03							
	Trafd1			2.94E-03	2.99E-02			4.16E-03	
	Txnip			1.85E-02				2.03E-02	
	Uba7			9.39E-03		1.04E-02			
	Usp18	1.31E-02		2.97E-02					
	Vcam1	2.04E-02		9.03E-04	1.40E-02				
T cell	Cd3d								
	Cd3e								
	Cd3eap								
	Cd3g								
	Cd4							2.20E-04	9.65E-03
	Cd40								
	Cd40lg								
	Cd69								
	Socs1								
	Socs3								
	Mmp12								
	Cxcl9								
	Icam1							1.68E-02	

Table 1: P-value and adjusted-p value (padj) <0.05 of fold changes in hippocampal differential gene expression analysis of Reg 9m, Irreg 9m, Acyc 9m and Acyc 15m with respect to Reg 6m for heatmap analysis in Figure 1. Pathway analysis focused on in microglial reactivity, complement, lipid metabolism, Transforming Growth Factor -β (TGF-β) signaling, Major Histocompatibility Complex (MHC) class I and class II, Tumor Necrosis Factor (TNF) signaling, type I and type II interferon signaling and T cell markers and signaling with respect to Reg 6m

Table S2. P-value of significant fold changes in gene expression with respect to Sham, related to Figure 5

	Gene names	OVX	E2 Prevention	E2 Treatment
Type I & II Interferon Response	Alox15	8.17E-04		
	B2m	4.35E-02		
	Casp1	4.74E-02		
	Casp4	4.55E-02		
	Fcer1g	6.49E-03		
	Ifit3	4.36E-02		2.14E-02
	Ifngr1	2.98E-02		7.14E-03
	Irf7	9.94E-03		
	Mx1	3.31E-03		
	RT1-A2	2.30E-02		3.08E-03
	RT1-CE9-ps1	2.00E-02		1.70E-02
	RT1-M10-2-ps	2.06E-02		
	RT1-T24-4	1.87E-04		
	Slc25a28	2.64E-02		
	Tlr7	2.70E-03	2.60E-02	1.47E-02
	Tnfaip2	1.16E-02		
	Usp18	1.54E-02		
Inflammation, myelin metabolism and neuronal markers	Abca3	1.27E-02		4.69E-02
	C1s	2.66E-02		7.34E-03
	C4a	3.29E-03		
	C4b	1.66E-02		
	Pla2g1b	4.08E-02		
	Map2	1.67E-03		
	Trem2	2.39E-02		

Table 2. P-value<0.05 of fold changes in hippocampal differential gene expression analysis of genes involved in type I and II interferon response genes and genes participating in inflammation, myelin metabolism and neuronal markers, with respect to Sham (OVX: ovariectomized, E2: 17-β-estradiol).

Table S3. Age and hippocampal sample information used for female aging analysis from the GSE11882 dataset, related to Figure 6

Sex	Age group	Accession number	Age (years)
Female	20-34 years	GSM300219	34
		GSM300272	26
		GSM300298	30
	35-59 years	GSM300187	45
		GSM300231	37
		GSM300290	44
		GSM300294	48
		GSM300321	47
	60-75 years	GSM300190	74
		GSM300197	74
		GSM300223	74
		GSM300239	70
		GSM300243	64

Table S4. Age and hippocampal sample information used for male aging analysis from the GSE11882 dataset, related to Figure 6

Sex	Age group	Accession number	Age (years)
Male	20-34 years	GSM300276	20
		GSM300280	20
		GSM300301	20
		GSM300305	33
		GSM300309	22
	35-59 years	GSM300262	52
		GSM300313	42
		GSM300317	45
		GSM300174	45
	60-75 years	GSM300255	69
		GSM300286	69
		GSM300325	69
		GSM300333	75

Transparent Methods

Animals

All animal studies and procedures were conducted using the National Institutes of Health guidelines for procedures on laboratory animals. The procedures were approved by the University of Southern California and University of Arizona Institutional Care and Use Committee. Rats were housed in a facility with 12h light/dark cycle and food and water was supplied *ad libitum*.

