Title: Early life psychosocial stress increases binge-like ethanol consumption and alters microglia and brain macrophage population density in early adulthood

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Highlights:

1. An early life psychosocial stress (PSS) exposure increases ethanol consumption
2. PSS and ethanol consumption alter microglia function in the ventral hippocampus
3. PSS and ethanol consumption have further consequences in other brain regions
4. Microglial inhibition during PSS trends towards reducing ethanol consumption
5. Microglial inhibition prevents microglia population changes in some brain regions

Abstract

As key regulators of brain homeostasis, microglia and brain macrophages are vital to development. However, early life stress (ELS) experiences, like bullying, can have lasting consequences on microglial function. During ELS, microglia and brain macrophages alter their engagement with cells and synapses. These alterations can result in lasting adaptations in circuit function that could lead to an increased risk to develop maladaptive drinking behaviors and alcohol use disorder (AUD) in adulthood. Whether microglia and brain macrophages truly mediate the relationship between ELS and AUD remains elusive. Here, we report: 1) a novel early life psychosocial stress exposure, PSS, increases binge-like ethanol consumption in early adulthood. 2) This increase in ethanol drinking is associated with alterations in microglia and brain macrophage populations in the ventral hippocampus and across other brain regions. 3) Inhibiting microglia and brain macrophages using a CSF1-r inhibitor, GW2580, results in a trend towards a reduction in binge-like ethanol consumption and normalizes microglia and brain macrophage populations. Therefore, our study suggests that acutely inhibiting microglia and brain macrophage function during periods of early life psychosocial stress may help reduce the risk to develop risky drinking behaviors and AUD.

Key Words: Early Life Stress, Alcohol, Microglia, Light-sheet Fluorescence Microscopy, CSF1-r inhibition, Macrophage

1. Introduction

Healthy brain development requires stable environments. In the clinical literature, it is reported that children exposed to early life stress (ELS) have rapid brain development (Hodel et al., 2018) and dysregulated executive function, emotion-regulation, and reward appraisal (Herzberg et al., 2020). This work has resulted in a large-scale push to better protect children from all forms of ELS. Over 50% of the US population has experienced at least one form of ELS during development (Swedo et al., 2023). ELS is experienced in a variety of ways including sexual, emotional, and physical abuse (Wade et al., 2022). One form of ELS, being bullied, yields all of the problematic outcomes listed above. Bullying is characterized as aggressive behavior that is repetitive and intentional in which a power differential exists between the victim and bully (Armitage, 2021). FMRI studies investigating the impact of adolescent bullying on brain activity demonstrated that individuals who experienced bullying had heightened activation of numerous brain regions, including the insula, dorsal striatum, hippocampus, and amygdala when presented with images of bullying. These changes in activation were associated with increased rates of social exclusion, anxiety, and depression (Perino et al., 2019). Further, bullying and other experiences of ELS are associated with an increased risk for problematic alcohol consumption such as binge drinking (Davis et al., 2018) and the development of AUD (Hallit et al., 2020).

Alcohol use remains one of the highest-ranked causes of death worldwide. Excessive and maladaptive use of alcohol, clinically defined as alcohol use disorder (AUD), affects nearly 14.4 million people in the United States and 107 million people worldwide (SAMHSA, 2018). While AUD comes with a heavy cost on society, with nearly

$249 billion spent in relation to excessive alcohol use per year in the United States, a vast majority of these costs ($194 billion) are accrued through a particularly dangerous behavior associated with AUD: binge drinking (SAMHSA, 2019). Identifying and characterizing early risk factors associated with binge drinking, and more broadly AUD, can provide clinicians with new approaches to prevent their onset.

In the context of AUD, ELS has been demonstrated to induce alterations in reward circuit function that can lead to increased preference for, and consumption of alcohol (Butler et al., 2016, Gilpin & Weiner, 2017). Although many studies have documented these ELS-mediated circuit adaptations, less is known about how these adaptations occur. Several preclinical models have been used to explore the effects ELS has on alcohol consumption and neurobiology. These include maternal separation (Bertagna et al., 2021), adolescent social isolation (Chappell et al 2013, Whitaker et al 2013, Skelly et al 2015), limited bedding and nesting (Okhuarobo et al., 2020), adverse social experiences (Surakka et al., 2021), and social defeat (Rodriguez-Arias et al., 2016). While results from these studies show a variety of outcomes and changes in neurobiology, few have been implemented during postnatal days (P) 14-21 (Catale et al., 2020). In mice, P14-21 represents the period of development associated with pre or early adolescence. This developmental window has been shown to be critical for the developing cortex (Andolina et al., 2011), immune system (Danese et al., 2017), and sculpting of the brain via synaptic pruning (Paolicelli et al., 2011).

Of note, ELS has been demonstrated to have lasting consequences on ventral hippocampal plasticity (Çalışkan et al., 2020). The ventral hippocampus (vHC) acts as a major relay point between numerous regions involved with affective state, motivation,

and reward (Turner et al., 2022). Further, chronic intermittent ethanol exposure alters vHC synaptic excitability resulting in increased anxiety-like behaviors (Ewin et al., 2019, Bach et al., 2021). Due to its role in regulating behaviors related to AUD, identifying what mechanisms underlie the effects of ELS on vHC plasticity can provide targets to prevent the longitudinal consequences of ELS on alcohol use. But how is vHC plasticity altered by ELS?

Microglia and brain macrophages are vital to healthy brain development and homeostasis across the lifespan (Paolicelli et al., 2022). During development, microglia regulate synapse formation and elimination. This process determines the fate of neuronal excitability and circuit function (Bolton et al., 2022, Surala et al., 2024, Zhao et al., 2024). Further, ELS is noted to alter microglia function and phenotype and these changes may lead to lasting consequences on behavior (Catale et al., 2020). A relatively novel model of ELS utilized to recapitulate the experience of a psychosocial stress (PSS), such as bullying, has been shown to increase conditioned place preference for cocaine, while altering dopamine neuron excitation and microglia and brain macrophage populations in adulthood (Lo Iacono et al., 2018). As a result, microglia and brain macrophages may be a key target in blunting the effects of ELS on vHC neurons and circuit function.

In this study, we sought to determine whether PSS exposure from P14-21 would facilitate an escalation in binge-like ethanol consumption. Next, we characterized how PSS and repeated binge-like ethanol consumption altered microglia and brain macrophage reactivity and phenotype in the vHC. We then used light-sheet fluorescence microscopy to conduct a broader, unbiased analysis of brain-wide effects

of PSS and repeated binge-like ethanol consumption on microglia and brain macrophage count and proximity. Finally, we administered a pharmacological intervention during PSS to inhibit microglia and brain macrophage function with the goal of determining whether we could prevent escalations in binge-like ethanol consumption.

2. Methods and Materials

2.1 Animals

Ten-week-old Hsd:ICR CD-1 outbred mice (CD1) male (n=10) (Inotiv Co), six-week-old C57BL/6J (c57) male (n=2) and female mice (n=4), and six-week-old male (n=4) and female (n=8) B6.129P2(Cg)-Cx3cr1tm1Litt/J (CX3CR1^{GFP+}) mice were purchased from Jackson Laboratories (Strain #:000664; Strain #:005582 respectively). CX3CR1^{GFP+} knock-in mice provide a means to label peripheral and CNS macrophages with green fluorescent protein (GFP). Further, the CX3CR1^{GFP+} mouse line is highly specific for microglia and macrophages in the brain (Chakraborty et al., 2019). Thus, this mouse line is ideal for experiments utilizing approaches like light-sheet fluorescence microscopy to ensure ubiquitous and consistent visualization of microglia and macrophages across the brain (Zhang et al., 2021). C57 and CX3CR1^{GFP+} male and female mice were mated strain independently at eight weeks of age and fathers were removed before parturition. PSS exposure and behavioral testing were performed during the dark cycle from 9:30 PM to 3:30 AM. Food and water were provided ad libitum, and mice were housed on a 12:12 light:dark cycle with lights on at 07:00 AM. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wake Forest University.

2.2 Psychosocial Stress (PSS) Procedure

Once pregnancy was determined, the female breeder was moved to individual housing until the litter was born. C57 and CX3CR1^{GFP+} pups from each litter were randomly assigned to an unhandled control or PSS at P14. In the PSS group, each pup was briefly isolated from the dam and litter, and housed in the home cage of an orchiectomized adult CD1 male mouse for 30 min per day from P14-21. Control and PSS-exposed pups were then weaned and randomly assigned to group housing with pups of the same strain, and of similar age until early adulthood (P60) (Lo Iacono et al., 2017).

