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Microglial sex differences in innate high anxiety and modulatory effects of minocycline

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ABSTRACT

Microglia modulate synaptic refinement in the central nervous system (CNS). We have previously shown that a mouse model with innate high anxiety-related behavior (HAB) displays higher CD68$^+$ microglia density in the key regions of anxiety circuits compared to mice with normal anxiety-related behavior (NAB) in males, and that minocycline treatment attenuated the enhanced anxiety of HAB male. Given that a higher prevalence of anxiety is widely reported in females compared to males, little is known concerning sex differences at the cellular level. Herein, we address this by analyzing microglia heterogeneity and function in the HAB and NAB brains of both sexes. Single-cell RNA sequencing revealed ten distinct microglia clusters varied by their frequency and gene expression profile. We report striking sex differences, especially in the major microglia clusters of HABs, indicating a higher expression of genes associated with phagocytosis and synaptic engulfment in the female compared to the male. On a functional level, we show that female HAB microglia engulfed a greater amount of hippocampal vGLUT1$^+$ excitatory synapses compared to the male. We moreover show that female HAB microglia engulfed more synaptosomes compared to the male HAB *in vitro*. Due to previously reported effects of minocycline on microglia, we finally administered oral minocycline to HABs of both sexes and showed a significant reduction in the engulfment of synapses by female HAB microglia. In parallel to our microglia-specific findings, we further showed an anxiolytic effect of minocycline on female HABs, which is complementary to our previous findings in the male HABs. Our study, therefore, identifies the altered function of synaptic engulfment by microglia as a potential avenue to target and resolve microglia heterogeneity in mice with innate high anxiety.

1. Introduction

Anxiety and depression are among the most common mental disorders in the world, and share up to 80 % of comorbidity (Klenk et al., 2011; Strand et al., 2021). Numerous studies have shown that females are more prone to anxiety-related disorders than males (Kessler et al., 2012; Strand et al., 2021; McLean et al., 2011; Moser et al., 2016) suggesting a potential moderating role of sex. However, cellular and molecular processes underlying these sex differences remain elusive.

Both stress and genetic predisposition have been recognized as significant risk factors for anxiety and/or comorbid depression (Weger and Sandi, 2018). The majority of clinical studies, which are primarily limited to peripheral cytokine levels, indicate an association between anxiety-related disorders and immune dysregulation (Passos et al., 2015; Rao et al., 2015; Fontenelle et al., 2012; Hou et al., 2017). Furthermore, rodents with stress-induced or innate high anxiety also

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exhibit dysregulations in neuroimmune systems, including an altered status of microglia (Rooney et al., 2020; Bollinger, 2021; Wohleb et al., 2018; Wang et al., 2018). Microglia, the resident macrophages and innate defensive system of the CNS, inhabit versatile functions that dynamically change in order to adapt to their surroundings (Kettenmann et al., 2011). As professional phagocytes, they sculpt neural circuits by contacting and engulfing synaptic components (Kettenmann et al., 2013; Schafer et al., 2012; Vainchtein and Molofsky, 2020; Vainchtein et al., 2018; Weinhard et al., 2018). This process is critical for normal brain development and synaptic plasticity (Furusawa and Emoto, 2020; Schafer et al., 2012; Paolicelli et al., 2011). Interestingly, altered brain connectivity has been reported in early innate anxiety (Kalin, 2017) and studies have indicated a link between synaptic pruning and anxiety-related behavior (Bolton et al., 2022; Socodato et al., 2020). Alterations in microglial density, reactivity and expression of markers associated to neuroimmune pathways have been reported in various rodent models of stress-induced anxiety (Kreisel et al., 2014; Liu et al., 2018). However, little attention has been given to innate high anxiety, especially regarding sex differences.

HAB (mice with innate high anxiety) represent a valuable model to study elevated anxiety that is not induced by specific stressors or immunological challenges. They intrinsically show higher anxiety and depressive-like behavior as an outcome of a selective breeding approach of CD-1 mice and exhibit high anxiety-related behavior compared to NAB (mice with normal anxiety), as evaluated through the elevated plus maze (EPM) and light–dark tests (LD) (Rooney et al., 2020; Sah et al., 2012). HAB mice have been reported to show altered basal neural transmission and long term potentiation (LTP) (Dine et al., 2015) in addition to neuroinflammatory alterations in the hippocampus (Salomé et al., 2004; Rooney et al., 2020). We have previously shown that HAB males have a higher density of microglial cells ($Iba1^+$ cells) as well as $CD68^+/Iba1^+$ microglia in the hippocampus than NAB males (Rooney et al., 2020). We also reported that minocycline, a broad-spectrum antibiotic, reduced both $Iba1^+$ and $CD68^+/Iba1^+$ microglia density in the hippocampus and had anxiolytic effects on the behavior of HAB males (Rooney et al., 2020). Various studies have also reported that minocycline dramatically reduces stress-induced anxiety in rodent models in addition to its anti-inflammatory effects (Wang et al., 2018; Levkovitz et al., 2015; Liu et al., 2018; Zhang et al., 2019). However, a great majority of these results including our prior findings (Rooney et al., 2020) have focused on male rodents, which leads to conclusions for both sexes based on the male-driven findings. Given that females show a higher prevalence of anxiety than males (Kessler et al., 2012), sex is a crucial biological variable that needs to be taken into account while investigating neurobiology of anxiety-related disorders. Additionally, the National Institute of Health has recognized the importance of sex in scientific knowledge and demands to include representation of both sexes among other variables influencing physiological processes.

Considering the alterations in neural connectivity, neural transmission and neuroinflammatory markers found in the context of anxiety (Dine et al., 2015; Salomé et al., 2004; Rooney et al., 2020), a potential role of microglia dysfunction in synaptic engulfment is yet to be investigated. Furthermore, multiple studies have reported the role of microglia heterogeneity in modulating their physiological functions and homeostasis (De Biase and Bonci, 2019; Silvin and Ginhoux, 2018; Hanisch, 2013; Tan et al., 2020), which has not been addressed yet in the context of either state- or trait- anxiety.

In the present study; we report heterogeneity and sex differences of microglia at single-cell resolution using mice with innate high anxiety. We report sex-associated differences in the transcriptional profile of microglia, and further provide evidence of altered microglial engulfment of synapses in the HAB female hippocampus. We further show the modulatory effects of minocycline treatment on this functional phenotype as well as on anxiety-related behavior. Our study reports a thorough characterization of microglia at transcriptional level and reveals sex-related differences in microglial engulfment of synapses at functional level along with modulatory effects of minocycline.

2. Material & Methods

2.1. Animals

All mice of both sexes used for the present study were on a CD-1 genetic background. All procedures including the handling of living animals were performed in strict accordance with the German Animal Protection Law and were approved by the Regional Office for Health and Social Services in Berlin (Landesamt für Gesundheit und Soziales, Berlin, Germany) under the license number G0061/21. The regulations followed the rules set in the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (revised 2019).

HAB and NAB mice were selectively inbred for their specific anxiety-related behavior at the Department of Pharmacology, Innsbruck Medical University, Innsbruck (Austria). The mice were group-housed in ventilated cages under standard laboratory conditions with a 12:12 light/dark cycle at the animal core facility of the MDC. Food and water were provided ad libitum. All mice used in the experiments were at post-natal day 84–91 (P84-91). Mice were sacrificed with an overdose of pentobarbital (Narcoren, Merial GmbH, Hallbergmoos, Germany) with intraperitoneal injection followed by decapitation.

2.2. Microglia isolation

Enzymatic dissociation of microglia was performed as previously described (Marsh et al., 2022) with minor alterations. Briefly, mice were deeply anesthetized and intracardially perfused with 15 ml ice-cold artificial cerebrospinal fluid (aCSF) (87 mM NaCl, 2.5 mM KCl, 1.25 mM $NaH2PO_4$, 26 mM $NaHCO_3$, 25 mM glucose, 1 mM $CaCl_2$, 7 mM $MgSO_4$, 20 mM HEPES) with a transcriptional & translational inhibitor cocktail, as previously described in detail (Marsh et al., 2022). Brains were removed out of skull and kept in ice cold Hibernate-A medium supplemented with B27 (Thermo Fisher Scientific, Waltham, USA, #A1247501, 1/50). Brains were quickly sliced into 8 even sections using surgical scissors, and placed into Miltenyi gentleMACS C Tubes (Miltenyi, Bergisch Gladbach, Germany, #130–093-237) including papain solution (Worthington Biochemical, Lakewood, NJ, USA, #LK003150) and the inhibitor cocktail (Marsh et al., 2022) for tissue dissociation at 37 °C for 30 min. Purpose of using the inhibitor cocktail was to prevent *ex vivo* microglial activation in response to the isolation procedure at the 37 °C (Marsh et al., 2022). Supplementary Fig. 1 shows that our isolation procedure did not trigger a major artificial *ex vivo* activation signature in the gene expression in the overall dataset, assessed by the expression of genes such as *Fos, Jun, Hsp1a* by microglia (Marsh et al., 2022). Samples were centrifuged for 5 min. at 300g and filtered through a 70-μm filter. 1 ml aCSF was added to each tube and cells were pelleted at 300g for 5 min (4 °C). Cell suspension including 22 % Percoll (GE Healthcare, Chicago, USA, catalog no. 17–0891-01) in 2 ml final volume was gently overlaid with 2 ml ice-cold calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, #J61196AP) and centrifuged for 10 min. at 3000 g with full acceleration and no breaks, as previously described in detail (Mattei et al., 2020). Upon a gentle myelin cloud removal, the pellets were washed once with aCSF, spun down at 400g for 5 min. and re-suspended in 100 μl DPBS.

2.3. Fluorescence-activated cell sorting (FACS)

All buffers and solutions were pre-chilled before use. Cell suspensions were incubated with a fixable viability dye (Thermo Fisher Scientific, #L34969, 1/1000 in PBS) for 30 min. at 4 °C. Upon washing with 1 ml ice cold FACS buffer (PBS 1X with 0.5 % BSA, 0.074 % EDTA), cells were pelleted at 300 g, 5 min. and incubated with anti-CD16/CD32 10 min. on ice (Thermo Fisher Scientific, #14–0161-82; 1/200 in FACS

buffer). Cells were next stained with antibody master mix (CD45-APC BD, # 559864; CD11b-PECy7 BD, #553311; CD44-BV421, BioLegend, San Diego, USA, #103040, Lineage-PE: Ly6C, BioLegend, #128026; Ly6G, BioLegend, #127606; B220, BioLegend, #103206; TCRb, BD Biosciences, New Jersey, USA, #553170; TCRgd, BioLegend, #118105; NK1.1, BioLegend, #108705; Ter119, BD, # 557915) for 25 min. at 4 °C and spun down for 5 min. at 300 g. 250 µl FACS buffer was used per sample to resuspend the cells after the washing step. Sterile 1.5 ml Eppendorf tubes were precoated with 50 % BSA overnight at 4 °C to collect the sorted microglia in 750 µl sterile aCSF. The gating strategy to sort microglia is given in Supplementary Fig. 5. BD FACS Aria III with 100-µm nozzle and purity mode was used to sort the final cell population to be loaded in a 10X chromium controller.

2.4. Single-cell RNA sequencing (scRNA-seq) using 10X Genomics

The quality and concentration of single-cell preparations were assessed with a hemocytometer and Keyence BZ-X800 microscope (Keyence GmbH, Neu-Isenburg, Germany). 16,500 cells per sample were loaded into the 10X Chromium controller (10X Genomics, Pleasanton, CA, USA) and library preparation was conducted using 10X single-cell 3′ v3 protocol according to manufacturer's instructions (paired-end reads, R1 = 28, i7 = 8, R2 = 91). All 8 libraries, each representing an individual mouse, were submitted to Berlin Institute for Medical Systems Biology (BIMSB) sequencing core facility and sequenced with an Illumina NovaSeq sequencer to a depth of around 70,000 reads per cell according to the 10X Genomics's recommendations.

