The role of locus coeruleus neuroimmune signaling in the response to social stress in female rats

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Abstract

Neuropsychiatric disorders that result from stress exposure, including post-traumatic stress disorder and substance abuse, are highly associated with central inflammation. Our previous work established that females selectively exhibit increased proinflammatory cytokine release within the noradrenergic locus coeruleus (LC) in response to witnessing social stress, which was associated with a hypervigilant phenotype. Thus, neuroimmune activation in the LC may be responsible for the heightened risk of developing mental health disorders following stress in females. Further, ablation of microglia using pharmacological techniques reduces the hypervigilant behavioral response exhibited by females during social stress. Therefore, these studies were designed to further investigate the impact of stress-induced neuroimmune signaling on the long-term behavioral and neuronal consequences of social stress exposure in females using DREADDs. We first characterized the use of an AAV-CD68-G_i-DREADD virus targeted to microglia within the LC. While the use of AAVs in preclinical research has been limited by observations regarding poor transfection efficiency in mice, recent data suggest that species specific differences in microglial genetics may render rats more receptive to chemogenetic targeting of microglia using a CD68 promoter. Therefore, clozapine-n-oxide (CNO) was used to activate the microglial expressed hM4Di to inhibit microglial activity during acute exposure to vicarious social defeat (witness stress, WS) in female rats. Neuroimmune activity within the LC, quantified by microglial morphology and cytokine release, was augmented by WS and prevented by chemogenetic microglial inhibition. Following confirmation of DREADD selectivity and efficacy, we utilized this technique to inhibit microglial activity during repeated WS. Subsequently, rats were tested in a marble burying paradigm and exposed to the WS cues and context to measure hypervigilant behaviors. Chemogenetic-mediated inhibition of microglial activity prior to each WS exposure prevented both acute and long-term hypervigilant responses induced by WS across multiple behavioral paradigms. Further, a history of microglial inactivation during WS prevented the heightened LC activity typically observed in response to stress cues. These studies are among

the first to use a chemogenetic approach to inhibit microglia within the female brain *in vivo* and establish LC inflammation as a key mechanism underlying the behavioral and neuronal responses to social stress in females.

Introduction

Stress exposure is a risk factor for many physiological and neuropsychiatric disorders including post-traumatic stress disorder (PTSD) and substance use disorder (Cohen et al., 2007; Liu et al., 2017). While up to 90% of the general population reports experiencing a highly stressful or traumatic event (Benjet et al., 2016), around 15% of these people will go on to develop conditions such as PTSD (Davidson, 2000). This disorder is unique in the fact that it requires exposure to a stressor or traumatic event for its initiation (Bisson et al., 2015). Interestingly, while males report greater levels of trauma exposure, females develop PTSD at twice the rate of males (Frans et al., 2005). Further, there are different symptom patterns between the sexes with the hyperarousal and hypervigilance aspects of PTSD symptomology (Miao et al., 2018) observed to a higher degree in females (Bangasser & Valentino, 2014; Irish et al., 2011). Early emergence of hyperarousal and hypervigilance are also associated with less symptom improvement over time and worse treatment outcomes when compared to traumatized individuals that did not exhibit this symptom profile (Marshall et al., 2006; Schell et al., 2004). Thus, there may be inherent neurobiological factors that promote hypervigilance and contribute to the higher rate of development of these stress-related disorders in females (Bangasser & Valentino, 2014).

One brain region highly involved in stress responses and hypervigilance is the noradrenergic locus coeruleus (LC) (Bangasser & Valentino, 2014; Borodovitsyna et al., 2020; Giustino et al., 2020; C. Naegeli et al., 2018; O'Donnell et al., 2004). Exposure to stress increases activity within this region, and LC hyperactivity is evident in patients with PTSD (Krystal et al., 2018). Further, the LC has been directly observed to promote PTSD-related behaviors including hypervigilance (McCall et al., 2015; C. Naegeli et al., 2018). As the main noradrenergic center of