Wild-type Sprague Dawley female rats of the ages of 5 months and 8 months were procured from Envigo laboratories (New Jersey, NJ, US). To establish the perimenopausal animal model used in this study (Yin et al., 2015), the animals were characterized for their reproductive cyclicity for a month using vaginal lavages conducted daily between 9 am and 11 am one week after they arrived(Bacon et al., 2019; Wang et al., 2020; Yin et al., 2015) . The vaginal lavages were fixed using 95% alcohol and stained using Giemsa stain for characterization of the cell types. A typical reproductive cycle of the female rat is defined by: Estrus (E) phase signified by large cornified cells, Metestrus (M) phase, which is marked by leukocytes, cornified cells and epithelial cells, Diestrus (D) phase marked by leukocytes and Proestrus (P) phase which can be identified by nucleated epithelial cells. During reproductively competent phases rats' cycle through the four phases (E, M, P and D) in 4-5 days, which is referred to as Regular cycling. Rats were enrolled into Regular 6-months (Reg 6m) or Regular 9-10-months (Reg 9m) group, if they had at least two consecutive regular cycles by the time of dissection. During the ages of 9-10 months, when the rats begin to transition to a reproductively incompetent phase the lengths of their cycles increase to 6-9 days, which is referred to as the reproductively irregular phase. Rats were enrolled into the Irregular 9-10-months (Irreg 9m) group if they had at least two consecutive irregular cycles. The onset of a reproductively senescent phase is established by 10-12-day long cycle usually composed of constant estrus, which is referred to as acyclic. Animals were enrolled into the Acyclic 9-10 months (Acyclic 9m) if they had been in constant estrus for 10 days or more. Animals that did not meet these criteria were aged further to 15-16 months. Animals were reproductively monitored for two weeks, and only animals that were constant estrus were used for the reproductively senescent group: Acyclic 15-16 months (Acyclic 15m). Animals were euthanized on the day of estrus to eliminate the confounding effects of the estrus cycle. The Reg 6m and Acyclic 15m groups were

used for studying the chronological aging phase preceding and succeeding the endocrinological transition phase. Comparison between Reg 6m and Reg 9m indicates early chronological aging group, whereas the comparison between Acyc 9m and Acyc 16m group indicates late-chronological aging phase. For understanding the effects of endocrinological aging without the confounding effects of aging the Reg 9m, Irreg 9m and Acyc 9m groups were used. Serum and cortical 17-β-estradiol ((8*R*,9*S*,13*S*,14*S*,17*S*)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol) (E2) and progesterone ((8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-17-acetyl-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one) (P4) levels of the perimenopausal animal model were established in a previous study (Yin et al., 2015). The steroidal concentrations were measured using LC/MS-MS. Cortical E2 levels were highest in the Reg 6m group, significantly reduced on early chronological aging (Reg 9m) and became almost negligent in Acyc 9m and Acyc 16m groups. Serum E2 levels were highest in the Reg 9m group, and steadily declined on endocrinological and late-chronological aging and were lowest in the Acyc 16m group. Interestingly, serum E2 levels increased with early chronological aging. Cortical P4 levels were highest in the Reg 6m group, and declined with early chronological and endocrinological aging, Serum P4 levels were highest in the Irreg 9m group. A significant correlation between cortical and serum P4 levels was observed. For RNA-Seq N=6/group was used.

Ovariectomy, estradiol treatment and prevention:

A total of 40 rats were used for this experiment. Ovariectomy (OVX) or sham (SHAM) surgery was conducted on 6-month-old Sprague Dawley rats. A subset of ovariectomized rats were enrolled in the 17-β-estradiol ((8*R*,9*S*,13*S*,14*S*,17*S*)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol) (E2) treatment (E2 Treatment) group. The treatment paradigm started 2 weeks after the surgery and included 3 weeks of estradiol treatment. Another subset of rats was enrolled in the estradiol prevention (E2 Prevention) group. The prevention paradigm started the day after the surgery and included 5 weeks of estradiol treatment. In the both the groups, 0.8 mg of E2 (10% E2 in cholesterol) was administered subcutaneously using silastic tubes (1.57mm inner diameter and 1cm length) and replaced every 30 days. Ovariectomized animals received cholesterol as vehicle treatment. Administration of treatment was based on a previously conducted study(Barron et al., 2015). Previous

pharmacokinetic studies have established that the serum estradiol concentration, with the dose of E2 administered, is around 40 pg/ml after 10 days of onset of treatment (Mannino et al., 2005). The serum estradiol levels match that of the proestrus stage of the reproductive cycle (Smith et al., 1975).Ovariectomized and sham animals were aged up to 5 weeks after the surgery. Animals used (N)= 5-7 animals/group.

Brain Dissection

Animals were anesthetized using intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). After a midline incision and lateral separation of the cranium, the whole brain was harvested from the skull. The brain was rapidly dissected on ice using a procedure previously described (Yin et al., 2015). Briefly, the meninges were peeled off following which the hypothalamus, cerebellum and brain stem were removed sequentially. The two hemispheres of the brain were separated. The cortex was peeled laterally, revealing the hippocampus which was rolled out. All the harvested brain regions were frozen on dry ice and stored at -80°C for further processing.