2.3 Orchiectomy Surgeries

To avoid physical harm to the pups, orchiectomy surgeries were performed on adult CD1 male mice 30 days prior to PSS exposure. Removal of the testes has been noted to reduce testosterone levels and aggression-related behavior in mice (Lo Iacono et al., 2017). Briefly, mice were anesthetized using vaporized isoflurane and placed on top of a heating pad. Mice were shaved using electric clippers along the abdominal cavity. Skin was then swabbed with 70% ethanol followed by sterile PBS. Surgical instruments including forceps and surgical scissors were sterilized with 70% ethanol swabs prior to each surgery. A horizontal incision was then made an inch above the scrotum and forceps were used to free the testicular fat pad. Testes were then removed using surgical scissors and the remaining fat pad was returned to the scrotum. Scrotal incisions were closed using an intermittent suturing technique. Antibiotic ointment was applied to the incision location, and mice were returned to sterile cages upon recovery from anesthesia.

2.4 GW2580 administration

A randomly-selected subset of CX3CR1 $^{GFP+}$ mice were administered colony stimulating factor 1 receptors (CSF1-r) inhibitor, GW2580. GW2580 is a selective antagonist for CSF1-r which are exclusively found on microglia and other cells from a myeloid lineage. Antagonism of these receptors inhibits microglia function and prevents cell cycle processes resulting in transient reductions in cell number (Neal 2020). GW2580 (Selleck Chem, Catalog #: S8042) was prepared as previously described (Olmos-Alonso et al., 2016). Intraperitoneal injections of GW2580 at a concentration of 75mg/kg or vehicle prepared with the same reagents were administered 30-minutes prior to each day of the stress exposure from P14-P21.

2.5 Drinking in the Dark (DID) procedure

At P60, mice were exposed to a modified DID procedure (Thiele & Navarro, 2014). Briefly, DID is a limited access ethanol exposure model where mice are isolated in separate drinking cages and provided access to 20% w/v ethanol without food or water during the dark phase. Mice are freely allowed to consume as much ethanol as desired. During the first three nights of the exposure, mice are provided access to ethanol for two hours. On the fourth night, mice are provided access to ethanol for four hours. This model has previously demonstrated that mice reach a clinically relevant point of intoxication (Thiele & Navarro, 2014). Our modified DID procedure sought to utilize the original model, but provided this four-day exposure repeatedly across four weeks to allow ample time for the mice to acclimate to the taste and psychoactive effects of ethanol. Mice were isolated in their drinking cages for one hour prior to DID to ensure perturbations in behavior brought on by relocation were minimized. Each night of

the exposure, mice were weighed, and the amount of ethanol consumed was measured as grams of ethanol (g) per kilogram (kg) of body weight.

2.6 Tissue Preparation for Histological Procedures

24 hours following completion of the DID procedure (P91), C57 mice were anesthetized using euthasol and prepared for transcardial perfusion. A vertical incision was made along the ventral mid-line of the mouse and the sternum and rib cage were removed exposing the heart. A 26-gauge butterfly needle was attached to a perfusion pump and the needle then punctured the left ventricle of the heart. Ice cold Del-Bucco's phosphate buffered saline (Thermofisher, Catalogue #14190136) (dPBS) was flushed into the system at a flow rate of 8mLs/minute for three minutes. Following the dPBS flush, 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, SKU# 15714) was perfused at 8mLs/minute for three minutes. Brains were then removed and stored in 4% PFA for 24 hours at 4° Celsius before being moved to a 30% sucrose solution for cryoprotection and stored for section preparation. Brains were sliced into 40µm sections using a Leica SM2010 R sliding microtome. Sections containing vHC were then selected and separated for immunohistochemistry (IHC).

2.7 IHC Procedure

Sections were rinsed 3 x 5 minutes in 1 x PBS + 1% tween-20 (PBS-T) while rocking. Following the rinses, sections were blocked in 3% normal goat serum (NGS) in PBS and .01% tween-80 for 1 hour at room temperature while rocking to prevent non-specific antibody binding. After blocking, sections were incubated in primary antibodies suspended in the above-described blocking agent overnight at 4° Celsius while rocking (Primaries included Biorad mouse anti-rat CD 68 (catalog #: MCA341GA at 1:1000;

Wako anti-rabbit IBA 1 (catalog #: 019-19741) at 1:1000; and Thermofisher anti-mouse Neun at 1:500 (Catalog # MA5-33103). The next morning, sections were washed 3 x 5 minutes in 1 x PBS before incubation in secondary antibodies for two hours at room temperature while rocking (Secondary antibodies included Thermofisher Alexafluors goat anti-mouse 405, goat anti-rabbit 488, and goat anti-rat 555; all secondary bodies were diluted to 1:1000). Sections were then mounted on slides (Thermofisher, Catalogue # 14190136), and cover slipped (Thermofisher, Catalogue # 12541057).

2.8 Image Acquisition and Analysis

Images of the CA 1 region of the vHC were acquired using a Nikon Eclipse Ci. Each individual channel (405, 488, 555) was captured and then merged using NikonAR software. Images were imported into Fiji (ImageJ). Image analysis included counting IBA 1^{+} microglia and brain macrophages with healthy and dystrophy-related phenotypes (Shahidehpour et al., 2021), and collecting CD 68 area fraction (Pait et al., 2024).

2.9 Tissue Preparation for Light-sheet Fluorescence Microscopy (LSFM) Experiments

24 hours following completion of the DID procedure (P91), $CX3CR1^{gfp+}$ mice were anesthetized using euthasol and prepared for transcardial perfusion. A vertical incision was made along the ventral mid-line of the mouse and the sternum and rib cage were removed exposing the heart. A 23-gauge butterfly needle was attached to a perfusion pump and the needle then punctured the left ventricle. Ice cold dPBS + 10 mg/L heparin was flushed into the system at a flow rate of 8mLs/minute for three minutes. Following the dPBS flush, 4% PFA + 10mg/L heparin was perfused at 8mLs/minute for three minutes. Brains were then removed and stored in 4% PFA for 24 hours at 4° Celsius before being preserved using a SHIELD-based preservation

protocol (kit and instructions located at www.Lifecanvastech.com) (Park et al., 2018). Following preservation, tissue was incubated in a SDS solution containing sodium dodecyl sulfate (final concentration 300 mM), boric acid (final concentration 10mM), sodium sulfite (final concentration 100mM) and sodium hydroxide (titrated until pH 9) at 37°C while rocking for 20 days to ensure complete clearing of tissue. Once cleared, tissue was indexed-matched using Easy Index (Life Canvas Technologies), and mounted in an Easy Index agarose gel solution as stated per manufacturer instructions located at www.Lifecanvastech.com.

2.10 Light-sheet Fluorescence Microscopy (LSFM) Imaging and Analysis

Following brain clearing, brains were mounted on a Life Canvas SmartSPIM light sheet microscope. Samples were illuminated with excitation wavelengths at 488 for CX3CR1^{GFP+} and 561 to act as a control channel and minimize background. Laser power was adjusted for each brain and channel. Images were captured at 4 µm steps. Following image capture, raw files were migrated to a local server for destripping and stitching. Stitched images were stored as TIFFs and converted into Imaris files using Imaris File Converter 10.1. Imaris files were then opened on Imaris 10.1 for visualization and thresholding before defining regions of interest (ROI) for each sample. Twelve brain regions were selected for analysis. Region dimensions were created using the Allen Brain Atlas 3D adult mouse brain viewer (atlas.brain-map.org). This 3D viewer included an adjustable scale bar and predefined region boundaries to enable ROI creation (see LSFM supplemental materials for ROI dimensions).

After ROI dimensions were determined, ROIs were drawn on each sample. Imaris spot detection and three nearest neighbor analyses were performed. Spot

detection is a semi-automated analysis provided by Imaris that counts the number of GFP-labelled cells within a defined ROI. Three nearest neighbor analysis is a semi-automated approach that provides proximity information based on the spot detection analysis. In short, after spot detection identifies cells in each ROI, the three nearest neighbor analysis measures the distance between each individual cell and its three nearest neighbor cells and averages the distances. From there, the program takes the average distance for each cell and then provides the average distance for all cells of each region. This analysis can provide an indirect measure of microglial reactivity in each region (Davis et al., 2017). Spot detection and three nearest neighbor analyses were performed in ROIs of both hemispheres. Collected CX3CR1^{gfp+} cell count and proximity values for both hemispheres were averaged within each animal. After data collection was completed within Imaris, results were exported and uploaded to Graphpad Prism 10 for all statistical analysis.