the brain, increased activity in the LC promotes norepinephrine (NE) release in multiple downstream brain regions that mediate stress-related behaviors (Borodovitsyna et al., 2020; McCall et al., 2017). Therefore, further research into the underlying mechanisms responsible for stress-induced increases in LC-NE activity is essential for understanding the role of this system in PTSD-related behavioral dysfunction. Importantly, the LC has been shown to be highly regulated by neuroimmune signaling, with proinflammatory cytokines selectively administered within this region leading to increased neuronal firing (Borsody & Weiss, 2002; Borsody & Weiss, 2004). This ability of neuroimmune signaling may serve underlying factor responsible for the increases in LC activity observed in response to stress. Importantly, PTSD, among other highly prevalent stress-induced neuropsychiatric disorders, is associated with increases in inflammatory factors in the brain and periphery (Hänsel et al., 2010; Ravi et al., 2021; Smiley & Wood, 2022). Further, neuroimmune signaling has been associated with psychiatric dysfunction following stress in rodent models (Finnell et al., 2018; B. S. Pate et al., 2023) and in clinical populations (Engler et al., 2016), especially in females (Bekhbat & Neigh, 2018; Levesque et al., 2023). Specifically, lipopolysaccharide (LPS) injections evoke greater inflammatory, noradrenergic, and cortisol responses in women which were associated with worsening mood symptoms that were not observed in men (Engler et al., 2016; Moieni et al., 2015). Overall, these data support the hypothesis that the predisposition to stress-related neuropsychiatric disorders in females may be due to an underlying sensitivity to the neuroimmune impact of stress.

Despite accumulating evidence that suggests a role for neuroimmune signaling in heightened stress susceptibility among females, studies examining a specific role for microglia are sparse, largely due to a lack of effective tools in the field. Our previous studies using clodronate, a compound designed to pharmacologically ablate microglial cells, supports the hypothesis that microglia within the LC play a major role in the female stress response (B. S. Pate et al., 2023). Therefore, by utilizing chemogenetics to transiently inhibit microglial activation during social stress exposure, these studies were designed to further explore the role of neuroimmune

signaling in the acute and long-term behavioral consequences associated with hypervigilance in females. Further, the impact of prior intra-LC microglial inhibition on neuronal activity during subsequent WS context exposure in female rats was assessed to determine how microglia shape stress context-dependent neuronal activation. DREADD viruses designed to chemogenetically inhibit microglia have been previously characterized for use in the spinal cord (Grace et al., 2016; Grace et al., 2018) and, through G-protein coupled receptors (GPCRs), can prevent microglial activation and subsequent release of proinflammatory cytokines (Gu et al., 2021; Haque et al., 2018; Kettenmann et al., 2011; Mizoguchi & Monji, 2017). However, there have been species specific differences observed with regard to the immune system and inflammatory reactivity in preclinical experiments. The genetic profile of microglia and cytokine release upon activation in response to inflammatory insults varies between murine species (Lam et al., 2017; Lively et al., 2018). Specifically, rats exhibit six times greater levels of CD68 in microglial cells when compared to mice, which may lead to a better transduction efficiency of viruses with a CD68 promoter when applied to rats compared to mice (Lam et al., 2017). Further, rats exhibit an immune system that is more genetically similar to humans than that of the mouse (Wildner, 2019). Therefore, when testing novel immune strategies for the translational treatment of neuropsychiatric disorders and new applications of preclinical techniques targeting immune cells, it is important to distinguish between results that have been achieved in different species. These experiments used these techniques to modulate microglial activity during acute and repeated psychosocial stress to assess the short- and long-term behavioral impact of stress-induced neuroimmune signaling in the LC of female rats.

Materials and Methods

Animals

Female Sprague-Dawley rats (~200 g and 9 weeks on arrival, witnesses/controls) and male Sprague-Dawley rats (~250 g on arrival, intruders) were obtained from Charles River

(Durham, NC) while male Long-Evans retired breeders (600-800 g, residents) were obtained from Envigo (Dublin, VA). All rats were housed individually in standard polycarbonate cages while female rats that underwent stress and all males were housed in a separate but adjacent room from control females. Rats were all maintained on a 12-hr light/dark cycle with the light cycle initiated at 0700 and received *ad libitum* access to food and water. Further, all cages, including the home cage and those used during behavioral testing, contained Teklad sani-chip bedding (Envigo). These studies were approved by the University of South Carolina's Institutional Animal Care and Use Committee and maintained adherence to the National Research Council's Guide for the Care and Use of Laboratory Animals.