The gene-cell count matrix was constructed using CellRanger count version 2.0.2 using the Ensembl GRCm38.p5 (mm10) reference genome. The R Seurat package (Stuart et al., 2019; Butler et al., 2018) was used for the analysis of Single Cell RNA-Seq data. Only genes found in a minimum of 5 cells were included in the study. In addition, we eliminated from the analysis any cell with fewer than 200 identified genes, as well as any cell with a mitochondrial percentage greater than 20 %. Data were log normalized and scaled as a standard Seurat (Butler et al., 2018) preprocessing. Default parameters were used for identifying anchor genes between the data sets. Dimensionality reduction was performed using t-SNE by using 24 principal components which were decided by using JackStraw analysis and ElbowPlot. The standard Seurat workflow consisted of data normalization/scaling, PCA analysis, and t-SNE clustering. We also used scCustomize R package (Marsh, 2021) for visualizations provided in Fig. 1c, Fig. 2a, Fig. 3a and b. To compare clusters, marker genes were identified using the Wilcoxon Rank Sum test of the Seurat. Marker genes from previous single cell studies (Masuda et al., 2020; Zeis et al., el 2015; Hammond et al., 2019; Saunders et al., 018; Mattei et al., 2020) were used for manual annotation of the microglia clusters. Differential expression analysis was carried out using DESeq2 (Love et al., 2014) and MAST algorithms (Finak et al., 2015) of the Seurat R package using FindMarkers function (pAdj < 0.001). 52.363 cells were analyzed, including 2 replicates per group (4 groups; n = 8 samples in total). Number of cells sequenced per group: HAB female: 15.548 cells; HAB male cells: 13.997; NAB female: 9853 cells; NAB male: 12.965 cells). Code required to reproduce Seurat object used for analysis and plotting can be found at: https://github.com/bugursu/Microglia-Anxiety-project.

2.5. Flow cytometry analysis of microglia surface markers

Mice were intracardially perfused with 15 ml ice-cold DPBS. Hippocampi were dissected on a cooled, smooth glass platform and placed in ice-cold Hibernate-A medium (Thermo Fisher Scientific, #A1247501). Mechanical dissociation at 4 °C was carried out with a Dounce homogenizer, with 30 gentle strokes using a loose pestle. Myelin debris was removed via percoll gradient centrifugation as described previously in detail (Mattei et al., 2020). Total cell pellets were used for staining by using monoclonal anti-mouse antibodies: CD45 (BD Bioscience, Heidelberg, Germany, #559864, 1/100), CD11b (Thermo Fisher Scientific, #25–0112-82, 1/100), TREM-2 (R&D Systems, Wiesbaden, Germany, #FAB17291C, 1/200), CX3CR1 (BioLegend #149019, 1/100), CD16/CD32 (1/200, Thermo Fisher Scientific, #14–0161-82) for 30 min at 4 °C. Cells were immediately acquired on BD FACS Aria II and the mean fluorescence intensity (MFI) for TREM2, and CX3CR1 from $CD11b^{++}/CD45^{+}$ microglia were analyzed with FlowJo v10 (BD Bioscience). MFI for CD11b (for CR3) were analyzed from the $CX3CR1^{++}/CD45^{+}$ population. The gating strategy to define and analyze microglia is given in Supplementary Fig. 6.

2.6. Microglial engulfment of $vGLUT1^{+}$ synapses

For detection of $vGLUT1^{+}$ microglia by flow cytometry, a mechanical cell isolation strategy was used as described previously in detail (Mattei et al., 2020). Upon percoll gradient centrifugation, total cell pellets were stained first with fixable live/dead staining (Thermo Fisher Scientific, #L34969, 1/1000, for 30 min.) and followingly for the microglial surface markers CD45-APC (BD Biosciences #559864, 1/100), CD11b-PECy7 (Thermo Fisher Scientific, #25–0112-82, 1/100), Ly6C-FITC (BD Biosciences, #553104), Ly6G-FITC (BD Biosciences, #551460). Stained samples were fixed and permeabilized using the BD Cytofix/Cytoperm kit according to manufacturer's instructions (#554714). Following fixation, intracellular staining for vGLUT1-PE Synaptic marker (1/200, Miltenyi Biotech, Bergisch Gladbach, Germany, #130–120-764, 1 h in 1x BD Permeabilization Buffer; Isotype Control: Miltenyi Biotech, #130–113-450) was performed. Samples were acquired on FACS ARIA II (BD Biosciences) and the microglial population was gated as $CD11b^{++}$/ $CD45^{+}$/ $Ly6C^{-}$/ $Ly6G^{-}$/ viable cells. Spleen macrophages were used as an internal biological negative control to set a threshold to gate vGLUT1 positive microglia fraction, since use of isotype controls are not ideal to distinguish the positive and negative populations, especially following the intracellular staining protocols (Maecker and Trotter, 2006). Gating strategy to define microglia and analyze microglia-specific vGLUT1 signal is provided in Fig. 5b. Comparison of the vGLUT1 positive signal to the isotype controls and spleen is provided in Supplementary Fig. 7. Specificity of the vGLUT1 antibody was tested on glutamatergic neurons from the Vglut-IRES-Cre//ChR2-YFP mice and 97.9 % of the cell fraction was detected as vGLUT1 positive (Supplementary Fig. 8). Gating strategy to define spleen macrophages is provided in Supplementary Fig. 9. Raw data were analyzed with FlowJo v10 (BD Bioscience).

Through this assay, we successfully quantified the engulfment of $vGLUT1^{+}$ synapses by microglia. It is important to note that microglia exhibit approximately 95 % viability after the isolation procedure (Supplementary Fig. 7a), and retain their ability to engulf synaptic material *ex vivo*. After the isolation procedure, we fixed the cells prior to the intracellular vGLUT1 staining. As a result, we measured a combination of *in vivo* and a short-term *ex vivo* engulfment until the fixation via this assay, which we have referred to as *in vivo** throughout the manuscript. An alternative *in vivo* approach to quantify microglial engulfment of synapses would be immunohistochemistry (IHC), where the tissue integrity is not compromised.

2.7. Crude synaptosome isolation and pHrodo^{TM}Red labeling

Hippocampal synaptic terminals were freshly isolated for each experiment, using Syn-PER Synaptic Protein Extraction Reagent (Thermo Fisher Scientific, #87793) as recommended by the manufacturer. In brief, hippocampi from BL6 male and female mice were dissected on ice-cold glass platform and homogenized by using a Dounce tissue grinder by ~ 30 gentle strokes. The homogenate was centrifuged at 1200g for 10 min. and the pellet was discarded. The supernatant was centrifuged at 15,000g for 20 min. and the synaptosome pellet was resuspended in 0.1 M Na_2CO_3. 0.2 mM pHrodo^{TM}Red (Thermo Fisher Scientific, #P36600) was used to label synaptosomes at room

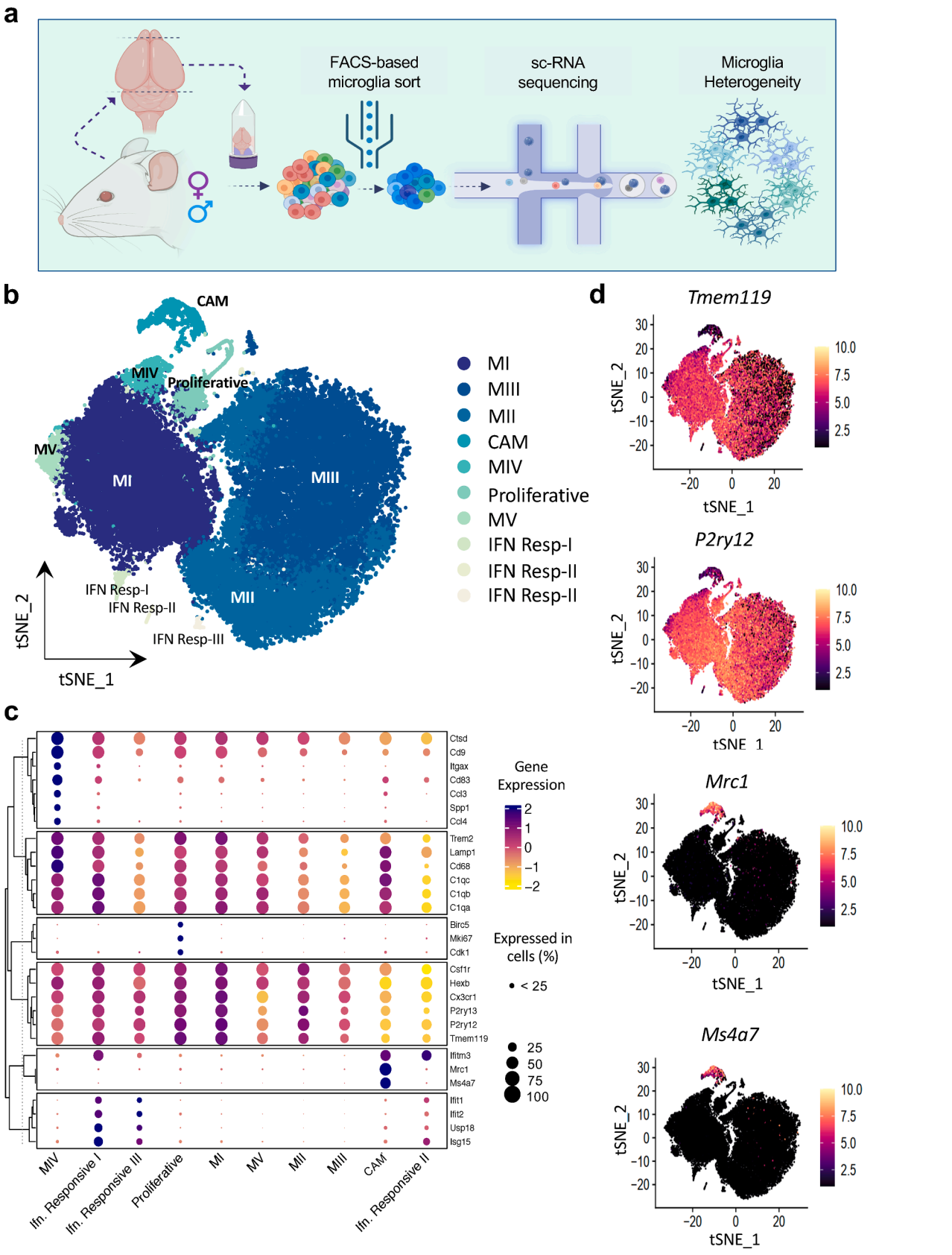

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Fig. 1. Single-cell RNA sequencing reveals nine microglia clusters diversified in their gene expression status in the HAB male and female brains. **a)** Experimental workflow for the single-cell RNA sequencing (Created by using BioRender.com). After the removal of brains, microglial cells were isolated by papain-based enzymatic dissociation and percoll gradient isolation, followed by FACS. The resulting cells were subjected to droplet-based separation and library preparation to perform single-cell RNA sequencing. **b)** t-distributed stochastic neighbor embedding (t-SNE) reveals 10 different clusters (9 microglia and 1 CNS-associated macrophages (CAM) in HAB and NAB brains of both sexes (n = 8, total 52.363 cells analyzed; HAB female: 15.548 cells; HAB male cells: 13.997; NAB female: 9853 cells; NAB male: 12.965 cells). **c)** Dot plot indicating the gene expression profile of different microglia clusters based on the expression of a selected set of genes indicating different microglia states. The colour scale codes the average gene expression level; purple tones indicate higher expression; and yellow tones indicate lower expression. The diameter of the circle codes for the percentage of cells expressing the gene. MI (homeostatic) cluster expresses high levels of microglia canonical marker genes (*Hexb, P2ry12, Tmem119*) and phagocytosis & synaptic pruning-associated genes (*Cd68, Trem2, Lamp1*). MII (intermediate) and MIII (pre-active) clusters show lower levels of *Tmem119, Hexb, P2ry12, P2ry13* as well as lower levels of phagocytosis & synaptic pruning-associated genes compared to the MI cluster. MII cluster is classified as an intermediate state of microglia, where the expression of homeostasis-related genes (*Hexb, Tmem119, P2ry12*) are lower than MI and higher than MIII clusters. M−IV cluster is related to the DAM signature, characterized by high expression of *Cd9, Spp1, Trem2 and Itgax*. It also shows low expression levels of *Tmem119, Cx3cr1, P2ry12, P2ry13, Hexb*. M−V cluster is at a similar state with the MIII cluster but shows relatively higher levels of the phagocytosis-associated genes such as *Cd68* and *Lamp1*, indicating a phagocytic/ pre-active state. The proliferative microglia cluster shows high expression of *Mki67, Birc5 and Cdk1* genes. Interferon (INF)-Responsive MI, MII and MIII clusters show high expression levels of *Ifit1,Ifit2,Isg15, Ifitm3*. The CAM cluster shows high expression of *Mrc1, Ms4a7 and Pf4* genes, distinguishing them from the rest of the microglia clusters. **d)** tSNE plots showing expression of microglial marker genes *Tmem119* and *P2ry12* to validate microglial identity of the cells in all the clusters. *Mrc1* and *Ms4a7* genes are highly expressed by the CAM population and distinguish them from the microglia clusters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

temperature for 1.5 h with gentle agitation. After seven times of washing with PBS to remove unbound excessive pHrodo^{TM}Red, synaptosomes were pelleted at full speed, 1 min. centrifugation and immediately snap frozen using liquid nitrogen.