2.11 Statistical Analysis

Mixed-effects analyses were utilized to measure differences in binge-like ethanol consumption between sex and exposure mice. Repeated measures ANOVA analyses were performed for body weight data (Figure 2a-b). Two-way ANOVA analyses were utilized to identify differences in macrophage count, dystrophy-related phenotypes, CD 68 area fraction, and LSFM data (Figure 3b-d; Figure 6a-h; Supplemental Figures 8a, c; 9-12). Mixed-effects analyses were performed for all drinking data (Figure 2c-d; Figure 4b-g; Supplemental Figures 1-7). One-way ANOVA analyses were performed to determine the effect of exposure on CX3CR1^{GFP+} count and proximity (Supplemental Figure 8b, d). Tukey's post-hoc comparisons were performed when main effects or

interactions were identified following any of the above-mentioned analyses. Correlations were performed to determine the strength of the relationship between CX3CR1^{GFP+} count and proximity (Supplemental Figures 13-18). All statistical methods were performed using Graphpad Prism 10. All statistical results are included in the supplemental materials.

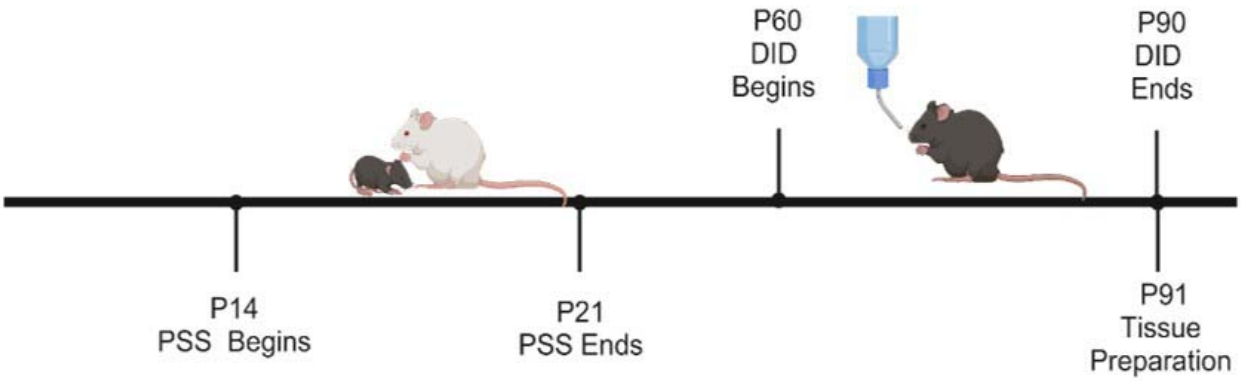

Figure 1. Experimental Timeline: Pups were randomly assigned to either control or PSS at P14. Following PSS at P21, control and PSS pups were weaned and group-housed until P60. At P60, control and PSS-exposed mice were briefly socially isolated for four nights a week across four weeks for DID. 24 hours following the final DID exposure, tissue was collected for immunohistochemistry.

3. Results

3.1 PSS-exposed C57 mice exhibit altered developmental body weights in a sex-dependent manner and increased binge-like ethanol consumption in early adulthood

We began our set of experiments by exposing C57 pups to PSS as described in the methods. Previous work demonstrated that PSS increases ultrasonic vocalizations, alters corticosterone levels, and lowers body weights directly following the exposure.

This work, however; did not explore whether the effects of PSS on body weight were sex-specific (Lo Iacano et al., 2017, Lo Iacono et al., 2018). In our hands, PSS exposure resulted in sex-dependent changes in body weight (Figure 2a-b). Repeated measures ANOVA analysis revealed a main effect of PSS exposure on body weight ($p=.015$) in male mice (control n=8; PSS n=6). Post-hoc analyses revealed PSS-exposed male mice had lower body weights than control male mice at P21 ($P=.0012$) (Figure 2a). In contrast, repeated measures ANOVA analysis revealed no effect of PSS on female mice (control n= 8; PSS n=7; $p=.46$), but a PSS x time interaction (.049). Prior to the exposure at P14, female mice assigned to PSS exhibited lower body weights than control female mice ($p=.0062$). Following PSS at P21, there was no difference between the body weights of control and PSS-exposed female mice (Figure 2b).

We then prepared a new cohort of C57 mice to determine whether PSS could lead to increased binge-like ethanol consumption in early adulthood (see Figure 1 for the experimental timeline). At P60, control (n= 7; 4 male and 3 female) and PSS-exposed (n=13; 9 male and 4 female) mice underwent DID as stated in the methods. Although this cohort was underpowered to perform sex-specific analyses, mixed-effects analysis revealed main effects of PSS ($p<.0001$) and time ($p<.0001$) on average daily ethanol consumption. Post-hoc analyses demonstrated PSS-exposed mice consumed more ethanol on the first, second, seventh, ninth, eleventh, twelfth, fourteenth, fifteenth, and sixteenth days of the DID exposure (Figure 2c). Further, mixed-effects analysis demonstrated main effects of PSS ($p=.0005$) and time ($p<.0001$) on cumulative ethanol

consumption. Post-hoc analyses revealed PSS-exposed mice consumed more ethanol throughout the study (Figure 2d).

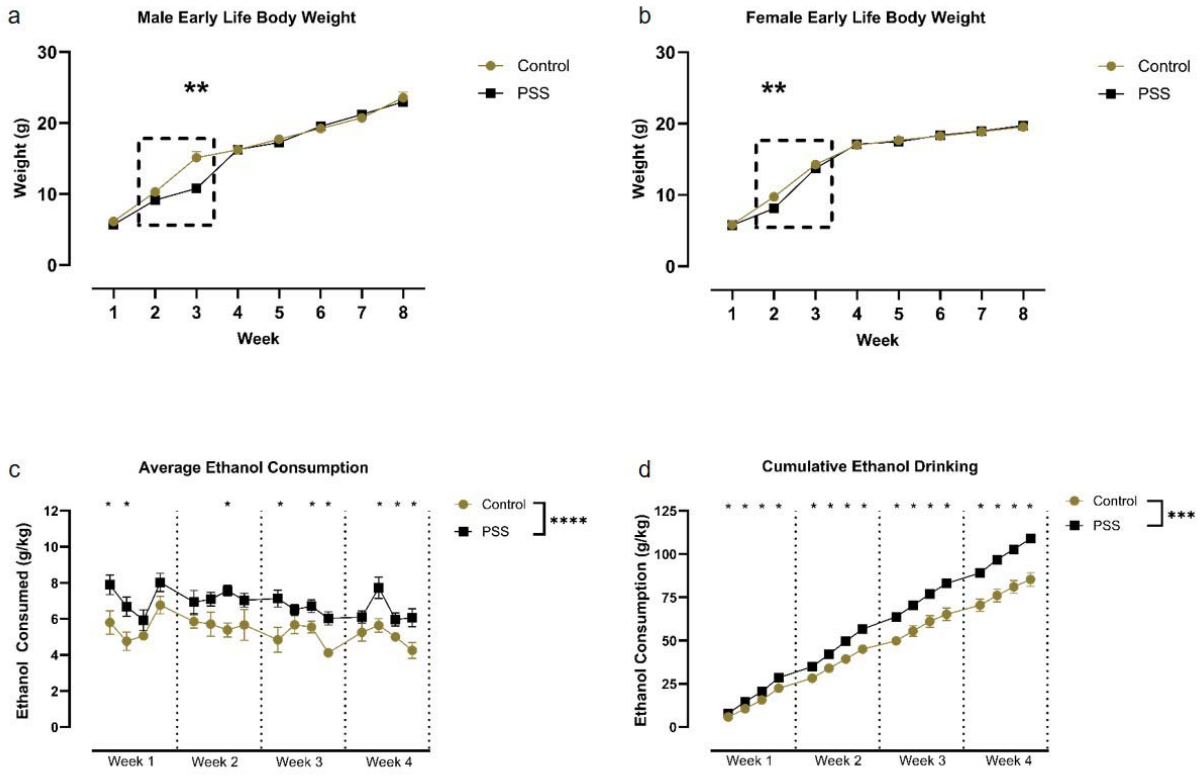

Figure 2. PSS alters developmental body weight in a sex-dependent manner and increases binge-like ethanol consumption in early adulthood. a) Developmental body weights for control and PSS-exposed male mice. b) Developmental body weights for control and PSS-exposed female mice. c) Average daily ethanol consumption for control and PSS-exposed mice. d) Cumulative ethanol consumption for control and PSS-exposed mice. Error bars indicate mean ± s.e.m. * indicates days that PSS-exposed mice consumed more ethanol than control mice. * Indicates p<.05; ** indicates p<.01; *** indicates p<.001; and **** indicates p<.0001.