There were two main experimental subsets completed to 1) characterize the use of chemogenetic techniques for the site-specific manipulation of microglial activity in the brain and 2) determine the role of LC microglia in the behavioral and neuronal responses to social stressors in females. Experiment #1A used 32 rats (n = 16/group, WS+VEH vs. WS+CNO) to determine the localization of the hM4Di expressing virus to microglia and establish its ability to prevent stress-induced neuroimmune signaling, while a second group of surgically naïve rats in Experiment #1B (28 rats, n = 7/group, CON+VEH vs. CON+CNO vs. WS+VEH vs. WS+CNO) were used to confirm the absence of off-target effects of CNO (**Figure 1A**). Experiment #2 comprised 34 rats with virus injected into the LC (n = 8-9/group, CON+VEH vs. CON+WS vs. WS+VEH vs. WS+CNO) to investigate the role of microglial activity during stress on hypervigilance and neuronal activity within the LC (**Figure 1B**).

Surgical Procedures/DREADD infusion

All experiments, excluding those designed to assess off-target effects of CNO, commenced with stereotaxic surgery to infuse a pAAV1 CD68-hM4D(G_i)-mCherry virus (Addgene viral prep #75033-AAV1 from Bryan Roth, Addgene, Watertown, MA) into the LC. While typically an AAV lacking the G_i component is utilized as the control condition for chemogenetic

experiments, these studies were limited by the lack of a commercially available pAAV1 CD68-mCherry virus and, therefore, all rats were injected with the active virus and systemic vehicle injections were used in parallel with CNO administration to control for viral activation. During surgery, electrophysiological recordings were used for precise identification of the LC to individual localize viral injection. Rats were anesthetized using isoflurane (5% for induction, 2-3% maintenance) and placed into a stereotaxic apparatus. The nose was angled down 10° and, starting at the following coordinates, in mm from bregma and the surface of the dura, A/P: -4.0; M/L: ± 1.2, D/V: -4.5, a tungsten coated electrode was lowered through the tissue while an oscilloscope relayed neuronal firing patterns. Once the LC was located, these coordinates were used to lower a microsyringe (Hamilton, Franklin, MA) to inject 500 nL of the pAAV CD68-hM4D(G_i)-mCherry virus (100 nL/minute) and the needle was left in place for at least 5 minutes following termination of the injection. These same coordinates were used for the contralateral LC to achieve bilateral infusion of the virus. Saline (10 mL/kg, s.c.) and flunazine (0.25 mg/kg, s.c.) were administered on the day of and day following surgery along with supplemental nutrition (Bacon Softies, Bio-Serv) as needed. Rats were given at least 10 days of recovery following surgery before initiation of pre-stress behavioral assays and at least 21 days prior to stress to allow for viral expression. During this recovery period, rats were given i.p. saline injections three times a week for habituation prior to drug delivery.

Drug Information

Water soluble clozapine-n-oxide (CNO) (HelloBio, Princeton, MA) was dissolved in sterile saline (0.9%) and rats were administered CNO (1 mg/kg) or vehicle (0.9% saline) intraperitoneally (i.p.) one hour before the start of stress either once (acute stress) or for five days in a row (repeated stress). A subset of surgically naïve animals that received CNO in the absence of viral injection was included to confirm that CNO alone did not impact microglial activation state or hypervigilant behaviors.

Behavioral Measurements

Marble Burying

Female rats in Experiment #2 were exposed to marble burying to assess behavioral responses to a novel stimulus known to induce hypervigilant behaviors in rodent models of PTSD (Kedia & Chattarji, 2014; Mikics et al., 2008; Sarkar, 2020; Smiley et al., 2023). A pre-stress test was completed during the 3-week viral incubation period to determine individual baseline levels of marble burying behavior. Subsequently, a post-stress test was done four days following the end of stress to determine the effect of WS and microglial inhibition on these behaviors in within-subjects comparisons. Rats were placed into a novel home-cage containing 5 cm of standard bedding with a 5x3 grid of glass marbles (1.5 cm in diameter) for 15 minutes during which behaviors were video recorded for retrospective quantification. The number of marbles with over 2/3 of their height covered by bedding were counted at the end of the 15-minute session and videos were assessed by a blinded experimenter for the following behaviors: burying (manipulation of the bedding by the fore- or hind-paws), marble interaction (any contact with the marble that is not during burying), rearing (extension of the forepaws with the hind paws remaining in contact with the bedding), and freezing (absence of all movement excluding respiration or vocalization). Data were calculated as a change from pre- to post-stress testing.