2.8. In vitro minocycline treatment and synaptosome engulfment assay

Under deep anesthesia, 12 weeks old mice were transcardially perfused with DPBS. Hippocampi were dissected on a cooled glass platform and placed in ice-cold Hibernate-A medium supplemented with B27 (Thermo Fisher Scientific # A1247501). Enzymatic dissociation was carried out using Worthington's papain dissociation kit (Worthington Biochemical, #LK003150) based on the manufacturer's instructions. Myelin debris was removed via percoll gradient centrifugation by overlaying 2 ml PBS on cell suspension including 22,5 % Percoll in 2 ml final volume, and centrifuged for 10 min. at 3000 g with full acceleration and no breaks (Mattei et al., 2020). Myelin cloud and rest of the supernatant were gently removed. Pellets were washed in ice-cold MACS-buffer (DPBS, 2 % BSA) and stained with anti-mouse CD11b magnetic beads (1/10, Miltenyi Biotech, #130–049-601) at 4 °C for 20 min. Total cells were passed through MS MACS columns (Miltenyi Biotech, #130–042-201). The flow through was discarded and the cells were flushed out of the column using a plunger into 1 ml Dulbecco's Modified Eagle Medium (DMEM; Gibco^{TM}, # 41965039) and pelleted down by centrifugation at 300g, 6 min. Resulted pellets were resuspended in DMEM complete (10 % FBS and 0.1 % PenStrep) and seeded in 24-well culture plates. After 1 h at 37 °C, 20 μM minocycline supplemented into the wells apart from the control (untreated condition) wells and cultivated for 24 h or 48 h. After the respective cultivation times, medium was gently taken out and cells were supplied with 500 μl fresh DMEM per well, supplemented with 3 μg PhRodo-labeled synaptosomes per well and incubated for 2 h at 37 °C. Cells supplemented with same amount of unstained synaptosomes were used as a negative control. For analyses, cells were washed with DPBS 3 times, detached using trypsin (Thermo Fisher Scientific, #R001100) and resuspended in DPBS including fixable viability dye (Thermo Fisher Scientific, #L34969, 1/1000) and kept at 4 °C for 25 min. Afterwards, cells were resuspended in FACS buffer including CD16/CD32 (1/200, Thermo Fisher Scientific, #14–0161-82) and kept for 10 min. on ice, and followingly 1/100 CD11b, 1/100 CD45 antibodies were added and incubated at 4 °C for 20 min. After the incubation, cells were washed once and centrifuged at 300g for 5 min. The resulting pellet was dissolved in 200 μl FACS buffer. Data were acquired using BD Aria II and PhRodo-PE MFI was analyzed using FlowJo v10 (BD Bioscience).

2.9. Minocycline treatment

Minocycline dosage was chosen according to our previously published study (Rooney et al., 2020). Minocycline (Sigma-Aldrich, Missouri, USA, #M9511) was dissolved in tap water and was kept in light-protected drinking bottles for oral administration. Each mouse received an average minocycline dosage of 40 mg/kg/day by adjusting the concentrations in the bottles according to the mean bodyweight and daily drinking volume per cage, twice a week for 28 days. A randomized block design was used in assigning groups to minocycline/tap water treatment.

2.10. Elevated plus maze (EPM)

The baseline anxiety was assessed using the elevated plus maze (EPM) test. Drug naïve mice (between 7 and 9 weeks of age) were placed into a plus maze elevated 72 cm from the ground, consisting of two closed arms (30 cm x 5 cm) illuminated with red light, and two open arms (30 cm x 5 cm) illuminated with white light at 100 lx, as previously described (Sah et al., 2022). The test started by placing the mouse on the center platform (5 cm x 5 cm) facing a closed arm. The total test duration was 5 min.

2.11. Open field test

Following 4 weeks of oral minocycline treatment, the open field (OF) test was conducted. The arena consisted of an open box (41 x 41 x 41 cm) with a center compartment illuminated at 150 lx. Mice were placed in a corner of the open field arena and allowed to explore the full arena for 10 min.

2.12. Light dark test (LD)

2 days after the OF test and by using the mice still on minocycline treatment, the light/dark test was performed. Here, the open field box was used but divided into a light chamber illuminated at 300 lx, as well as a closed dark chamber illuminated at < 20 lx (black box is one third the size of the whole arena) and that was accessible through a small door (assigned as transition zone). In brief, mice were placed at the door of the dark chamber and, when it entered the dark chamber, they were allowed to explore the full arena for 10 min.

A recent study (Bosch et al., 2022) demonstrated that stress-induced modulation of anxiety-like behavior, as assessed by the EPM, was only observed during initial testing. Subsequent repetitions of the test resulted in the abolition of this behavioral effect. Repetative use of the same tests on the same mice would have little success since the primary mandate of novelty will be lost when the test is repeated (Prevot et al.,

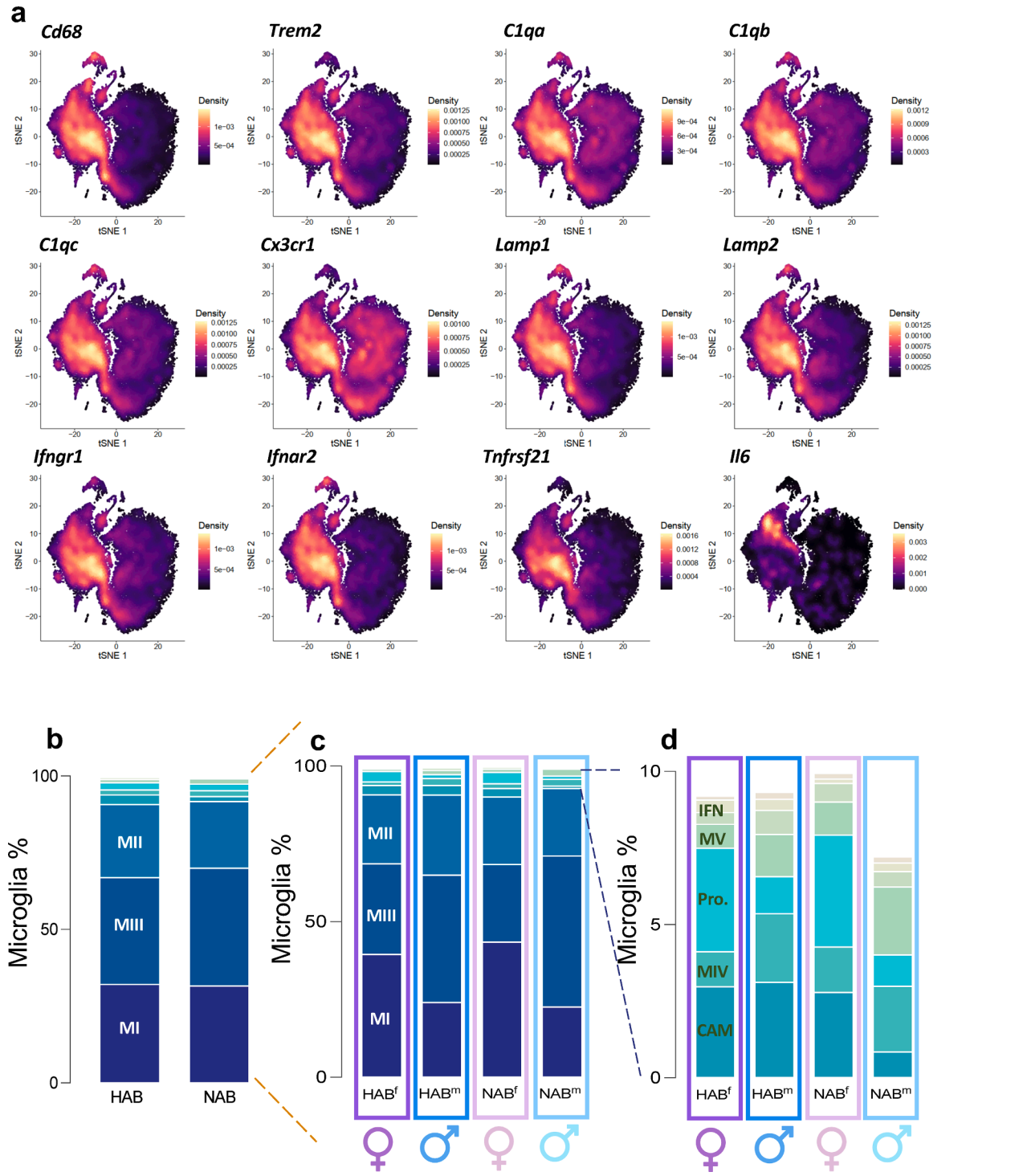

Fig. 2. MI cluster shows high expression of genes related to phagocytosis, synaptic pruning as well as interferon response. **a**) Density plots showing higher expression (orange) of genes associated with synaptic engulfment and phagocytosis such as *Cd68*, *Trem2*, *C1qc, Cx3cr1* in the MI cluster. Genes associated with interferon response such as *Ifngr1*, *Ifnar2* also show higher expression in the MI cluster. **b)** Main microglia clusters (MI-MII-MIII) indicate minor differences in their percentage between HAB and NAB brains. Distribution of MI, MII, MIII, CAM, MIV, Proliferative, MV, Interferon (IFN)-responsive I, IFN. Responsive II and IFN. Responsive III. in the HAB brain: 32.11 %, 34.78 %, 23.84 %, 3.03 %, 1.66 %, 2.35 %, 1.06 %, 0.57 %, 0.39 %, 0.17 %; NAB brain: 31.6 %, 38.33 %, 21.67 %, 1.67 %, 1.86 %, 2.16 %, 1.72 %, 0.54 %, 0.22 %, 0.19 %, respectively. **c and d)** Microglia clusters indicate varying percentages in HAB and NAB brains of male and females. Distribution of MI, MII, MIII, CAM, MIV, Proliferative, MV, Interferon (IFN)-responsive I, IFN. Responsive II and IFN. Responsive III. in HAB male: 23.94 %, 41.02 %, 25.71 %, 3.11 %, 2.23 %, 1.21 %, 1.37 %, 0.77 %, 0.37 %, 0.22 %; HAB female: 39.47 %, 29.16 %, 22.15 %, 2.97 %, 1.14 %, 3.37 %, 0.77 %, 0.38 %, 0.41 %, 0.12 %; NAB male: 22.61 %, 48.49 %, 21.67 %, 0.84 %, 2.14 %, 1.02 %, 2.21 %, 0.50 %, 0.27 %, 0.20 %; NAB female: 43.42 %, 24.95 %, 21.67 %, 2.78 %, 1.49 %, 3.65 %, 1.07 %, 0.59 %, 0.15 %, 0.18 %, respectively. Major microglia clusters (MI, MII and MIII) are highlighted in (c) and represent ~ 90 % of the total microglia analyzed. Rest of the microglia clusters and CAM are separately shown with higher resolution in (d) and represent ~ 10 % of the total microglia analyzed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