3.2 PSS exposure and repeated binge-like ethanol consumption alter IBA 1$^+$ microglia and brain macrophage count, dystrophy-related phenotype, and reactivity in the ventral hippocampus of C57 mice

Following completion of DID, vHC sections were selected from a new cohort of mice including control (n=8; 4 male and 4 female), PSS-exposed (n=8; 4 male and 4 female), ethanol-exposed (n=10, 5 male and 5 female), and PSS and ethanol-exposed (n=10, 5 male and 5 female) mice. vHC slices were stained with IBA 1 to identify macrophage number and phenotype; CD 68 to determine changes in microglial and brain macrophage reactivity, and NEUN to label neurons and ensure region-specificity (Figure 3a). The number of IBA 1$^+$ microglia and brain macrophages were counted within the target region of interest and morphology was characterized as described by previous work (Shahidepour et al., 2021).

In relation to microglia and brain macrophage count, mixed-effects analysis revealed main effects of PSS ($p=.019$) and ethanol consumption ($p<.001$), but no PSS x ethanol interaction ($p=.15$). Post-hoc analyses revealed ethanol-naïve control and PSS mice exhibited significantly more IBA 1$^+$ microglia and brain macrophages than ethanol-exposed control and PSS-exposed mice (ethanol-treated controls vs ethanol naïve controls: $p<.0001$, ethanol-treated controls vs. ethanol naïve: PSS $p<.0001$, ethanol-treated PSS vs. ethanol naïve controls: $p<.0001$, ethanol-treated PSS vs ethanol naïve PSS: $p<.0001$). Further, ethanol-naïve control mice trended towards having more IBA 1$^+$ cells when compared to ethanol-naïve PSS-exposed mice ($p=.06$; Figure 3b).

Next, we sought to determine whether PSS and repeated binge-like ethanol consumption would alter IBA 1$^+$ microglia and brain macrophage dystrophy-related

phenotypes. Dystrophic phenotypes in microglia and brain macrophages are associated with a loss of function and senescence. Typical features associated with dystrophy include beaded somas and fractured processes (Shahidepour et al., 2021). (Fig 3a). Mixed-effects analysis revealed main effects of PSS ($p=.0016$), and ethanol consumption ($p<.0001$), and a PSS x ethanol interaction ($p=.02$). Post-hoc analyses demonstrated that both control and PSS mice that consumed ethanol had significantly more dystrophic microglia and brain macrophages compared to ethanol-naïve mice (ethanol-treated controls vs ethanol naïve controls: $p<.0001$, ethanol-treated controls vs. ethanol naïve PSS: $p<.0001$, ethanol-treated PSS vs. ethanol naïve controls: $p<.0001$, ethanol-treated PSS vs ethanol naïve: PSS $p<.0001$). Further, ethanol-treated PSS-exposed mice had significantly more dystrophic microglia and brain macrophages than ethanol-treated control mice ($p<.0001$; Figure 3c).

We then sought to determine the extent of microglia and brain macrophage reactivity within the vHC by measuring the area fraction (area coverage) of CD 68. CD 68 is a type 1 membrane protein found in macrophages in the periphery and CNS that binds to apoptotic cells. As microglia and brain macrophages engulf and clear apoptotic neurons from the brain, CD 68 area fraction increases, thus providing an indirect measure of microglia and brain macrophage reactivity (Chistiakov et al., 2017). Mixed-effects analysis revealed a main effect of ethanol ($p=.01$), no effect of PSS, and a PSS x ethanol interaction ($p=.05$). Post-hoc analyses demonstrated that ethanol-treated control mice had higher area coverage of CD 68 when compared to ethanol-naïve control ($p<.01$), PSS-exposed mice ($p=.02$) as well as ethanol-treated PSS-exposed mice ($p=.04$; Fig 3d).

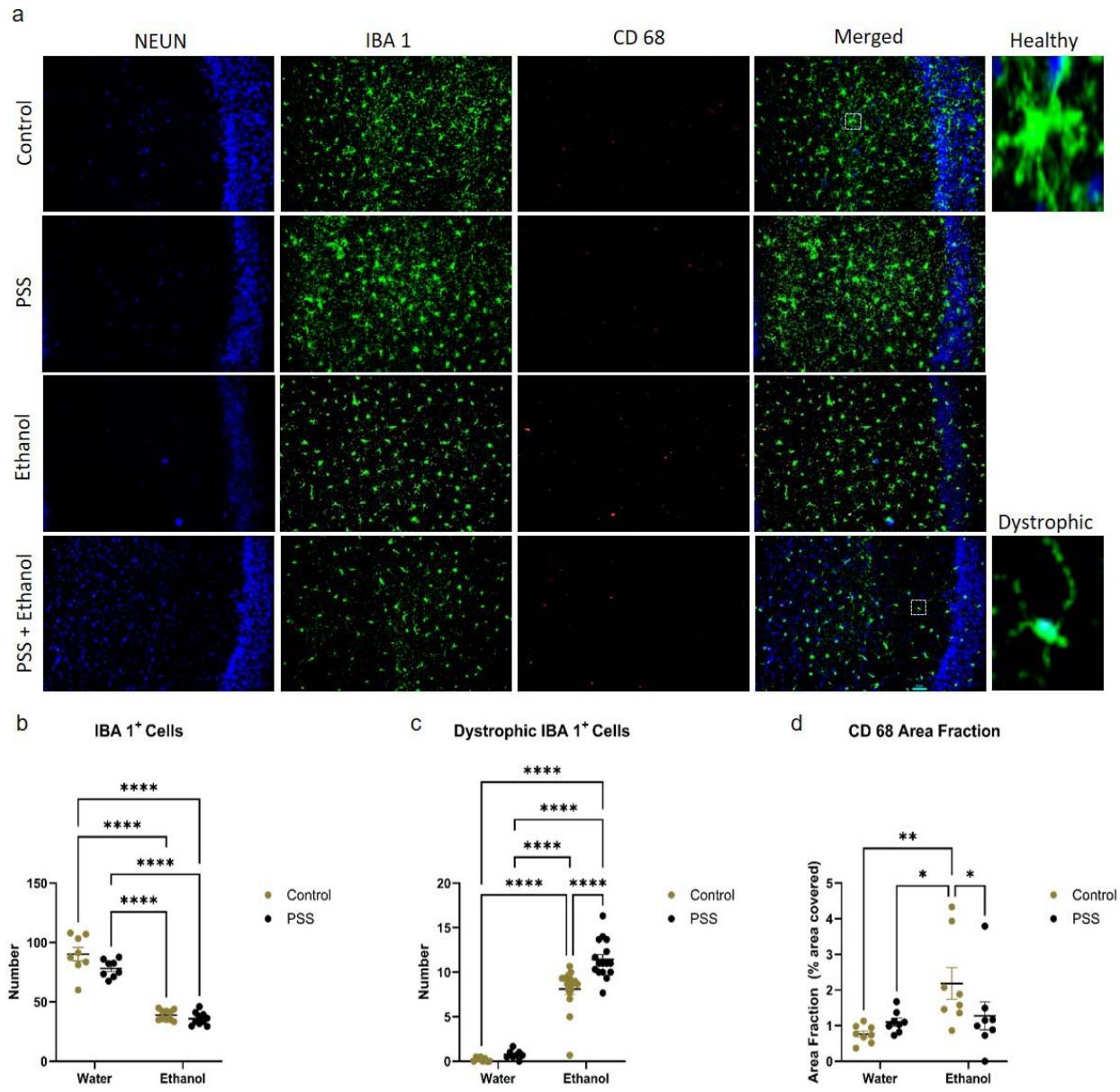

Figure 3. PSS and repeated binge-like ethanol consumption alter IBA 1+ cell number, phenotype, and reactivity in the ventral hippocampus. a) Representative images of the vHC collected from control, PSS, Ethanol, and PSS + Ethanol mice. Neuronal marker, Neun, in blue; microglia and brain macrophage marker, IBA 1, in green; and microglia reactivity marker, CD68, in red. Representative images of healthy and dystrophic microglia are included in the final column. b) The average number of IBA 1+ cells for

each exposure. c) The number of IBA 1+ cells exhibiting dystrophy-related phenotypes for each exposure. d) The area fraction of CD 68 for each exposure. * Indicates p<.05; ** indicates p<.01; *** indicates p<.001; and **** indicates p<.0001.