Witness Stress or Control Handling

Female rats in all experiments were exposed to witness stress paradigms (WS) as previously described (J. E. Finnell et al., 2018; B. S. Pate et al., 2023; Smiley et al., 2023). Briefly, a clear perforated Plexiglas partition was placed into the cage of a male Long-Evans retired breeder (resident) to allow for the transfer of visual, olfactory, and auditory cues from one side of the cage to the other (described in detail in (Finnell et al., 2018)). The female witness was placed in the smaller side followed immediately by the male intruder into the side containing the resident and witnesses observed the aggressive social defeat encounter for 15 minutes. Female rats were

either exposed to a single acute stressor (Experiment #1A & B) or this paradigm was completed once a day for a total of five days for those that received repeated stress (Experiment #2), and CNO or vehicle was administered one hour prior to the start of each stress exposure. Witnesses were paired with the same male intruder rat for daily WS, but the resident was novel each day. Control rats (CON) were briefly handled and returned to their home cage where behaviors were recorded. Previous experiments from our lab established that females exposed to the resident's cage behind the partition, with the intruder on the opposite side (Finnell et al., 2018) or in absence of the intruder (Smiley et al., 2023), do not exhibit behavior that differs from rats handled and returned to their home cage, thus, this procedure was utilized as the control. Sessions were video recorded on the first and fifth days of exposure to allow for retrospective manual scoring of stress-evoked behaviors. Briefly, behaviors including burying, freezing, and rearing were quantified according to the following attributes and our previous publications (Finnell et al., 2018; Pate, 2023; Smiley et al., 2023). Burying was counted when the female rat exhibited spontaneous and rapid manipulation of the bedding. Freezing consisted of complete immobility with the exception of respiratory movement or vocalization. Rearing was displayed when the animal raised their forepaws off of the bedding, with or without extension of the hindlimbs. The latency to begin each behavior along with the total duration was quantified and analyzed.

Witness Stress Context Exposure (WS CXT)

WS CXT consisted of placing the female rat behind the partition in the residents' cage with their paired intruder on the opposite side but in the absence of the resident to expose the females to the sensory cues and environment in which they originally experienced social stress. Previous studies from our lab have determined that a history of stress exposure leads to increased burying in the WS CXT and this paradigm serves as a method of translationally measuring hypervigilant behaviors (Smiley et al., 2023). Behaviors were scored identically to those assessed

during WS (described above) and were compared to CON rats that were handled and recorded in their home cages.

Euthanasia and Tissue Collection

Rats in Experiment #1A and B were euthanized 120 minutes after the start of acute stress while those in Experiment #2 were euthanized 120 minutes following the start of WS CXT exposure. Rats were either transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) or simply decapitated after isoflurane anesthesia overdose in a bell jar. Perfused brains were extracted, blocked into anterior and posterior sections, post-fixed in 4% PFA for at least 2 hours, and saturated with 20% sucrose + 1% azide upon which they were flash frozen using ice cold isopentane and stored at -80°C until further analysis while non-perfused brains were flash frozen prior to dissection.

Brain Analysis

Perfused brains were sliced at 30 microns on a cryostat (Leica) and slices from the LC were collected and stored in cryoprotectant at -20°C until immunohistochemical analysis while non-perfused brains were biopsied for discrete brain regions. Sections were analyzed for either 1) mCherry localization to the LC, 2), mCherry selectivity for microglial cells, 3) microglial morphology with Iba1 staining (Kedia & Chattarji, 2014), or 4) LC neuronal activation with colabeled cfos and tyrosine hydroxylase (TH) which can be used to label neurons within the LC (Fan et al., 2014). Tissue biopsies were assessed via Western Blot for the effects microglial inhibition on cytokine release during stress. Visual confirmation of mCherry and Iba1 colabeling within the LC was completed with confocal microscopy (Leica Stellaris) to ensure that mCherry was selectively localized to microglial cells. Microglial responses were quantified using morphological analysis to assign reactivity status, either homeostatic or reactive (nomenclature based on (Paolicelli et al., 2022)) individually to each cell based on cell body and projection shape

(**Supplementary Figure 1**) (Karperien et al., 2013). Molecular experiments were completed following the protocols outlined below.