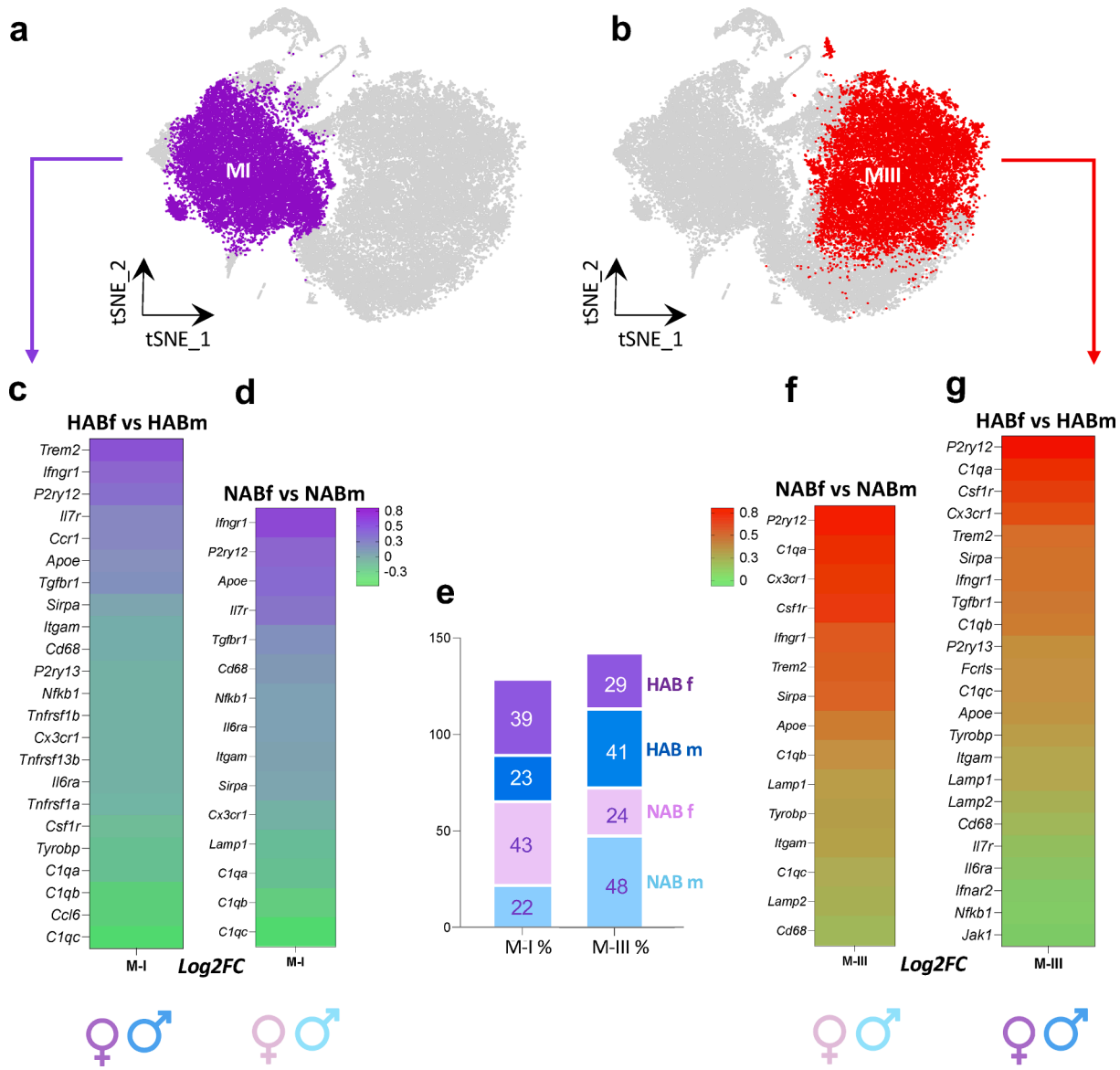

Fig. 3. Phagocytosis and inflammation-associated genes in the MI and MIII clusters show higher expression levels by HAB female compared to the HAB male. **a and b)** Feature plots highlighting the MI (purple) and MIII (red) clusters, respectively. **c and d)** Heatmaps indicating a selected set of genes that are differentially regulated in the MI cluster of HAB female and HAB male brains(c) and NAB female and NAB male brains (d). The scale indicates the log2 fold-change values between the groups compared. Genes associated with synaptic pruning and phagocytosis (*Trem2, Cx3cr1, Itgam, Cd68*) and inflammatory response (*Nfkbi, Il6r, Ifngr1*) show higher expression in female compared to male in the MI cluster for both HAB and NAB groups (Computed by using DESeq2 in Seurat differential expression testing workflow, pAdj < 0.001). **e)** Bar plot indicates a higher proportion of MI cluster yet lower proportion of MIII cluster in the female brain compared to the male for both HAB and NAB groups (blue: HAB male; light blue: NAB male; purple: HAB female; light purple: NAB female). **f and g)** Heatmap indicating a selected set of genes that are differentially regulated in the MIII cluster of NAB female and NAB male brains (f) and HAB female and HAB male brains (g). The scale indicates the log2 fold-change values between the groups compared. Genes associated with synaptic pruning and phagocytosis (*Trem2, C1qa, C1qb, Cd68, Cx3cr1, Itgam*) and inflammatory response (*Nfkbi, Il6r, Ifngr1, Jak1*) show higher expression in HAB female compared to HAB male in the MIII cluster (Computed by using DESeq2 in Seurat differential expression testing workflow, pAdj < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2019). That is the reason why we employed different anxiety tests for evaluating baseline (EPM) versus treatment (LD) to avoid the confounding effect of memory.

2.13. Statistical analysis

Data analyses were performed with GraphPad Prism 8.0 software (GraphPad Software Inc., Boston, MA, USA). A 95 % confidence interval was used for statistical evaluation, and $P < 0.05$ was considered as a statistically significant difference in all sampled groups, if not specified otherwise in the figure legends. Data are presented as means ± standard error of the mean (S.E.M.), and were all tested for homoscedasticity and outliers using GraphPad Prism 8 Software. No statistically significant outliers were detected in the datasets and no outliers were removed during the data analysis. The respective statistical tests are mentioned in the figure legends as well as provided below.

Fig. 3*c, d, f and g:* Differential expression analysis between different groups was performed using the DeSeq2 of the Seurat R package. pAdj < 0.001 was considered as statistically significant.

Figs. 4–7: Two-way ANOVA with Sidák's multiple comparison post-hoc test was used to compare HAB male, HAB female, NAB male and NAB female groups with each other. Number of replicates (n) and p-

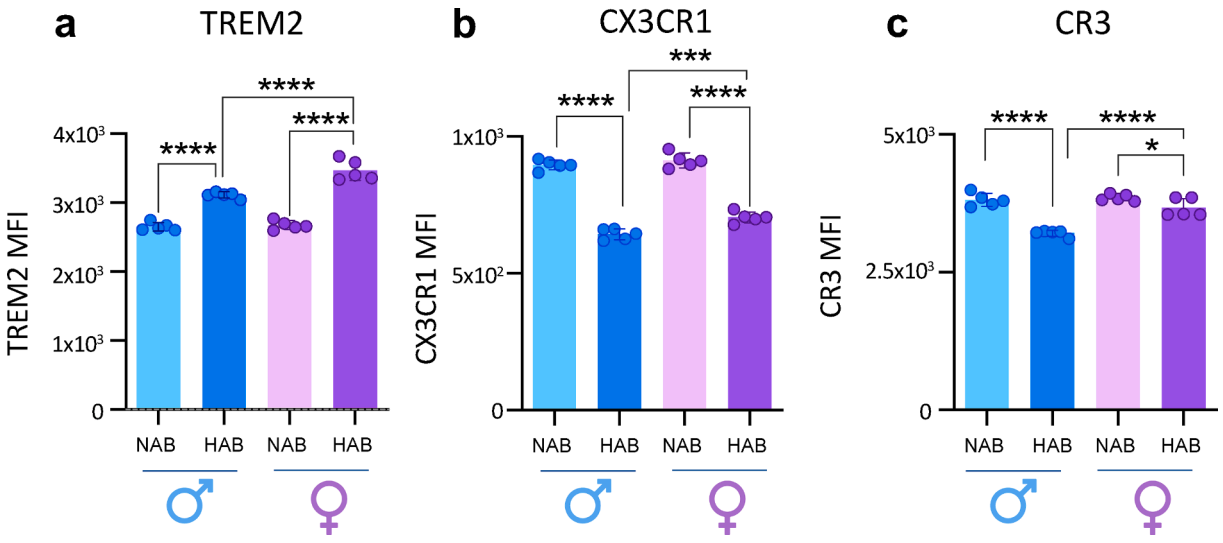

Fig. 4. HAB female microglia show higher surface levels of TREM-2, CX3CR1, CR3 in the hippocampus. **a, b, c)** Surface expression levels of microglia-specific TREM-2 (a), CX3CR1 (b), and C3R (c) are significantly higher in the HAB female hippocampus compared to HAB male, indicated by mean fluorescence intensity (MFI) from $CD11b^{++}$/ $CD45^{+}$ microglia in the hippocampus. No sex differences comparing male and female of the NAB group were detected. Higher expression level of TREM2 were detected in HAB compared to NAB in both sexes, whereas lower expression levels of CX3CR1 and CR3 were detected in HAB compared to NAB microglia in both sexes in the hippocampus. (Two-way ANOVA with Sidák's multiple comparison post-hoc test, HAB/NAB male: $p^{TREM2} < 0.0001$, $p^{CX3CR1} < 0.0001$, $p^{CR3} < 0.0001$; HAB/NAB female: $p^{TREM2} < 0.0001$, $p^{CX3CR1} < 0.0001$, $p^{CR3} = 0.042$; HAB male/ HAB female: $p^{TREM2} < 0.0001$, $p^{CX3CR1} = 0.0007$, $p^{CR3} < 0.0001$; NAB male/NAB female: $p^{TREM2} = 0.905$. $p^{CX3CR1} = 0.449$, $p^{CR3} = 0.836$,; $n^{male} = 5$; $n^{female} = 5$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, $^{****}P < 0.0001$).

values are provided in the figure legends for each experiment.

Fig. 8: Unpaired *t* test was used to compare HAB female vehicle control versus HAB female minocycline-treated groups.

3. Results

3.1. Single-cell RNA sequencing reveals heterogeneity and sex difference of microglia in mice with innate high anxiety

To address microglia heterogeneity in HAB compared to NAB, we first performed single-cell RNA sequencing of HAB and NAB brains (whole brain excluding cerebellum and olfactory bulb) including both sexes to perform an exploratory analysis (Fig. 1a). Using various genes related to a particular microglial function or subset, we identified 10 clusters including a cluster of CNS-associated macrophages (CAM) (Fig. 1b–d). We confirmed the microglial identity of these clusters by analyzing their expression levels of the canonical microglial marker genes *P2ry12, Tmem119 and Hexb* (Masuda et al., 2020) (Fig. 1d, and Supplementary Figs. 2, 3).

3.2. Microglia holding a high potential of synaptic engulfment and phagocytosis are enriched in the female brain

MI cluster shows high expression of canonical microglial marker genes such as *Hexb*, *Tmem119*, *P2ry12*, suggesting a homeostatic state (Fig. 1c and Supplementary Figs. 2 and 3). On the other hand, MIII cluster indicates a pre-active state given the lower expression of genes related to homeostasis (Fig. 1c and Supplementary Figs. 2 and 3). MII cluster represents a transitory state between the MI and MIII clusters, based on the expression levels of the canonical microglial marker genes, which is higher than the MIII and lower than the MI (Supplemetary Fig. 2). We also found a higher expression of genes related to phagocytosis and synaptic engulfment such as *Cd68*, *Trem2*, *C1qc*, *Lamp1* in the MI cluster compared to the MII and MIII (Fig. 2a). Furthermore, we detected a higher expression of genes related to interferon response such as *Ifngr1* and *Ifnar2* in the MI cluster (Fig. 2a).