3.3 PSS exposure increases binge-like ethanol consumption in CX3XR1^{GFP+} mice

After performing these pilot experiments and observing the effects of PSS on binge-like ethanol consumption and microglia and brain macrophages in the vHC of C57 mice, we sought to determine whether PSS would have similar effects on other mouse strains and brain regions outside of the vHC. We chose a CX3XR1^{GFP+} mouse line due to its utility in labelling microglia and brain macrophages (Chakabroty et al., 2019). Mice were exposed to either control + vehicle (n=21; 13 male and 8 female), PSS + vehicle (n=18; 9 male and 9 female), or PSS + GW2580 (n=18; 10 male and 8 female) from P14-P21 (Fig 4; see timeline). At P60, CX3XR1^{GFP+} mice began DID as described in the methods.

Mixed-effects analysis revealed main effects of PSS (p=.006) and time (p<.0001) on average daily ethanol consumption, but no exposure x time interaction (p=.19). PSS-exposed mice consumed more ethanol than control mice (p=.003), while there was a trend towards PSS-exposed mice drinking more ethanol than GW2580-treated mice (p=.06) (Figure 5a). Looking at average ethanol consumption in male mice, mixed-effects analysis demonstrated a main effect of time (p<.0001), a trend towards a main effect of exposure (p=.09), but no exposure x time interaction (p=.15). However, there was a trend toward PSS-exposed male mice consuming more ethanol than control male

mice (p=.06) (Figure 5c; Supplemental Figure 3). Consistent with male mice, mixed-effects analysis revealed a main effect of time (p<.0001), a trend towards a main effect of exposure (p=.08), but no exposure x time interaction (p=.32) on average daily ethanol consumption in female mice. However, PSS-exposed female mice consumed more ethanol than control mice (p=.029), and PSS-exposed female mice trended toward having higher average daily ethanol consumption than GW2580-treated female mice (p=.14) (Figure 5e; Supplemental Figure 3).

We next sought to determine the effect of these exposures on cumulative ethanol consumption. Mixed-effects analysis revealed main effects of exposure (p=.015) and time (p<.0001); and an exposure x time interaction (p<.0001). PSS-exposed mice consumed more ethanol than control mice (p=.0064) and trended towards consuming more ethanol than GW2580-treated mice (p=.097) (Fig 5b). In male mice, mixed-effects analysis demonstrated a main effect of time (p<.0001), no effect of exposure (p=.17), and an exposure x time interaction (p<.0001) (Figure 5d). In female mice, mixed-effects analysis revealed a main effect of time (p<.0001), a trend towards an effect of exposure (p=.12), and an exposure x time interaction (p<.0001). Post-hoc analyses revealed PSS-exposed female mice consumed more ethanol across the study when compared to control female mice (p=.036) (Figure 5f; Supplemental Figure 3). Overall, PSS exposure had a more robust effect on female binge-like ethanol consumption when compared to male mice and GW2580 treatment was more effective at reducing ethanol consumption in female mice. For all statistics and additional group comparisons view Supplemental Figures 2-7.

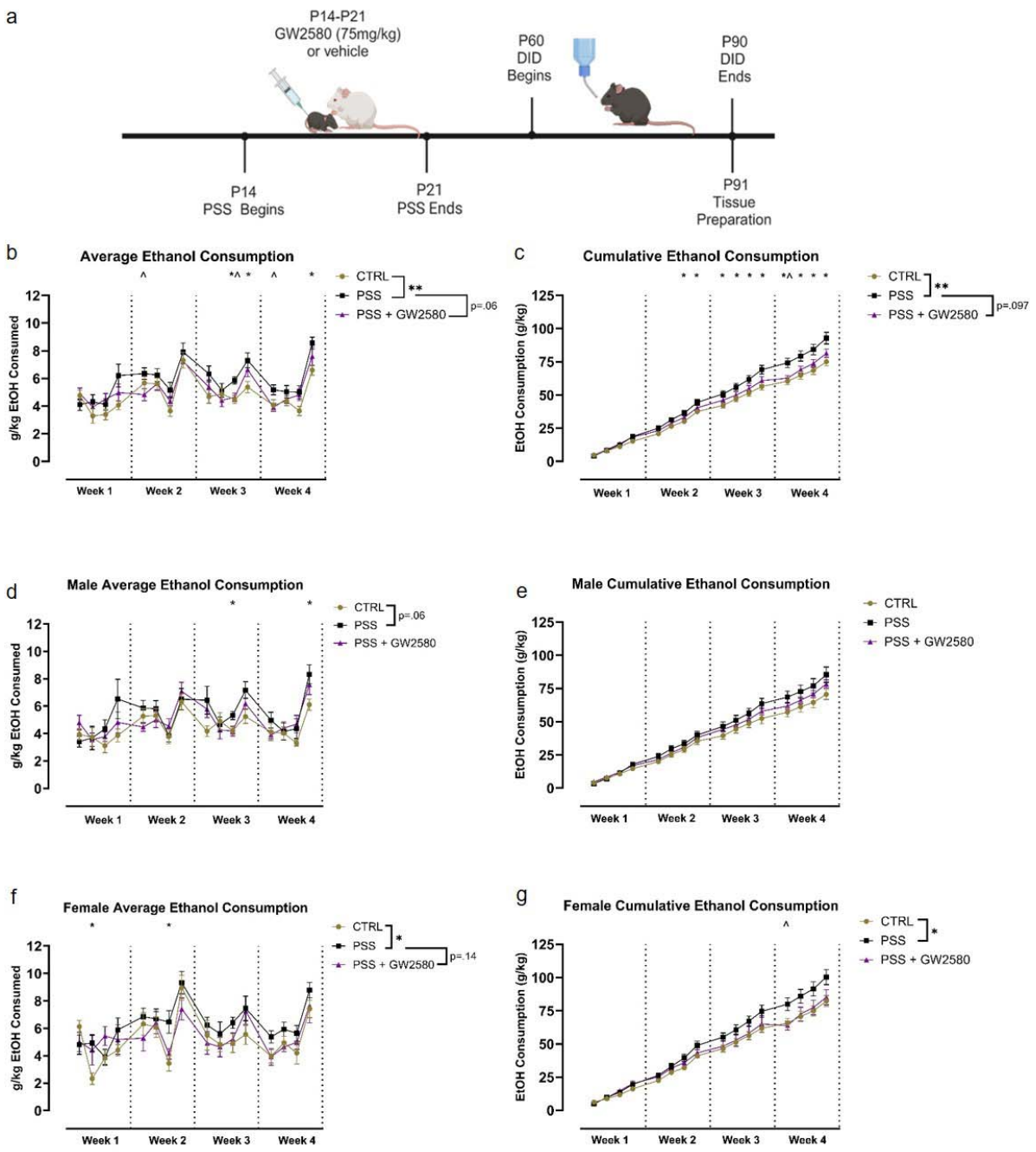

Figure 4. PSS exposure increases binge-like ethanol consumption in CX3CR1GFP+ mice. a) Experimental timeline: CX3CR1^{GFP+} pups were randomly assigned to either control or PSS exposure at P14. Control (n=21) and PSS-exposed (n=18) mice were administered a vehicle, while a cohort of PSS-exposed (n=18) mice were administered

GW2580 30 minutes prior to each PSS exposure. Following PSS at P21, control and PSS pups were weaned and group-housed until P60. At P60, control and PSS-exposed mice were briefly socially isolated for four nights a week across four weeks for DID. 24 hours following the final DID exposure, tissue was collected for LSFM. b) Daily average ethanol consumption for control, PSS-exposed, and GW-2580-treated mice. c) Cumulative ethanol consumption for control, PSS-exposed, and GW-2580-treated mice. d) Daily average ethanol consumption for control, PSS-exposed, and GW-2580-treated male mice. e) Cumulative ethanol consumption for control, PSS-exposed, and GW-2580-treated male mice. f) Daily average ethanol consumption for control, PSS-exposed, and GW-2580-treated female mice. g) Cumulative ethanol consumption for control, PSS-exposed, and GW-2580-treated female mice. Error bars indicate mean ± s.e.m. * indicates days that PSS-exposed mice consumed more ethanol than control mice. ^ indicates days that PSS-exposed mice consumed more ethanol than GW-2580-treated mice.