RNA extraction

The left hippocampus was cryopulverized and aliquoted for further processing. The tissue was homogenized in TRIzol™reagent (Invitrogen™, cat# 15596026) using 0.5 mL of regent per 20-30 mg of tissue. The tissue was homogenized using Bullet Blender™ and RNAase-free silicon beads for 3-5 mins at speed 6. The homogenized tissue was incubated with TRIzol™reagent for 5-7 minutes at RT. Chloroform was added to extract RNA, using a 1:5 ratio of chloroform: TRIzol™ reagent, and vigorously mixed. The mixture was centrifuged at 12,000 *g*, for 15 minutes at 4°C. The upper chloroform phase was separated and further purified using the PureLink® RNA mini kit (Invitrogen™, cat# 12185010) using the manufacturer's protocol. RNA was eluted using UltraPure™ water (Invitrogen™, cat# 10977015). RNA concentration and ratios to estimate RNA integrity, was measured on NanoDrop™ One (Thermo Scientific™,cat# ND-ONE-W).

RNA Sequencing (RNA-Seq)

For conducting unbiased discovery-based assessment of differentially expressed genes during female aging, RNA-Seq was conducted at Active Motif (Active Motif, Inc.) for the perimenopausal animal model and Vanderbilt Technologies for Advanced Genomics (VANTAGE), Vanderbilt University for the ovariectomy, estradiol prevention and estradiol treatment experiment. Quality control was performed on the RNA samples, and samples with RNA Integrity Index (RIN) > 8 were used for further processing. Enrichment of poly A tailed RNA (m-RNA and some long non coding RNA) and cDNA library preparation was conducted using stranded mRNA (poly A selected) sample prep kit. Sequencing was conducted on Illumina HiSeq 2500 at 50 bp paired-end for the perimenopausal animal model and NovaSeq6000 at 100 bp paired-end for the ovariectomy, estradiol prevention and estradiol treatment experiment. Demultiplexed FASTQ files were developed containing reads on average at 50 million reads/samples for the perimenopausal animal model and 30 million reads/sample for the ovariectomy, estradiol prevention and estradiol treatment samples. The FASTQ files were mapped to cDNA library of the rat genome (Ensemble release 95) to retrieve the count information using Salmon (Patro et al., 2016). To generate counts table from the Salmon output TIxmportV.16.0 was used (Soneson et al., 2015) and DeSEQ2 (Love et al., 2014) was utilized to generate differentially expressed gene list comprised of normalized read counts for each gene/transcript (DEG). Using the normalized counts, inter-sample variability was evaluated by conducting Pearson's correlation in R to identify outliers. Fold changes were established by computing the ratio of the experimental group's average normalized read count versus the control group's average normalized read count. P-values were corrected using the Benjamini and Hochberg False Discovery Rate, total number of significantly DEGs with p-adjusted values less than 0.05 were determined.

Ingenuity pathway analysis (IPA)

IPA was used to conduct discovery-based assessment of the transcriptomic changes between female aging groups. DEG (gene id, p-values, false discovery rate and log expression fold change) files developed from RNA-Seq were uploaded into IPA and only DEGs that had a p-value less than 0.05 were considered for assessment using core analysis. Top canonical pathways and predicted activation and inhibition of upstream regulators generated on the basis of the log expression fold change of the selected genes were used to guide further analysis. Gene lists were defined based on the canonical pathways most pertinent to

the biology of the central nervous system and that have been implicated in the age-related neurodegenerative disorders, especially Alzheimer's disease were investigated(Deczkowska et al., 2018; Heneka et al., 2015; Keren-Shaul et al., 2017; Masuda et al., 2020; Mathys et al., 2017; Von Bernhardi et al., 2015). Comparison analysis was conducted to identify systems most affected by female aging.

Heatmap analysis

Fold changes were computed relative to Reg 6m group and analyzed using heatmap function using Morpheus (https://software.broadinstitute.org/morpheus). For the ovariectomy, estradiol treatment and estradiol prevention, the Sham group was used as the control.