Most microglia detected in the HAB and NAB brains belonged to the MI, MII and MIII clusters, constituting approximately 90 % of the total microglia analyzed (Fig. 2b). Comparing HAB and NABs, we found only slight differences in the proportion of the major microglia clusters (Fig. 2b). Interestingly, when we separated different sexes of HAB and NABs in the analysis, we found more pronounced differences in the proportion of these clusters indicating a sex difference in the distribution of different microglia clusters (Fig. 2c).

The proportion of the MI cluster in the HAB female brain is ~ 39 %, whereas ~ 24 % in the HAB male (Fig. 2c). A similar sex difference is also reflected in the NAB brains with ~ 43 % in NAB females versus ~ 22 % in NAB males (Fig. 2c). MIII, on the other hand, constitutes ~ 30 % of HAB female, ~40 % of HAB male, 25 % of NAB female and 48 % of NAB male microglia (Fig. 2c). These data together suggest that microglia clusters indicating homeostatic (MI) and pre-active (MIII) states represent varying percentages in male and female brains.

Apart from these major microglia clusters, the rest represent 10 % of the total microglia analyzed and their percentages also suggest sex difference, especially for the microglia at the proliferative state, showing high expression of cell cycle-associated genes such as *Cdk1*, *Mki67* and *Birc5,* as well as for the M−IV cluster indicating a DAM signature (Fig. 1c and Fig. 2d). Altogether, present percentage dynamics of the microglia clusters detected in the HAB and NAB brains suggest a sex difference along with a great degree of heterogeneity, which we could show in 9 distinct clusters and 1 CAM cluster based on their gene expression profile.

3.3. MI and MIII clusters express higher levels of genes related to synaptic pruning and inflammatory response in the HAB female brain compared to the HAB male

We have shown that the MI and MIII clusters, which are among the major microglial clusters detected in our dataset, indicate sex difference in their percentage dynamics comparing male and female brains. When we compared the gene expression profiles of these two clusters between the HAB male and HAB female brains, we found that in both HAB and NAB groups, the genes related to inflammatory response such as *Nfkb1*, *Il6ra*, *Ifngr1* show higher expression in female as compared with male microglia in the MI cluster (Fig. 3a,c and d). Interestingly, we also observed that in this cluster, which shows a high phagocytic capacity

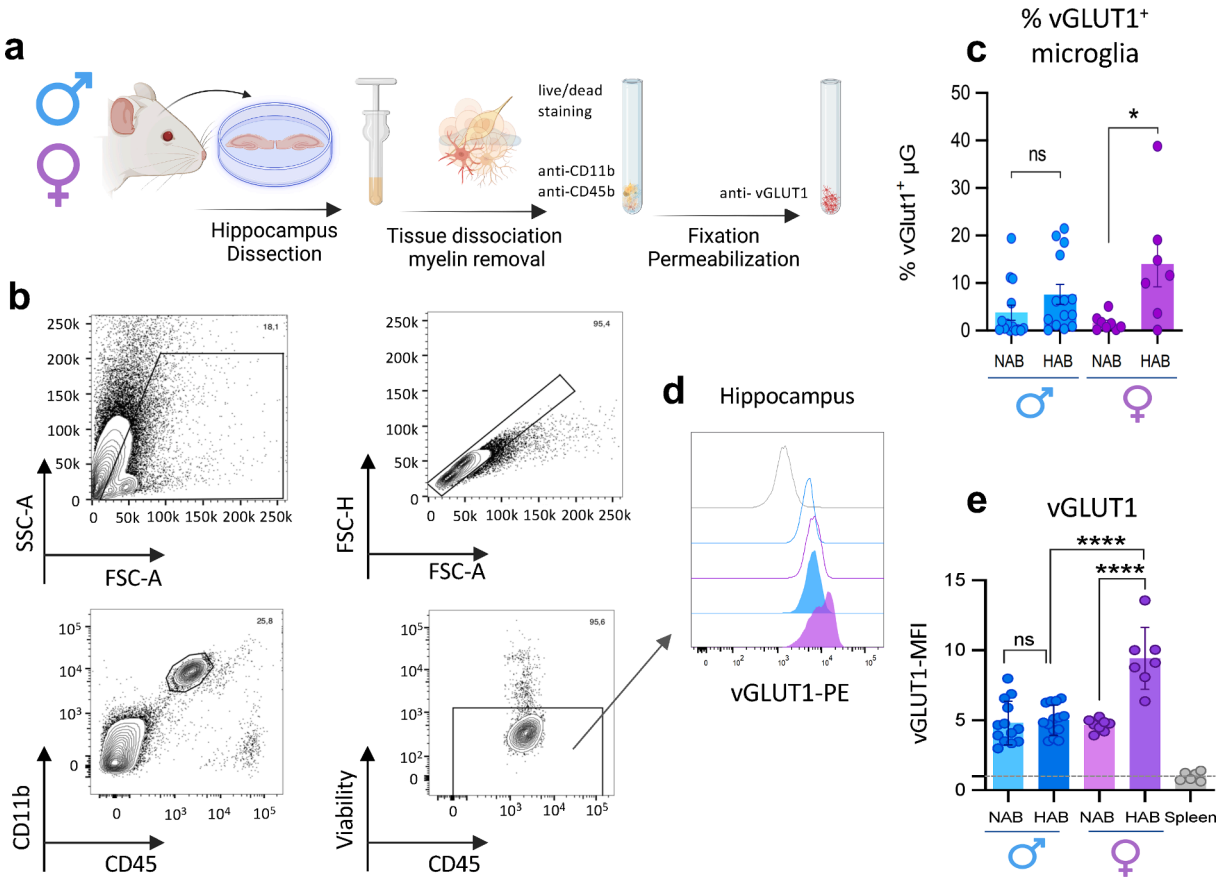

Fig. 5. Microglia engulf more vGLUT1^{+} synapses in the hippocampus of HAB females in comparison to the other groups. **a)** Schematic representation of the experimental workflow (Created by using BioRender.com). Cells were freshly isolated from hippocampus and stained for microglial markers (CD11b/CD45) followed by intracellular vGLUT1 staining. **b)** Gating strategy to define microglia as CD11b^{++}, CD45^{+}, viable population to analyze vGLUT1-MFI. **c)** % of vGLUT1^{+} microglia (CD11b^{++}/CD45^{+}/Ly6C^{-}/Ly6G^{-}) is significantly higher in the HAB female hippocampus compared to NAB female (2-way ANOVA with Šídák's multiple comparisons test, $n^{HABmale} = 14$, $n^{NABmale} = 14$, $n^{HABfemale} = 7$, $n^{NABfemale} = 8$; $p^{HABmale/NABmale} = 0.717$, $p^{HABfemale/NABfemale} = 0.018$, $p^{HABmale/HABfemale} = 0.375$, $p^{NABmale/NABfemale} = 0.998$, $p^{HABmale/NABfemale} = 0.402$, $p^{HABfemale/NABmale} = 0.034$). **d)** Representative histogram depicting vGLUT1 fluorescence intensity from HAB and NAB microglia (CD11b^{++}/CD45^{+}/Ly6C^{-}/Ly6G^{-}/Viability^{-}) of both sexes (Histogram: Grey line histogram: spleen; blue line histogram: NAB male; purple line histogram: NAB female; blue-filled histogram: HAB male; purple-filled histogram: HAB female). **e)** vGLUT1-mean fluorescence intensity (MFI) indicating the engulfment of vGLUT1^{+} synapses by CD11b^{++}/CD45^{+}/Ly6C^{-}/Ly6G^{-} hippocampal microglia reveals significantly higher vGLUT1-MFI of HAB female microglia compared to NAB female and HAB male. vGLUT1-MFI values are normalized to that of spleen macrophages, which were used as negative control (2-way ANOVA with Šídák's multiple comparisons test, $n^{HABmale} = 14$, $n^{NABmale} = 14$, $n^{HABfemale} = 7$, $n^{NABfemale} = 8$; $p^{HABmale/NABmale} = 0.916$, $p^{HABfemale/NABfemale} < 0.0001$, $p^{HABmale/HABfemale} < 0.0001$, $p^{NABmale/NABfemale} = 0.962$, $p^{HABmale/NABfemale} = 0.993$, $p^{HABfemale/NABmale} < 0.0001$; µG: microglia). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

based on its gene expression profile, the expression of genes related to phagocytosis as well as to synaptic engulfment such as *Cd68, Trem2*, *Itgam*, *Cx3cr1* are higher in the female compared to the male in both HAB and NAB groups (Fig. 3c and d). Therefore, this data suggests that microglia belonging to this cluster might have a higher potential to engulf synapses in the female compared to the male brain in the HAB and NAB (Fig. 3e). Comparing HAB male to NAB male, we similarly found higher expression of genes such as *Trem2, Ifngr1*; and lower expression of *C1qc, Lamp1* in the MI cluster (Supplementary Fig. 4a). When we compared HAB females to NAB females, we detected only C1qa among the marker genes associated with synaptic pruning and inflammatory response, and it has higher expression in the HAB (Supplementary Fig. 4b). This data, therefore, indicates that in the MI cluster, the difference between HAB and NABs is not as pronounced as the sex differences between the same groups.

When we analyzed the MIII cluster, which has a lower expression of genes related to phagocytosis, we observed a higher expression of genes related to an inflammatory response in the female compared to the male, which was more pronounced in the HAB group (Fig. 3b, f and g). Moreover, we showed that female microglia express higher levels of *Trem2*, *C1qa*, *C1qb*, *Cx3cr1*, *Itgam*, *Cd68* compared to male, indicating that also the cells in the MIII cluster show a higher potential to engulf synapses in the female brain compared to the male in both groups (Fig. 3b, f and g). This cluster shows a higher percentage in the male compared to the female brain (Fig. 3e). Comparing HAB males to NAB males in the MIII cluster, we detected lower expression of *C1qb* and *C1qc* by HAB male microglia (Supplementary Fig. 4c), and there was no significant difference between the expression of genes related to phagocytosis, synaptic engulfment and inflammatory response between HAB females and NAB females (Supplementary Fig. 4d). Concerning these particular pathways, we overall detected minor differences between the lines of matching sexes in terms of gene expression profile of the main microglial clusters. We observed more pronounced differences comparing different sexes of the same lines. While comparing different sexes, analysis of these major microglia clusters indicated potential markers such as *Trem2*, *Itgam* and *Cx3cr1*, which could possibly drive an elevated microglial pruning of synapses in the female brain compared to male. Furthermore, the gene expression profile of these clusters indicate a state that is more prone to inflammation in the female compared to the male brain (Fig. 3).