3.4 PSS exposure and repeated binge-like ethanol consumption alter CX3CR1^{GFP+} microglia and brain macrophage population density in a region-dependent manner

Following completion of DID, brains (n=27) were dissected from control (n=6; 3 male and 3 female), PSS-exposed (n=4; 2 male and 2 female), ethanol-exposed (n=5; 2 male and 3 female), PSS + ethanol-exposed (n=6; 3 male and 3 female), and GW2580-treated (n=6; 3 male and 3 female) mice for LSFM. While PSS and repeated binge-like ethanol consumption altered microglia and brain macrophage populations in the vHC, microglia are present heterogeneously throughout the brain. Further, microglia migrate

to different regions to respond to environmental insults with the purpose of maintaining brain homeostasis (Paolicelli et al., 2022). Therefore, we sought to determine how PSS exposure, repeated binge-like ethanol consumption, the combination of PSS exposure and repeated binge-like ethanol consumption, and GW2580 treatment altered microglia and brain macrophage populations within the vHC and across eleven additional brain regions including the prefrontal cortex, primary motor cortex, secondary motor cortex, somatosensory cortex, caudate putamen, hypothalamus, nucleus accumbens, amygdala, dorsal hippocampus, ventral tegmental area (VTA), and cerebellum. First, Imaris spot detection analysis was performed to count the number of CX3CR1^{GFP+} microglia and brain macrophages within each region (Figure 5a). Second, a nearest neighbor analysis was performed to identify the average distance between each cell and its three nearest neighbors (Figure 5b). This unbiased brain wide analysis revealed numerous changes that varied in a region, treatment, and sex dependent manner (Supplemental Figures 8-18). Further, we have provided movies of a 3D reconstructed brain as well as an individual CX3CR1^{GFP+} cell in the supplemental materials. Below we highlight the effect of these various exposures on microglia and brain macrophage population densities in the ventral hippocampus, somatosensory cortex, ventral tegmental area, and cerebellum.

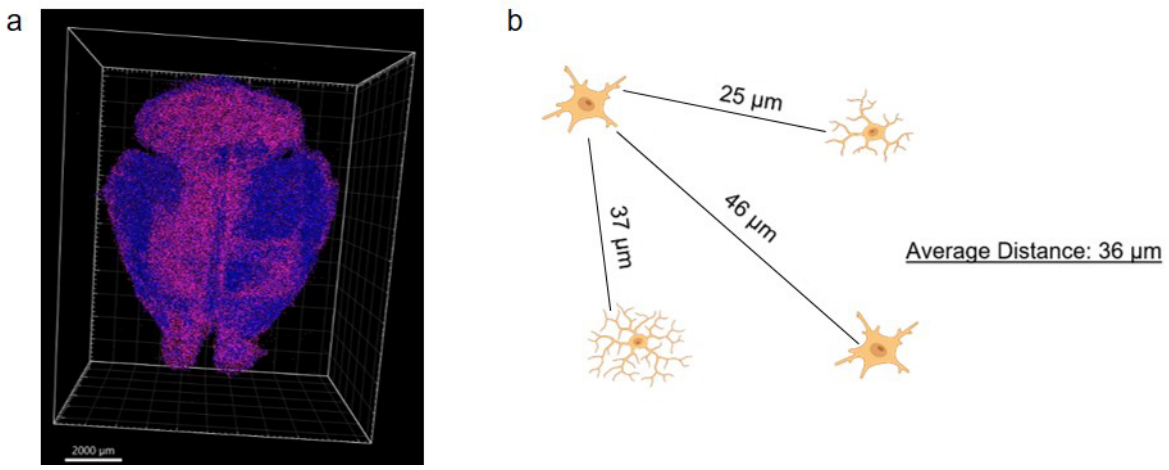

Figure 5. Light-sheet fluorescence microscopy analyses. a) Following 3D reconstruction, microglia and brain macrophage counts were collected using Imaris 10.1 spot detection software. Spots detection thresholds were set based on size (30µm+/-15µm) and circularity. b) Along with cell counting, nearest neighbor analysis was performed. This approach analyzes the average distance of each CX3CR1GFP+ cell to its three nearest neighbors. Nearest neighbor analysis provides an indirect measure of microglial reactivity.

In the vHC, two-way ANOVA analysis revealed a main effect of exposure (p=.04), no effect of sex (p=.27), and an exposure x sex interaction (p=.02). Post-hoc analyses demonstrated control male mice had more CX3CR1^{GFP+} cells than PSS + ethanol-exposed male (p=.006) and control female (p=.05) mice (Figure 6a). Further, two-way ANOVA analysis revealed a main effect of exposure (p<.0001), a trend towards a main effect of sex (p=.07), and no exposure x sex interaction (p=.75) on microglia and brain macrophage proximity. PSS + ethanol-exposed male mice had greater distance between CX3CR1^{GFP+} cells than control (p=.02; p=.0007) and ethanol-treated (p=.01;

p=.001) male and female mice. GW2580-treated male mice had greater distance between CX3CR1^{GFP+} cells than control female mice (p=.05). While PSS + ethanol-exposed female mice exhibited greater distance between CX3CR1^{GFP+} cells than control (p=.02) and ethanol-treated (p=.04) female mice (Figure 6b).

In the somatosensory cortex, two-way ANOVA analysis revealed a main effect of exposure (p=.0005), no effect of sex (p=.52), and a trend towards an exposure x sex interaction (p=.11) on CX3CR1^{GFP+} cell count. Post-hoc analyses demonstrated that PSS + ethanol-exposed male mice had fewer CX3CR1^{GFP+} cells than control (p=.03) and ethanol-treated (p=.006) male mice as well as PSS-exposed (p=.04) and ethanol-treated (p=.01) female mice (Figure 6c). Further, two-way ANOVA analysis revealed a main effect of exposure (p=.003), but not sex (p=.12) or an exposure x sex interaction (p=.64) on microglia and brain macrophage proximity. Post-hoc analyses demonstrated that PSS + ethanol-exposed male mice had greater distance between CX3CR1^{GFP+} cells than control (p=.007) and ethanol-treated (p=.03) female mice (Figure 6d).

In the ventral tegmental area, two-way ANOVA analysis revealed a main effect of exposure (p=.001), but not sex (p=.72) or an exposure x sex interaction (p=.31) on CX3CR1^{GFP+} cell count. Post-hoc analyses demonstrated ethanol-treated male mice had more CX3CR1^{GFP+} cells than control female mice (p=.05). While ethanol-treated female mice had more CX3CR1^{GFP+} cells than control male (p=.04) and female (p=.002) mice and PSS + ethanol-exposed (p=.04) male mice (Figure 6e). Further, two-way ANOVA analysis revealed a main effect of exposure (p=.05), but not sex (p=.19) or an exposure x sex interaction (p=.47) on microglia and brain macrophage proximity. Post-hoc analyses demonstrated no further group differences (Figure 6f).

In the cerebellum, two-way ANOVA analysis revealed main effects of exposure (p=.006), but not sex (p=.27) or an exposure x sex interaction (p=.18) on CX3CR1^{GFP+} cell count. Post-hoc analyses demonstrated ethanol-treated female mice had more CX3CR1^{GFP+} cells than PSS-exposed (p=.04), PSS + ethanol-exposed (p=.004), and GW2580-treated (p=.02) male mice as well as control (p=.004), PSS-exposed (p=.04), and GW2580-treated (p=.03) female mice (Figure 6g). Further, two-way ANOVA analysis revealed main effects of exposure (p=.0003) and sex (p=.04), but no exposure x sex interaction (p=.26) on microglia and brain macrophage proximity. Post-hoc analyses demonstrated that ethanol-treated female mice had shorter distance between CX3CR1^{GFP+} cells than PSS-exposed (p=.003; p=.02), PSS + ethanol-exposed (p=.005; p=.007), and GW2580-treated (p=.002; p=.003) male and female mice (Figure 6h).