Single tube quantitative Real Time-PCR

To further corroborate findings from the RNA-seq and pathway analysis, single-tube PCR was conducted on select targets. Single-tube PCR was conducted using TaqMan® probes (Thermo Fisher Scientific) for specific targets: MHC-II (*Rt1-Db*: Rn01429350_m1, *Rt1-Da*: Rn01427980_m1, *Rt1-Ba*: Rn01428452_m1, *Rt1-Bb*: Rn01429090_g1). A total of 25 ng of m-RNA was used to convert to c-DNA. Further amplification was done on Applied Biosystems QuantStudio™12K Flex. Ct values generated were converted to ΔCt, on normalization with β- actin Ct values. $\Delta\Delta$Ct values for each sample were generated by normalization with mean values from Reg 6m group. Fold changes with respect to Reg 6m group were generated using $-2^{\Delta\Delta Ct}$.

Tissue sectioning & Immunohistochemistry

Animals were anesthetized after an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Animals were transcardially perfused with phosphobuffered saline (PBS) for 5 minutes and then perfused-fixed with 4% paraformaldehyde (FujiFilm Wako Pure Chemical corporation, cat# 163-20145) for 15-20 minutes, until the peripheral limbs stiffened. A midline incision was made on the cranium and whole brain was harvested from the skull. Meninges were peeled and the brain was immersion fixed in 4% paraformaldehyde overnight, then washed and stored in PBS overnight at 4°C. To cryopreserve the brains were transferred to a 20% sucrose solution (in PBS) for 2 days at 4°C to remove the excess water. The

brains were then stored in PBS at 4°C until sectioned. The brains were embedded in gelatin using the MultiBrain® Technology (Neuroscience Associates, Knoxville, TN) and were coronally sectioned into 40μ thick sections. The sections were immunostained with microglial marker Iba-I (FujiFilm Wako Pure Chemical corporation, cat# 019-19741, 1:500, 4°C, overnight) and reactivity marker MHC-II (Abcam, cat# ab23990, 1:500, 4°C, overnight) followed by secondary antibodies anti-rabbit Alexa Fluor 555 (viewed under Cy3) (Thermo fisher cat# A-21428,1:500, RT, 1 hr) and anti-mouse Alexa Fluor 488 (viewed under FITC)(Thermo fisher cat# A-11001,1:500, RT, 1 hr). Three-dimensional (3D) stacked fluorescent images were taken on Axiovert 200M Marianas Digital Microscopy Workstation, using Intelligent Imaging Innovation, SlideBook6 digital microscopy software (Denver, CO). The images were deconvoluted to the nearest neighbors and a projection was created. Extent of colocalization of the Cy3 and FITC channel was computed using the Pearson's correlation by defining a background region. For the corpus callosum including the cingulum, 7-9 10x images were obtained for each animal. For the Fimbria and Hippocampus CA2 region 3-6 10x images were taken per animal. For each group, average of correlation of the images was computed, and the standard error of mean was computed. P-values were calculated using one-way ANOVA followed by unpaired student t-test. N=3-6 animals/ group.

Adult brain dissociation

Animals of the age 6 month, 9-10 months and12 months were used for primary cell culture and assays. As mentioned earlier, animals from the age groups 6 months and 9-10 months were monitored for reproductive cyclicity for 1 month by vaginal lavages and enrolled into Reg 6m, Reg 9-10m, Irreg 9-10m or Acyc 9-10m groups by the definitions stated above. The 12-month group was not monitored for reproductive cyclicity, as the group was considered reproductively senescent due to age. Euthanasia of the animals was not subjective to the day of cycling in the estrus cycle, to minimize inter-day variability in the functional assays performed. Animals were sedated and the brain was harvested from the skull and kept in ice-cold D-PBS (Gibco™, cat# 14287072). The meninges were peeled off and cortical regions, hippocampi and the white matter were separated on ice. The brain was dissociated using the Miltenyi Biotec adult brain dissociation kit (cat# 130-107-677), using the manufacturer's protocol.

Microglia and astrocyte isolation

Using the MACS Miltenyi Biotec CD11b/c magnetic microbeads (cat# 130-105-634) for rat microglial cells, microglia from the single neural cell suspension generated from the adult brain dissociation kit were magnetically tagged. They were isolated using the manufacturer's protocol. Briefly, separation column was placed in a magnetic field and the magnetically tagged cell suspension was applied to the column. The column was washed and the flow through was collected. The column was removed from the magnetic field, and the microglia were eluted out. The cells collected from the flow through were resuspended in media containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco™, cat# 11039021) and 10% fetal bovine serum (ATCC, cat# 30-2020) and seeded in T25 flask (Thermo Scientific™, cat# 156367) coated with poly-d-lysine (37°C, 2-3 hours) to selectively culture astrocytes. Astrocytes were cultured until 80-90% confluency and were trypsinized and seeded for the metabolic flux assays. Isolated microglia were resuspended in DMEM/F-12 and 10% fetal bovine serum and seeded for metabolic flux assays.