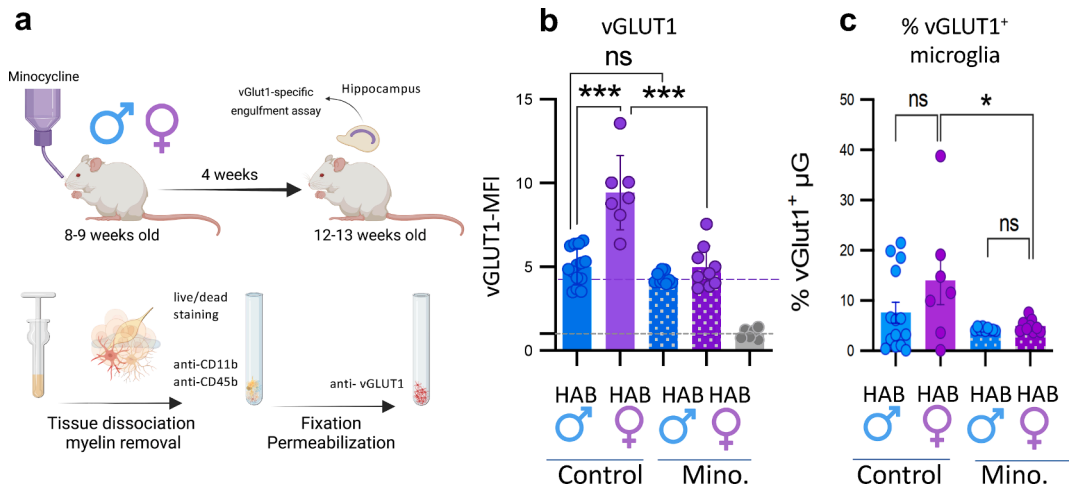

Fig. 6. *In vivo* minocycline treatment alleviates the higher microglial engulfment of vGLUT1^{+} synapses in the HAB female hippocampus. **a)** Experimental workflow demonstrating chronic oral minocycline treatment and vGLUT1-specific synaptic pruning assay (Created by using BioRender.com). Minocycline was supplemented in drinking water of the mice for 4 weeks. Cells were freshly isolated from the hippocampus and intracellular vGLUT1 staining was performed following the treatment, as described in the legend to Fig. 5. **b)** Significant reduction in the engulfment of vGLUT1 positive synapses by HAB female microglia in response to minocycline treatment is shown. Data indicates no difference between HAB female (magenta) and HAB male (blue) after 4 weeks of minocycline treatment. Grey dashed line indicates the average vGLUT1-MFI from spleen macrophages that were used as negative control (y = 1). Purple dashed line indicates the average vGLUT1-MFI from NAB microglia (y = 4.73). (2-way ANOVA with Šídák's multiple comparisons test, $n^{HABmale} = 14$, $n^{HABfemale} = 7$,$n^{HAB\ male}_{mino} = 9$, $n^{HAB\ female}_{mino} = 9$, $p^{HABm/HABf} < 0.0001$, $p^{HABm/HABm\text{-}mino} = 0.394$, $p^{HABf/HABf\text{-}mino} < 0.0001$, $p^{HABm\text{-}mino/HABf\text{-}mino} = 0.488$). **c)** Significant reduction in the percentage of vGLUT1 positive microglia in the HAB female hippocampus in response to minocycline treatment is shown. Data indicates no difference between HAB female (magenta) and HAB male (blue) after 4 weeks of minocycline treatment (2-way ANOVA with Šídák's multiple comparisons test, $n^{HABmale} = 14$, $n^{HABfemale} = 7$,$n^{HAB\ male}_{mino} = 9$, $n^{HAB\ female}_{mino} = 9$, $pAdj^{HABm/HABf} < 0.116$, $pAdj^{HABm/HABm\text{-}mino} = 0.493$, $pAdj^{HABf/HABf\text{-}mino} < 0.032$, $pAdj^{HABm\text{-}mino/HABf\text{-}mino} = 0.976$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. HAB female microglia express higher levels of markers related to synaptic engulfment in the hippocampus

So far, we reported different gene expression profiles of microglia comparing males and females of HAB and NAB groups in the whole brain. Moreover, differential expression analysis revealed changes in the regulation of genes related to phagocytosis and synaptic pruning in the whole brain. After these first steps of exploratory analysis using single-cell transcriptomics, we narrowed our focus down on one of the promising pathways that were indicated in the single-cell RNA sequencing dataset and picked the synaptic pruning as our first target considering it has a promising potential to modulate synapses. We focused on the hippocampus since we can functionally test synaptic pruning by microglia in this region at the adult stage due to its plasticity in terms of synaptic remodeling (Weerasinghe-Mudiyanselage et al., 2022). We first checked the expression of CX3CR1(encoded by *Cx3cr1*), TREM2 (encoded by *Trem2*) and CR3 (encoded by *Itgam*) proteins that have previously been shown to modulate pathways that regulate engulfment of synapses by microglia (Paolicelli et al., 2011; Stevens et al., 2007; Filipello et al., 2018) and were detected as differentially regulated in our transcriptomics dataset. We found no sex-specific differences in the NAB group, yet detected significantly higher expression of these three proteins by microglia in the HAB female compared to the HAB male (Fig. 4). These findings point to an elevated potential for microglial pruning of synapses in the HAB female hippocampus compared to the HAB male, which was also reflected in the transcriptomics dataset (Fig. 3). Interestingly, we found higher expression of TREM2 but lower levels of CX3CR1 and CR3 in the HABs of both sexes compared to sex-matching NAB controls in the hippocampus (Fig. 4a–c). These findings deviate from our transcriptional findings in the whole brain (Fig. 3), and indicate differences between HAB and NAB groups in the hippocampus versus the whole brain. Our findings in the hippocampus indicate a potential difference in the microglial engulfment of synapses especially between sexes in the HAB group, since all three markers show higher expression by HAB female microglia compared to the HAB male. Therefore, we next focused on the functional validation of our hypothesis in the hippocampus using both sexes of HAB and NAB.

3.5. HAB female microglia engulf more vGLUT1^{+} excitatory synapses in the hippocampus

We next functionally examined *in vivo** (*referred in the Methods section 2.7) synaptic engulfment by microglia in the hippocampus (Fig. 5a), and showed that HAB female microglia engulf more vGLUT1^{+} synapses than either HAB males or NAB females (Fig. 5b–e). We also detected a higher percentage of vGLUT1^{+} microglia in the hippocampus of HAB female compared to NAB female (Fig. 5c). These findings suggest that higher engulfment of vGLUT1^{+} excitatory synapses by hippocampal microglia is sex-specific and more pronounced in the HAB females than in any of the other groups. Yet, we found no differences in the engulfment of vGLUT1^{+} synapses by NAB male and NAB female microglia (Fig. 5b–e). Therefore, this data supports the sex difference reflected in the transcriptomics dataset at a functional level only in the HAB female; whereas, it does not indicate any differences between NAB female and NAB male.

3.6. Minocycline treatment alleviates higher engulfment of synapses by HAB female microglia and reduces anxiety-related behavior of HAB female

Previous reports have indicated that minocycline alleviates high anxiety-related behavioral symptoms in HAB mice and influences microglia in models of innate anxiety (Rooney et al., 2020) and other psychiatric disorders (Mattei et al., 2017). We therefore next tested the *in vivo* effects of minocycline on microglia of HAB male and HAB female mice by supplying it in the drinking water for 4 weeks (Fig. 6a). We focused only on HAB males and HAB females since the HAB group reflects a striking sex difference both at transcriptional and functional level in the hippocampus. We first checked engulfment of vGLUT1^{+} synapses by microglia in response to the treatment, and found a

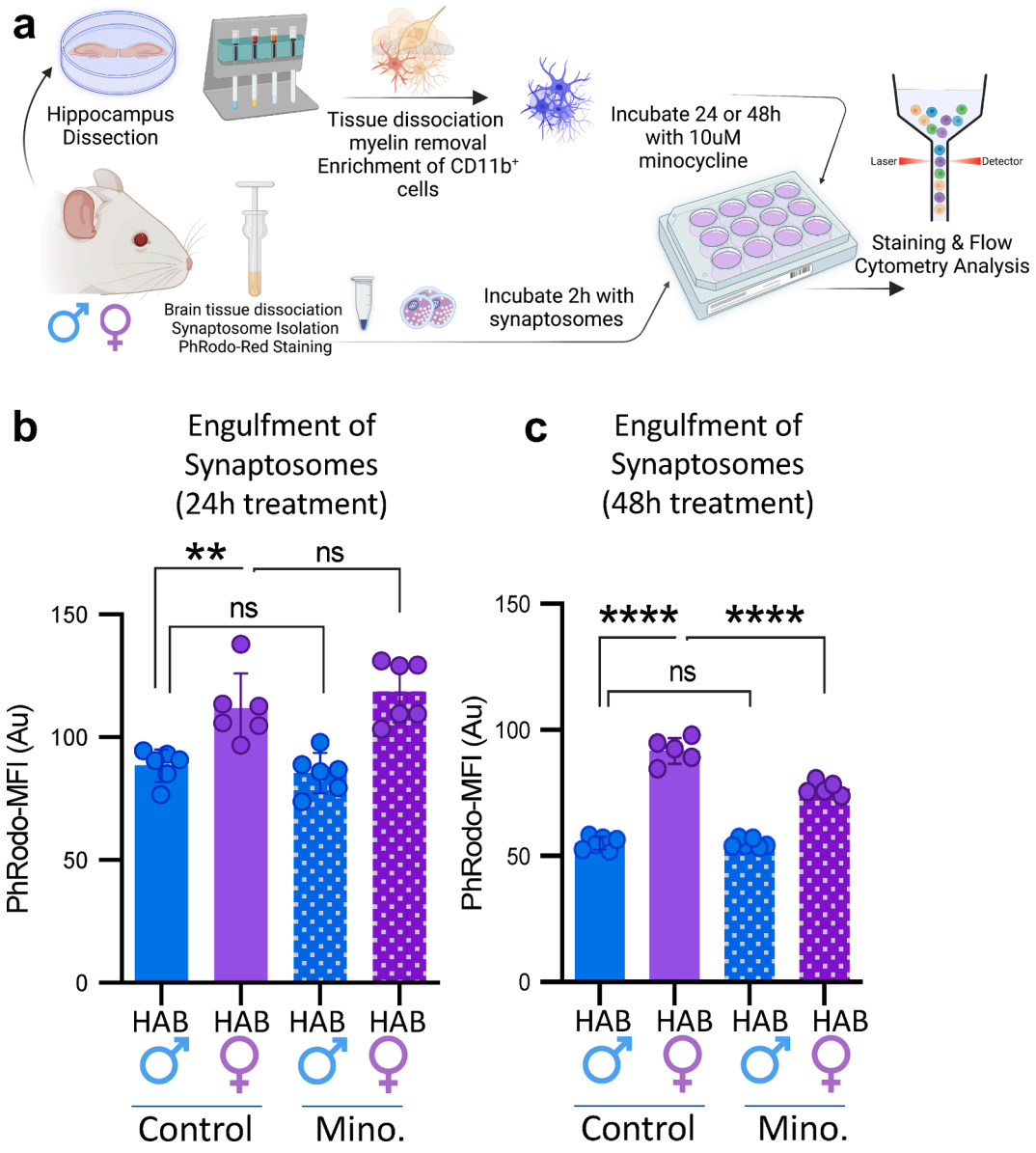

Fig. 7. *In vitro* minocycline treatment alleviates higher microglial engulfment of synaptosomes in the HAB female. **a)** Experimental workflow depicting the *in vitro* minocycline treatment and the synaptosome engulfment assay (Created by using BioRender.com). Freshly isolated adult hippocampal microglial cells were treated with minocycline in culture for 24 and 48 h. The treatment was followed by incubation with synaptosomes to measure their engulfment compared to untreated controls. **b)** HAB female microglia engulf more pHrodo^{TM}Red-labeled synaptosomes compared to HAB male after 24 h in culture. *In vitro* minocycline treatment (24 h) do not show an effect on total synaptosome engulfment by HAB male and female microglia compared to the untreated controls (2-way ANOVA with Šídák's multiple comparisons test, $n^{HABmale} = 6$, $n^{HABfemale} = 6$, $p^{HABm/HABf} = 0.002$, $p^{HABm/HABm\text{-}mino} = 0.873$, $p^{HABf/HABf\text{-}mino} = 0.497$, $pAdj^{HABm\text{-}mino/HABf\text{-}mino} < 0.0001$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). **c)** HAB female microglia engulf more pHrodo^{TM}Red-labeled synaptosomes compared to HAB male after 48 h in culture. *In vitro* minocycline treatment (48 h) decreases the engulfment of total synaptosome by HAB female microglia compared to the untreated control. Treatment did not show a significant effect on HAB male microglia compared to the untreated control (2-way ANOVA with Šídák's multiple comparisons test, $n^{HABmale} = 6$, $n^{HABfemale} = 5$, $p^{HABm/HABf} < 0.0001$, $p^{HABm/HABm\text{-}mino} = 0.997$, $p^{HABf/HABf\text{-}mino} < 0.0001$, $p^{HABm\text{-}mino/HABf\text{-}mino} < 0.0001$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

significant reduction of synaptic engulfment by the HAB female microglia to the level of HAB male (Fig. 6b). We also showed that the treatment reduced the percentage of vGLUT1^{+} microglia in the female HAB hippocampus compared to the untreated female HAB controls (Fig. 6c).