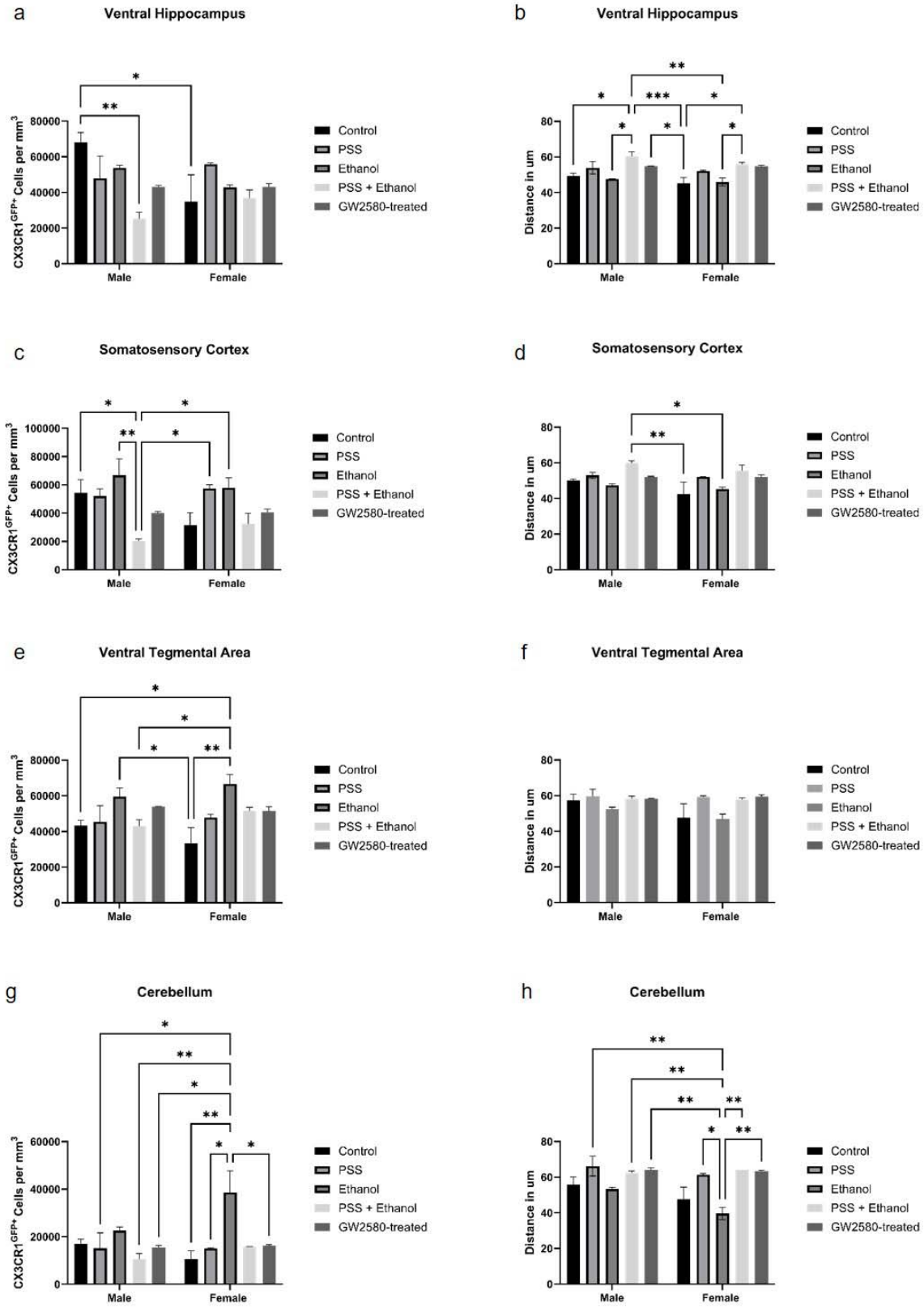
a
Ventral Hippocampus
CX3CR1GFP+ Cells per mm3
Male
Female
b
Ventral Hippocampus
Distance in um
c
Somatosensory Cortex
d
Somatosensory Cortex
e
Ventral Tegmental Area
f
Ventral Tegmental Area
g
Cerebellum
h
Cerebellum
Control
PSS
Ethanol
PSS + Ethanol
GW2580-treated

*Figure 6. PSS and repeated binge-like ethanol consumption alter microglia and brain macrophage count and proximity in a region and sex-dependent manner. a) Exposure-dependent changes in CX3CR1^{GFP+} cell number in the ventral hippocampus. b) Exposure-dependent changes in CX3CR1^{GFP+} cell proximity in the ventral hippocampus. c) Exposure-dependent changes in CX3CR1^{GFP+} cell number in the somatosensory cortex. d) Exposure-dependent changes in CX3CR1GFP+ cell proximity in the somatosensory cortex. e) Exposure-dependent changes in CX3CR1^{GFP+} cell number in the ventral tegmental area. f) Exposure-dependent changes in CX3CR1^{GFP+} cell proximity in the ventral tegmental area. g) Exposure-dependent changes in CX3CR1^{GFP+} cell number in the cerebellum. h) Exposure-dependent changes in CX3CR1^{GFP+} cell proximity in the cerebellum. * Indicates p<.05; ** indicates p<.01; *** indicates p<.001; and **** indicates p<.0001.*

4. Discussion

In this study, we sought to determine the effects of a novel ELS paradigm, PSS, on binge-like ethanol consumption, identify changes in microglia and brain macrophage populations across the brain, and determine whether microglia and brain macrophage inhibition was sufficient to prevent PSS-evoked binge-like ethanol consumption.

In C57 mice, we found that PSS altered developmental body weight in a sex-dependent manner and increased binge-like ethanol consumption (Figure 2). While prior work did not explore sex-dependent effects of PSS exposure on developmental body weight (Lo Iacono et al., 2017), our findings suggest that PSS exposure transiently reduced body weight gain in male mice. In contrast, female mice assigned to the PSS

had lower starting body weights, but gained weight at a faster rate than control female mice. These findings are consistent with a growing body of literature suggesting that outcomes related to ELS can be sex-dependent (Bath et al., 2020; Catale et al., 2020). Previous work investigating the effect of ELS and other forms of stress on palatable food seeking behaviors indicate that female mice are more likely to seek out and consume high fat chow following a stress exposure (Pankevich & Bale, 2008; Ruigrok et al., 2021). We hypothesize that PSS could be promoting sex-specific changes in feeding behavior or metabolism during the exposure window leading to these outcomes.

Following completion of the DID exposure, we collected and prepared vHC tissue to identify changes in microglia and brain macrophage function. We found that repeated binge-like ethanol consumption altered microglia and brain macrophage count, phenotype, and reactivity. Further, the combination of PSS exposure and repeated binge-like ethanol consumption exacerbated changes in microglia and brain macrophage phenotype (Figure 3c) and reduced ethanol-related increases in reactivity (Figure 3d). Consistent with our findings within the vHC, previous work has demonstrated that repeated binge-like ethanol consumption can reduce the number of microglia and brain macrophages in the dentate gyrus, as well as the CA 1, 2, and 3 regions of the dorsal hippocampus. (Nelson et al., 2021). This result is supported by existing literature demonstrating that ethanol exposure can lead to cell cycle arrest resulting in apoptosis and cell death (Do et al., 2013). In homeostatic conditions, microglia self-renew to maintain consistent region-specific population densities (Li & Barres, 2017). Due to the repeated ethanol exposures, microglia and brain

macrophages may not have an opportunity to self-renew resulting in the reduction in number observed in our study (Figure 3).

But what about the combination of PSS and binge-like ethanol consumption? Previous work has suggested that microglia may be primed by early life environmental insults resulting in lasting functional changes that could mediate an increased risk for problematic drinking behaviors and AUD following ELS (Melbourne et al., 2017). Further, early life corticosterone treatment can alter microglia function in response to future environmental factors (Frank et al., 2014). In this study, we found that PSS alone did not alter microglia count, phenotype, or reactivity. However, the combination of PSS and repeated binge-like ethanol consumption increased the number of microglia and brain macrophages exhibiting dystrophy-related phenotypes and blunted microglial reactivity (Figure 3c-d). We hypothesize that PSS primed microglia and brain macrophages leading to increased reactivity to the ethanol exposure and a greater susceptibility to the cytotoxic effects of ethanol. Following repeated ethanol exposures, PSS-exposed microglia and brain macrophages entered a chronic reactive state that resulted in more cells exhibiting dystrophy and reducing their functional viability.

In CX3CR1^{GFP+} mice, PSS increased binge-like ethanol consumption in early adulthood. After confirming that PSS facilitated an escalation in binge-like ethanol consumption in C57 and CX3CR1^{GFP+} mice, we compared the effect of PSS on ethanol consumption between the two strains. We found that C57 and CX3CR1^{GFP+} control mice did not differ in average daily ethanol consumption. However, PSS-exposed C57 mice consumed more ethanol than PSS-exposed CX3CR1^{GFP+} mice (Supplemental Figure 1). Although the CX3CR1^{GFP+} mouse line has the same genetic background as the C57

line, we believe that this difference in consumption may, in part; be due to seasonal variations in physiology and behavior. Recent work argues that variance in results across cohorts can be explained by internal biological rhythms (Suckow & Tirado-Muñiz, 2023). As such, future work will have to be performed to determine whether this is the case. Regardless of the underlying reasons for the cohort variance in ethanol consumption, PSS significantly increased binge-like ethanol consumption in both strains. Therefore, PSS may be an effective model of ELS for alcohol studies across strain and species.