Metabolic flux assays

Using the XFe24 flux analyzer, mitochondrial function was assessed by the measurement of oxygen consumption rate (OCR). Primary microglia and astrocytes cultured from 6-month and 12-month-old animals were seeded in XFe24 cell plates at the densities of 50,000 cells/well and 75,000 cells/well respectively. Assay design and method was developed from studies published in the literature (Irwin et al., 2011a; Irwin et al., 2011b; Orihuela et al., 2016; Sarkar et al., 2017). Microglia were cultured in the plate for 3 days after isolation and, astrocytes for 24 hrs. The day before the assay, the calibration plate was hydrated overnight in a non-CO_2 incubator at 37°C. On the day of the assay, the media was substituted to DMEM (Sigma Aldrich, cat# D5030), which was supplemented with 25mM glucose, 1mM sodium pyruvate and 2mM Glutamine (Gibco™, cat# 25030081). The pH of the medium was adjusted to 7.4 ± 0.05. Following the substitution in medium, the plates were incubated at 37°C in a non-CO_2 incubator for 1 hour. The assay includes baseline measurement of OCR, and serial injections of Oligomycin (1 μM for microglia, 4 μM for astrocytes) (MP Biomedicals, cat# 02151786), FCCP (1 μM for microglia, 2 μM for astrocytes) (Tocris Bioscience, cat# 0453) and rotenone/antimycin (0.5 μM for microglia, 1 μM for astrocytes) (Sigma Aldrich, cat# A-8674). Each injection was followed by the 3 measurements of OCR. This method is used to compute

the baseline respiration, maximal respiration, spare respiratory capacity, ATP production and proton leak. Each assay plate was normalized to protein content to reduce the effect of variances due to cell seeding, cell death or proliferation and a total of 6-8 wells/ group were used.

Measurement of oxidative stress and microglial reactivity

For measurement of microglial oxidative stress and microglial reactivity. MitoSOX™ (Invitrogen™, cat# M36008) staining was conducted by incubating the single cell suspension with 13 μM MitoSOX™ for 10 mins (37°C, non-CO_2 incubator), washed with PBS + 0.5% BSA. The cells were Fc Blocked using Anti-CD32 antibody (BD Pharmingen, cat# 550271) then immunostained with an antibody cocktail: CD11b (Miltenyi Biotec, cat# 130-105-273), CD45 (Miltenyi Biotec, cat# 130-111-774) and MHC-II (Miltenyi Biotec, cat# 130-108-776), for 30 mins on ice. Flow cytometry was conducted on MACSQuant Analyzer 10. Data was analyzed using Flowlogic™V7 (Miltenyi Biotec, cat# 150-000-381). Animals used per group were n =6-8/ group.

Phagocytic capacity assay

For measurement of phagocytic capacity, single cell suspension was incubated with pHrodo™ Red *S. aureus* Bioparticles™ conjugate (Invitrogen™, cat# A10010) at 37°C in a non-CO_2 incubator for 2 hours. Following which, the cell suspension was washed with PBS +0.5% BSA. The cells were immunostained with CD11b (Miltenyi Biotec, cat# 130-105-273), CD45 (Miltenyi Biotec, cat# 130-111-774). The cells were analyzed using MACSQuant Analyzer 10. Data was analyzed using Flowlogic™V7 (Miltenyi Biotec, cat# 150-000-381). Animals used per group were n =4-8/ group.

Gene Expression Omnibus (GEO) dataset analyses

To validate the gene expression data from the perimenopausal animal model, a clinical gene expression GEO dataset GSE11882 was used (Berchtold et al., 2008). Using GEO2R tool, the top 250 gene differentially expressed in the hippocampus were analyzed between 20-34 years (early aging), 35-59 years (mid-aging) and 60 -75 years (late-aging) in females and males (Table S1 & Table S2). Using the early aging group as a sex-matched control, fold changes during mid-aging and late-aging were computed using

GEO2R tool, using default settings, for males and females. Parameters used for analysis are false discovery rate, p-value and log fold change.

Statistical analyses

Statistical analysis was conducted using GraphPad Prism version 8.1. For statistical comparison, one-way ANOVA was conducted unless otherwise mentioned. Correction for multiple test was conducted by Tukey's. For the transcriptomic analyses the statistical comparisons are listed under the RNA-Seq section.

Graphical abstract

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