Minocycline is also acting on other cells and organ systems following systemic administration (Möller et al., 2016). Therefore, we next explored the direct effect of minocycline on microglial engulfment of synapses by directly supplying it on MACS- isolated CD11b^{+} microglia from the hippocampus of HAB male and HAB female at postnatal day 90 (P90) *in vitro*. We incubated freshly isolated pHrodo^{TM}Red-labeled synaptosomes with both HAB male and HAB female microglia, and analyzed their engulfment upon 24 h and 48 h *in vitro* minocycline treatment (Fig. 7a). We found higher levels of synaptosome engulfment by untreated HAB female microglia compared to the untreated HAB male, both after 24 h and 48 h in culture (Fig. 7b and c), which shows that HAB female microglia engulf more synaptosomes also *in vitro* and that is in line with our vGLUT1-specific findings. After 24 h of minocycline treatment, we detected no significant difference in the synaptosome engulfment, comparing the treated HAB male and HAB female microglia to the untreated controls (Fig. 7b). However, after 48 h of treatment, we found a significant decrease in the engulfment of total synaptosomes specifically by the female HAB microglia compared to the untreated control (Fig. 7c).

Together these findings indicate that female HAB microglia engulf

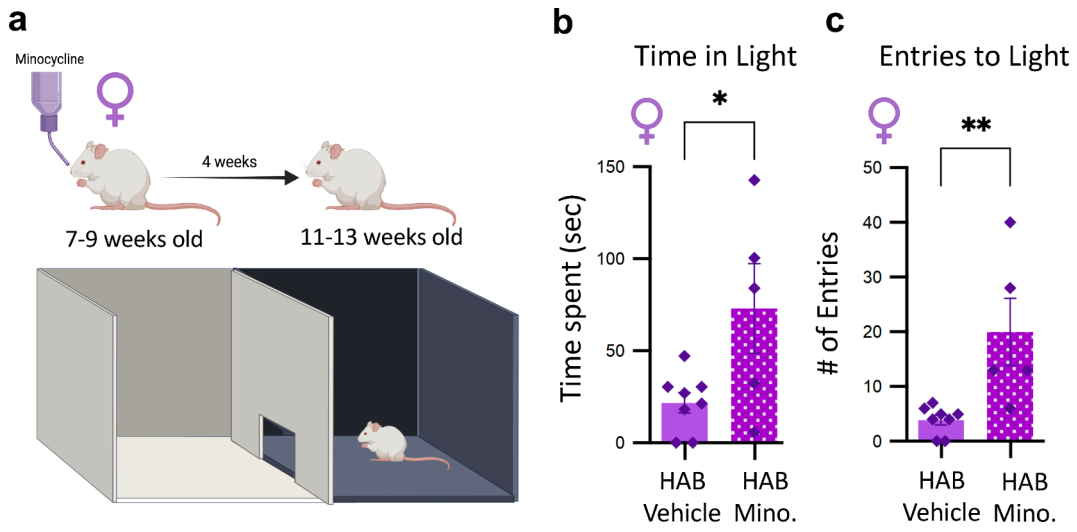

Fig. 8. Chronic oral minocycline administration reduced anxiety-related behavior of HAB female mice. **a)** Experimental workflow depicting the chronic oral minocycline administration via supplementing it in the drinking water and the light–dark box test to assess anxiety and depressive-related behavior of female HABs (Created by using BioRender.com). **b)** HAB females that undergone 4 weeks of minocycline treatment spent significantly longer time in the light box compared to the untreated controls (Unpaired *t* test, $n^{control} = 8$, $n^{minocycline} = 5$, p value = 0.027). c) HAB females that undergone 4 weeks of minocycline treatment made significantly higher number of entries to the light box compared to the untreated controls (Unpaired *t* test, $n^{control} = 8$, $n^{minocycline} = 5$, p value = 0.007; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

more vGLUT1^{+} excitatory synapses and total synaptosomes compared to the male HAB microglia, indicating a clear sex difference in the function of microglia. We also provided evidence that both *in vitro* and *in vivo* minocycline treatments significantly alleviate this intrinsic state of HAB female microglia to engulf more synapses but show no significant effect on the HAB male microglia. *In vitro* assays do not fully recapitulate the dynamic functionality of microglia *in vivo* (Timmerman et al., 2018). That is why, we complemented these *in vitro* experiments with *in vivo** findings to obtain a more comprehensive understanding of the effect of minocycline treatment on microglial engulfment of synapses.

At last, we investigated whether the effect of minocycline on microglial engulfment of synapses is associated with changes in behavior, and tested anxiety-related behavior of HAB females that were subjected to oral minocycline treatment. We report significantly longer time spent in the light arena as well as a higher number of entries to the light arena in the minocycline-treated group (Fig. 8). No differences in locomotor activity were observed between the treated and untreated groups (Supplementary Fig. 11). This data indicates an anxiolytic effect of minocycline on behavior of female HABs and that is in parallel with our microglia-specific findings in the female HAB hippocampus.

4. Discussion

Many psychiatric disorders, including anxiety and depression, exhibit gender differences in their prevalence, clinical manifestation as well as at the cellular level (Bangasser and Valentino, 2014). Moreover, these differences are often associated with dysregulations in the immune system and alterations of neural circuits (Ressler and Mayberg, 2007; Gaspersz et al., 2017; Tubbs et al., 2020). Considering the critical role of microglia in immune responses (Kettenmann, 2011) and synaptic refinement (Schafer et al., 2012; Vainchtein and Molofsky, 2020; Vainchtein et al., 2018; Paolicelli et al., 2011; Ji et al., 2013; Miyamoto et al., 2016); it is surprising that very few studies addressed sex differences in microglial cells in the context of innate high anxiety. To address this gap, our study presents three pertinent findings: 1) we report heterogeneity and sex difference of microglia at single-cell resolution in a context of innate high anxiety and comorbid depressive-like behavior. Microglia clusters bearing a prominent signature of high synaptic engulfment and phagocytosis showed higher percentage in the HAB female brain compared to the HAB male. We moreover found that genes related to synaptic engulfment such as *Trem2, C1qa, Cd68* showed higher expression by HAB female in the major microglia clusters compared to the HAB male. 2) We functionally supported these findings by demonstrating that the hippocampal microglia of HAB females engulfed more vGLUT1^{+} excitatory synapses and total synaptosomes than those of HAB males. Our findings revealing altered synaptic engulfment by microglia align with other studies that found a link between altered brain connectivity and anxiety-like behavior (Kalin, 2017). However, further investigation is needed to determine the precise effect of altered microglial engulfment of synapses on synaptic connectivity. Anxiety-related behavior has been associated with alterations in excitatory synaptic transmission in the paraventricular nucleus of the hypothalamus (Bolton et al., 2022) and prefrontal cortex (Socodato et al., 2020); therefore, from the outset we focused on vGLUT1^{+} excitatory synapses but further showed an effect also on the engulfment of total synaptosomes by microglia. 3) We report that *in vitro* and *in vivo* minocycline treatment modulates synaptic engulfment by microglia, and in particular, the treatment significantly reduces the synaptic engulfment by female HAB microglia.

Our data indicate that the MI cluster, in particular, holds a great capacity to modulate synapses based on its gene expression profile. MII and MIII clusters on the other hand represent a different state by showing lower expression of genes related to synaptic engulfment, phagocytosis (*Trem2, C1qb, C1qc, Cd68*) as well as homeostasis (*Hexb, Tmem119, P2ry12*). Higher expression of lysosomal markers in microglia may as well suggest an altered lysosomal function and/ or lysosomal biogenesis (Holtman et al., 2015). Recent studies also highlight the contribution of autophagy in regulating lysosomal function (Martinez et al., 2011). Therefore, it is possible that higher expression of lysosomal markers in microglia may indicate multiple alterations at the functional level. Therefore, we performed a phagocytosis assay using synaptosomes to ensure that higher expression of these markers in the HAB female brain is related to phagocytic activity in the context of synaptic engulfment.

The higher expression of *Trem2, Tyrobp, Cd9, Spp1, Ccl3, Ccl4* in MIV cluster indicates similarities to the previously reported disease-associated microglia (DAM) signature, which initially is associated with Alzheimer's disease (AD) models (Keren-Shaul et al., 2017). However, such signatures should be cautiously assessed since the definitions of different microglial states or clusters are highly context-

dependent. Microglia display diverse states and responses based on the signals they receive from their microenvironment (Hanisch and Kettenmann, 2007). These states can be represented by a variety of transcriptional changes that do not always correspond to a function (Paolicelli et al., 2022). For instance, downregulation of *P2ry12, Tmem119*, *Hexb* as shown in the MIII cluster, has been linked to a particular microglia state called white matter-associated microglia (WAM) in the Alzheimer context as well as regarded as an activated state of microglia (Schlepckow et al., 2017; Safaiyan et al., 2021). However, these core microglia marker genes are also under regulation of various signals, and do not show a steady expression (Paolicelli et al., 2022). Therefore, changes in their expression levels are not always sufficient for defining a similar microglial state in the context of anxiety, unless it is functionally validated. Their lower expression in one context may suggest myelin debris clearance (Safaiyan et al., 2021), while in another, may indicate a physiological state based on the cues received from the microenvironment. For this reason, we refrain from relating these clusters to those observed in earlier publications in the context of different diseases.

We found that many critical pathways were upregulated in HAB females compared to HAB males, particularly in the MIII cluster, including '*regulation of immune response*', '*synaptic pruning*', '*microglial activation', and 'neurogenesis'* (data not shown). In the current study, we mainly focused on synaptic engulfment by microglia among these pathways, since it holds a solid potential for modulating synapses. We functionally tested it in the hippocampus, and showed that HAB female microglia engulfed more vGLUT1^{+} synapses compared to the HAB male which is as well reflected in the transcriptomics data from the whole brain. According to autopsy reports (Duric et al., 2013; Zhao et al., 2012) and functional magnetic resonance imaging (fMRI) studies (Zeng et al., 2012), patients with depression and comorbid anxiety showed altered brain connectivity, particularly synapse loss. Additionally, preclinical research has also indicated synapse loss in depression and anxiety context (Han et al., 2022). The high microglial engulfment of synapses presented here could potentially drive loss of synapses, which remains to be investigated in future studies.

Although females are repeatedly reported to have a higher prevalence of anxiety-related disorders (Kessler et al., 2012; Strand et al., 2021; McLean et al., 2011; Moser et al., 2016), pre-clinical research mostly relies on data from males. Sex differences in microglia have been reported by several groups, in contexts of both health (Guneykaya et al., 2018; Villa et al., 2018; Hanamsagar et al., 2017) and disease (Kodama and Gan, 2019; Kalm et al., 2013; Acaz-Fonseca et al., 2015; Bodhankar et al., 2015). Such differences in the context of innate high anxiety have largely remained unexplored, but studies have shown that chronic stress exert different effects on male and female microglia (Bollinger, 2021; Wohleb et al., 2018; Wilber et al., 2011). The significance of these findings lies in the fact that chronic stress has been implicated as a profound risk factor for anxiety and depression-related behavior (Bouter et al., 2020; Conrad et al., 2011). It also alters microglia morphology and the expression of immunoregulatory factors by microglia (Tynan et al., 2010; Hinwood et al., 2012) differently in males and females (Bollinger, 2021). Furthermore, Wohleb et al. (2018) reported enhanced microglial engulfment of synapses in the medial prefrontal cortex of male mice, but not female, in response to chronic high stress (Wohleb et al., 2018). As sentinels of the CNS, microglia are overly sensitive to signals from their surroundings (Kettenmann, 2011; Kettenmann et al., 2013). Chronic stress is also known to alter neural activity (Wilber et al., 2011), which can be detected by microglia and could drive such pruning-associated results in the stress-induced anxiety models. Similarly, it has been shown that HABs display changes in basal neurotransmission at the ventral CA3-CA1 synapses of the hippocampus (Dine et al., 2015), which could have an impact on microglia-mediated synaptic pruning. Yet this particular study investigated only the male mice and we were unable to detect a functional phenotype in the HAB male microglia in terms of the engulfment of synapses. HAB females, on the other hand, show an evident phenotype of higher synaptic engulfment compared to HAB males and NAB females. Another study that exclusively focused on HAB females found that high innate anxiety with comorbid depression and anhedonia-related behavior were accompanied by a lower rate of neurogenesis and impairments in the functional integration of newly generated neurons in the hippocampus (Sah et al., 2012). We add to these findings by reporting elevated engulfment of synapses by HAB female microglia and speculate that microglia with a higher potential to engulf synapses could have a potential effect on integration of newly born neurons in the HAB female hippocampus. These findings are also in parallel with clinical data reporting decreased hippocampal connectivity and volume in patients with depression (Campbell et al., 2004; Ge et al., 2019).