In addition to validating the efficacy of PSS to evoke binge-like ethanol consumption, we sought to identify whether inhibiting microglia and brain macrophage function with GW2580 was sufficient to prevent escalations in ethanol consumption. We found GW2580 treatment trended towards reducing PSS-evoked increases in binge-like ethanol consumption. When taking sex into consideration, results suggest that PSS had a more robust effect on female mice when compared to male mice. Although not statistically significant, GW2580 treatment also appeared to be more effective at blunting the effects of PSS on binge-like ethanol consumption in female mice (Figure 4). Therefore, PSS may have a more robust effect on females than males resulting in the observed increased efficacy of the GW2580 treatment. While the effects of GW2580 treatment on binge-like ethanol consumption were not statistically significant, our data suggest that microglia and brain macrophage inhibition during PSS may reduce ethanol consumption in a study designed over a longer period, especially in female mice (Figure 4 f-g).

Following completion of DID, we created 3D reconstructions of CX3CR1^{GFP+} brains from each exposure to identify changes in microglia and brain macrophage population density across the brain. The initial goal of this portion of the study was to determine whether the effects PSS and ethanol consumption on vHC microglia and brain macrophages observed in C57 mice would generalize to CX3CR1^{GFP+} mice. Then, we sought to determine how these exposures altered microglia and brain macrophage population densities in other regions of the brain. In C57 mice, we observed that ethanol consumption decreased the number of microglia and brain macrophages regardless of PSS (Figure 3b). However, the combination of PSS and repeated binge-like ethanol consumption exacerbated the number of microglia and brain macrophages exhibiting dystrophy-related phenotypes and altered their function (Figure 3c-d). In contrast with these findings, our LSFM data collected from the vHC found that control male mice had higher CX3CR1^{GFP+} cell number when compared to PSS + ethanol exposed male mice and control female mice. While we were able to replicate the reduction in microglia and brain macrophage number in male mice exposed to PSS + ethanol, ethanol alone was not sufficient to reduce the number of microglia and brain macrophages in either sex (Figure 6a).

We propose the difference in outcomes is, in part, due to the difference in scale between the observations. In C57 mice, images were acquired from 40µm sections. In CX3CR1^{GFP+}, ROIs generated for LSFM were much larger and provided a more comprehensive look at how these exposures altered microglia and brain macrophage count. Despite this, LSFM data collected from the vHC further supports the notion that ELS experiences such as PSS may prime microglia to be more reactive to future

environmental insults, like ethanol consumption, resulting in increased rates of dystrophy and issues with function. Proximity data gathered from the vHC corroborates this notion. Regardless of sex, PSS + ethanol exposed mice exhibited greater distance between cells when compared to ethanol alone mice (Figure 6c). This suggests that PSS + ethanol exposed microglia and brain macrophages are not as reactive as microglia and brain macrophages from ethanol alone mice implying a reduction in functional viability. However, the most exciting outcome observed in the vHC was that GW2580 treatment prevented the effect of PSS on microglia and brain macrophage count and proximity (Figure 6a-b). In turn, this data highlights the importance of preventing the effects of ELS on microglia to ensure stable function later in life.

After comparing our findings in the vHC, we went on to perform population density analyses in eleven other regions of the brain (Supplemental Figure 8). While we found interesting outcomes in relation to these exposures in most regions, we discussed three regions: the somatosensory cortex, the ventral tegmental area, and the cerebellum.

We chose the somatosensory cortex for several reasons. First, ELS is known to alter the developmental trajectory of the somatosensory cortex (Takatsuru & Koibuchi, 2015). The somatosensory cortex's primary function is to process sensory information including nociception (Kropf et al., 2018). The PSS exposure we utilized in this study involves a lot of tactile stimulation from the foreign adult male. Previous work suggests that tactile stimulation between P14-P16 can alter longitudinal neuronal activity within the somatosensory cortex (He et al., 2017). Further, alcohol intoxication increases functional connectivity between the somatosensory cortex and the brain stem which is

related to issues with coordinating motor function (Shokri-Kojori et al., 2017). If PSS can alter the developmental trajectory of the somatosensory cortex leading to individuals being more prone to injury due to issues with motor impairment while under the influence of alcohol, preventing this outcome via microglia inhibition during PSS could reduce the risks associated with alcohol consumption. We found that male mice that were exposed to PSS and that engaged in repeated binge-like ethanol consumption had fewer CX3CR1^{GFP+} cells than control and ethanol-treated male mice and PSS or ethanol-treated female mice. Mice treated with GW2580 during PSS did not exhibit this change in CX3CR1^{GFP+} cell number suggesting that microglia inhibition was sufficient to prevent this long-term outcome (Figure 6c-d).

We then assessed how PSS and repeated binge-like ethanol consumption altered microglia and brain macrophage population density in the ventral tegmental area. The ventral tegmental area is a key regulator of environmental stimuli salience (You, Vandegrift, & Brodie, 2018). Existing literature widely supports the notion that ELS can blunt ventral tegmental area development leading to issues with reward appraisal (Park et al., 2021). PSS increases the number of microglia and brain macrophages within the ventral tegmental area following cocaine administration in adulthood (Lo Iacono et al., 2018). Further, previous work suggests that alcohol consumption can trigger adaptations within the ventral tegmental area reducing some of the aversive features of alcohol use (You, Vandegrift, & Brodie, 2018). As a result, the logic behind our decision to investigate the VTA was two-fold: 1) this region is susceptible to ELS and is an important regulator of alcohol use and 2) we wanted to determine whether we could replicate previous findings. We found PSS did not influence CX3CR1^{GFP+} cell

number or proximity in the ventral tegmental area. However, ethanol-treated female mice exhibited an increase in the number of CX3CR1^{GFP+} cells when compared to male and female control mice and PSS and ethanol-treated male mice (Figure 6e-f). Consistent with previous work, PSS alone did not lead to an increase in microglia and brain macrophage number. However, we could not replicate the effect of the combination of PSS and cocaine use on cell count. This to some extent was expected. The pharmacokinetics and pharmacodynamics of cocaine and alcohol are unique. Although both cocaine and alcohol can increase inflammation (Sil et al., 2019; Van de Loo et al., 2020), the impact these substances have on other regions of the brain may be distinct. This could explain the difference in microglia and brain macrophage number observed between the studies.

The last region we included in this commentary is the cerebellum. Although the cerebellum is most widely known for its involvement in motor function, a growing body of work suggests that it plays central roles in executive function, affective processing, and the establishment of motivational behaviors (Moulton et al., 2014). ELS is known to alter the developmental trajectory of the cerebellum in a sex-dependent manner (Moussa-Tooks et al., 2020). Like other regions of the brain, alcohol consumption increases inflammation and promotes cell death within the cerebellum (Mitoma et al, 2021). Due to the relationship between the somatosensory cortex and cerebellum in coordinating motor function and the suggested role of the cerebellum in executive function and reward, we determined this region to be a good addition to our discussion. We found that ethanol-treated female mice had more CX3CR1^{GFP+} cells and less distance between cells when compared to other exposures (Figure 6g-h). We were

surprised to find such a robust increase in the number of CX3CR1^{GFP+} cells exclusively in female mice. Recent work investigating the effects of adolescent intermittent ethanol exposure on cerebellar gene expression suggests that the effect of ethanol on gene expression is not sex-dependent (Healey et al., 2023). To the best of our knowledge, we are the first group to report a finding like this.

5. Conclusion

Our study demonstrates that early life PSS elicited increased binge-like ethanol consumption in two different mouse lines. Microglia and brain macrophage inhibition via GW2580, during PSS, trended towards reducing binge-like ethanol consumption. In the vHC, PSS exposure exacerbated the effects of repeated binge-like ethanol consumption on microglia and brain macrophage phenotype and reactivity. The effects of PSS and repeated binge-like ethanol consumption on microglia and brain macrophage count and proximity are sex and region-dependent. Finally, GW2580 treatment during PSS prevents long term changes in microglia and brain macrophage population density in the vHC and several other regions. Future work will seek to identify whether targeted inhibition of vHC microglia and brain macrophages during PSS is sufficient to reduce binge-like ethanol consumption.

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