Given that our findings on synaptic engulfment at the functional level are female-specific, what could explain differences between the sexes? Gonadal hormones appear to have a role driving some of these sex differences (Bollinger, 2021; Bollinger et al., 2019). Many adult sex differences in behavior and brain function are known to be developmentally programmed by steroid hormones, such as estrogen and testosterone (McCarthy et al., 2012; Gorski et al., 1986). Particularly during critical developmental pre- and postnatal time points, sex steroids can have long-lasting impact on the organization of brain circuits (McCarthy et al., 2017). Overall, the developmental programming of adult sex differences by steroid hormones contributes to the complex interplay between genetics, hormones and environmental factors in shaping sex differences. Receptors for glucocorticoid, estrogen, and androgen are expressed on microglia, which makes them responsive to variations and fluctuations in these hormonal signals (Carrillo-de Sauvage et al., 2013; Horchar and Wohleb, 2019; Sierra et al., 2008). A number of studies have reported that hormones play a crucial role in mediating sex-specific neural (Garrett and Wellman, 2009; Shansky, et al., 2010) and microglial responses (Bollinger et al., 2019). In a highly intriguing study, Caetano et al. (2017) used prenatal exposure to glucocorticoids (GC) to induce high anxiety behavior in male and female rats (Caetano et al., 2017). They found that microglia in the medial prefrontal cortex exhibit sexually dimorphic morphologies; those of females are less complex, and those of males are hyper-ramified (Caetano et al., 2017). When an experimental anxiolytic adenosine A2AR agonist was administered to these rats, males showed a drop in anxiety and the hyper-ramified morphology of male microglia was normalized, while there were no effects on either behavior or microglial morphology in females. This study reports striking sex differences in the morphology of microglia and their response to stimuli, even though there were no sex-linked differences detected in the high anxiety-related behavior of males and females, which both exhibited elevated anxiety compared to normal controls (Caetano et al., 2017). Likewise, no behavioral differences have been detected in HAB males and HAB females using classical behavioral tests to assess anxiety-like behavior such as the elevated plus maze (EPM) and light–dark test (LD) (data not shown). Classical behavioral tests are useful to detect changes in the anxiety-related behavior while comparing anxious groups to the normal controls. However, as both the HAB male and HAB female groups exhibit high anxiety-related behavior compared to the normal NAB controls, it becomes more complex when attempting to detect sex differences by comparing two anxious groups to each other. Classical tests such as EPM or LD demonstrate certain limitations, which include introducing a novel environment and inducing a stress response (Bailey et al., 2009; Börchers et al., 2022). In different studies, they reveal some sex differences with varying results amongst tests and show deviations from human findings indicating that these classical tests have poor predictive power for both detecting rodent sex differences and for reflecting human findings (Börchers et al., 2022; Lee, et al., 2022). These tests may potentially understate sex differences, particularly in females, because most of them were validated only in males (Börchers et al., 2022; Donner and Lowry, 2013). This means that our findings at the cellular and molecular level might have numerous sex-specific behavioral manifestations, whose detection calls for more

comprehensive behavioral tests such as 3D spontaneous behavior mapping (Huang et al., 2021). However, such in-depth behavioral characterization was not within the scope of this study and remains to be explored in our future studies.

Microglia are critical players in the organization of neural circuits through phagocytosis of synapses mainly via fractalkine receptor (CX3CR1), complement receptor 3 (CR3) and the triggering receptor expressed on myeloid cells 2 (TREM2) (Furusawa and Emoto, 2020; Paolicelli et al., 2011; Filipello et al., 2018; Qin et al., 2022). These three signaling pathways have been widely reported to modulate microglial engulfment of synapses in different brain regions (Furusawa and Emoto, 2020; Paolicelli et al., 2011; Filipello et al., 2018; Qin et al., 2022). Liu et al. (2020) reported that CX3CR1-CX3CL1 signaling is also associated with depressive-like behavior, anxiety and anhedonia (Liu et al., 2020). Similarly, C3 deficient mice display an excessive number of synapses and resilience to anxiety, which confirms a role for the C1q-C3-CR3 axis in synaptic pruning (Furusawa and Emoto, 2020; Stevens et al., 2007) and anxiety-related behavior (Crider et al., 2018). While these observations are not explicitly focused on the context of innate high anxiety, they support our functional findings concerning the higher engulfment of synapses by female HAB microglia, which express higher surface levels of TREM2, CR3 and CX3CR1 than HAB male microglia. We therefore hypothesize that these signaling pathways, especially TREM2, might potentially contribute to the reported functional differences in microglia in the hippocampus of HAB, in a sex-specific way. Surface levels of TREM2 are higher in the HAB female microglia compared to the NAB female and HAB male. We also reported higher synaptic engulfment by HAB female microglia compared to these two groups. Yet, we observed similarly higher expression of TREM2 by HAB male microglia compared to NAB males in the hippocampus, wherein we detected no functional difference in the synaptic engulfment. Therefore, we can speculate on a potential interplay between TREM2 levels and sex to regulate microglial engulfment of synapses in this particular context, highlighting a potential effect of sex. A recent study indeed indicated that TREM2 deletion in microglia has a sex-dependent impact on glioma growth, suggesting a relationship between TREM2 levels and sex in a different context (Chen et al., 2023). We found no significant differences in the regulation of these markers, including TREM2, comparing NAB male and NAB female microglia, and we detected no functional difference between them. Additionally, we could show that some of our findings such as higher expression of these markers by HAB female microglia compared to HAB male were represented both in the whole brain and hippocampus. Whereas, for instance, the sex difference in the NAB group was not reflected in the hippocampus although detected in the whole brain by single-cell RNA sequencing. Variation in these findings indicate another degree of variation in the state of microglia depending on the brain region, which was also highlighted by numerous other studies (De Biase and Bonci, 2019; Silvin and Ginhoux, 2018; Tan et al., 2020; Masuda et al., 2020). Therefore, our exploratory analysis indicates overall changes in the HAB and NAB brains of both sexes in the whole brain, whereas our hippocampus-specific findings indicate synaptic pruning-associated changes at the functional level. Our data therefore indicate different levels of complexity at transcriptional, spatial and functional levels and provide a valuable resource to further dissect the sex differences in innate high anxiety.

Several studies have shown that minocycline, a lipophilic broad-spectrum antibiotic, ameliorates stress-induced anxiety in rodent models (Wang et al., 2018; Levkovitz et al., 2015; Liu et al., 2018; Zhang et al., 2019; Molina-Hernández et al., 2008). Minocycline also exerts unspecific, complex effects on microglia regarding cellular density, morphology and reactivity in a context-dependent manner (Wang et al., 2018; Yrjänheikki et al., 1998; Wang et al., 2017; Strahan et al., 2017; Mattei et al., 2017). It crosses the blood–brain barrier (Garrido-Mesa et al., 2013) and has been clinically tested on patients with major depressive disorder and anxiety with promising results (Zazula et al., 2021; Dean et al., 2017). Paralleling these findings, we have previously reported that minocycline has anxiolytic effects on HAB males upon 28 days of oral administration (Rooney et al., 2020). We further found that the treatment reduced the density of $CD68^{+}$ microglia in the dentate gyrus of hippocampus, and concluded that minocycline might modulate the phagocytic potential of HAB microglia (Rooney et al., 2020). In addition to these findings, we herein provide functional data showing that hippocampal female HAB microglia engulf more synapses compared to the male HAB and NAB, which was reduced in response to minocycline treatment. In parallel to this, we showed a significant anxiolytic effect of chronic oral minocycline treatment on HAB female behavior (Fig. 8), which associates with our microglia-specific findings. The anxiety level in the treated HAB females did not decrease completely to the level of NAB female controls (data not shown). Therefore, we conclude that minocycline exhibits an anxiolytic effect on HAB female behavior, albeit without fully normalizing the anxiety levels to that of the NAB females. Furthermore, it is crucial to note that our findings do not indicate a direct causal relationship between synaptic pruning and anxiety-related behavior. Instead, we report an association between high anxiety-related behavior and microglial engulfment of synapses specifically within the HAB female group. Thus, our findings provide valuable insights into the changes in behavior and the phenotype of microglia, emphasizing the need for careful interpretation of the data.

Interestingly, HAB male microglia neither exhibited differences in synaptic engulfment compared to NAB, nor responded to minocycline treatment, despite the previously observed effect on behavior. We underline that the phagocytic potential of microglia is not only restricted to the engulfment of synapses, but it extends to the engulfment of apoptotic and necrotic cells (Green et al., 2016), bacteria and viruses (Nau et al., 2014), neural precursors (Sierra et al.,2010), and amyloid plaques (Huang, 2021). Therefore, phagocytosis of other targets cannot be ruled out, even though a significant difference in the microglial engulfment of synapses was not observed in the HAB males. Given that a higher percentage of $CD68^{+}$ microglia was previously detected in the HAB male hippocampus (Rooney et al.,2020), future studies should test the phagocytosis of other potential targets by HAB microglia. Interestingly, Han et al. (2022) recently reported elevated engulfment of synapses by microglia in the hippocampus of a chronic social defeat stress (CSDS)-induced mouse model of depression. They further showed that minocycline suppresses the synaptic engulfment by microglia (Han et al., 2022), which is strikingly parallel to our findings. In a different study using the poly(I:C) model for schizophrenia, we could also show the effect of minocycline to functionally restore microglial phagocytosis to normal levels in the hippocampus (Mattei et al., 2017). These results, albeit in different contexts, point to a potential impact of minocycline on microglial phagocytosis, which we also found in the present study.

In the absence of a stressor, genetic modification or immune challenges, our mouse model allowed us to obtain a thorough understanding of the microglial heterogeneity in the context of innate high anxiety and comorbid depression. We herein provide an overview of the microglia phenotype along with sex differences and show evidence of higher synaptic engulfment by microglia in the HAB female brain. These findings suggest microglial engulfment of synapses as a critical potential target, which can be affected by minocycline treatment. We further underline the necessity of including both sexes in the anxiety research given the known differences in risk and prevalence, as well as the sex-linked differences we have reported in microglia phenotype and function. Our findings establish a foundation for future studies that will focus on sex differences regarding the interplay of microglia and synapses as well as the modulatory effects of minocycline.

CRediT authorship contribution statement

Bilge Ugursu: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Anupam Sah:** Writing – review & editing, Investigation,

Funding acquisition, Data curation, Conceptualization. **Simone Sartori:** Investigation, Formal analysis. **Oliver Popp:** Formal analysis. **Philip Mertins:** Formal analysis. **Ildiko R. Dunay:** Writing – review & editing, Writing – original draft, Methodology. **Helmut Kettenmann:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Nicolas Singewald:** Project administration, Funding acquisition, Data curation, Conceptualization. **Susanne A. Wolf:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability Statement.

Data supporting the findings of this study are available within the article or Supplementary material. Additional data will be made available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2024.03.035.

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