Immunohistochemistry

Tissue slices were washed 4x10 minutes in 0.1M PBS, immersed for 30 minutes in 0.9% hydrogen peroxide + 4% Triton-X 100 (TX) in 0.1M PBS, and incubated in 1° antibodies overnight at room temperature in antibody buffer consisting of 1% normal donkey serum (NDS) + 0.25% TX in 0.1M PBS. Antibodies were used at the following concentrations: anti-Iba1 1:1000 (rabbit, FujiFilm Wako Chemicals #019-19741), anti-cfos 1:500 (rabbit, Millipore #ABE-457), and anti-TH 1:2500 (mouse, Millipore #MAB-5280). The following day, tissue slices were washed 3x10 minutes in 0.1M PBS prior to a 2-hour incubation at room temperature in secondary antibody (diluted in antibody buffer) that corresponds with the species of the primary: biotinylated anti-rabbit 1:200 (Vector Labs #BA-1000) or biotinylated anti-mouse 1:1000 (abcam #ab208001). Tissue was then washed in PBS (3x10 minutes) and incubated in avidin biotin complex (Vectastain Elite ABC Kit, Vector Laboratories) diluted in 1% NDS in 0.1M PBS for 1 hour. Three subsequent washes (10 minutes each in 0.1M PBS) occurred prior to staining in diaminobenzidine (DAB) or metal enhanced DAB (Vector Laboratories) for double labeling experiments. The DAB development times were optimized for each antibody, with 1 minute and 30 seconds for Iba1, 2 minutes for cfos, and 15 seconds for TH. Tissue was placed in a final set of 3x10 minute washes before mounting on slides (SuperFrost). Once dried, slides were dehydrated in increasing concentrations of ethanol (70%, 95%, 100% x2, each for 1 minute) and placed into HistoClear (2 and 5 minutes) prior to coverslipping with Permount (Fisher Scientific). Slides were imaged using a Nikon E600 microscope and manually quantified using ImageJ for microglial morphological state or counted for cfos/TH+ cells by an experimenter blinded to the treatment conditions.

Immunofluorescence (IF)

IF techniques were improved by utilizing antibody signaling enhancing buffers to increase the permeabilization of cells (Piña et al., 2022). Brain slices were washed 4x10 minutes in 0.5% Tween-20 in 0.1M PBS and incubated for 30 minutes in blocking buffer consisting of 2% NDS, 50 mM glycine, 0.05% Tween-20, 0.1% TX, and 0.01% bovine serum albumin (BSA) in 0.1M PBS. Sections were then placed into primary antibody overnight at 4°C in antibody buffer containing 10 mM glycine, 0.05% Tween-20, 0.1% TX, and 0.1% hydrogen peroxide in 0.1M PBS along with either anti-Iba1 1:1000 (rabbit, FujiFilm Wako Chemicals #019-19741), anti-NeuN 1:500 (mouse, Millipore #MAB-377), or anti-GFAP 1:1000 (rabbit, Cell Signaling #12389T) primary antibody. Subsequently, tissue was rinsed in 0.5% Tween-20 in 0.1M PBS (3x10 minutes) and incubated in the secondary antibody (anti-rabbit Alexa-Fluor 488, 1:1000, abcam #150073) diluted in 0.1% Tween-20 in 0.1M PBS. Slices were then washed 3x10 minutes in 0.1M PBS, mounted onto slides (SuperFrost), and coverslipped with Prolong Diamond Media (Thermo Fisher). Slides were imaged on a confocal microscope (Leica Stellaris) for localization of viral-mediated mCherry with Iba1+ microglial cell staining.

Western Blot Analysis

LC tissue biopsies were collected for Western Blot analysis of IL-1β protein concentration. Flash frozen brains were sliced on a cryostat (Leica) starting at the brain stem, until reaching the most posterior point of the LC, at which time a biopsy punch (1 mm in diameter, 1 mm depth) was used to selectively dissect out the LC. Post punch slices were collected and stained with Neutral Red to confirm that all LC cells were removed. Tissue was homogenized with 200 µL of lab-made lysis buffer (37 mM NaCl, 20 mM Tris, 1% Igepal, 10% glycerol, and 1X Halt Protease Inhibitor Cocktail [Thermo Scientific, #87786]) and 100 mg of zirconium oxide beads (Next Advance, Inc., Troy, NY) in a Bullet Blender followed by centrifugation at 1400 rcf for 15 minutes at 4°C. Supernatant was collected and a 12 µL aliquot was diluted 1:4 with 0.1 PBS + 1% azide for protein concentration analysis using a Pierce Bicinchoninic Acid (BCA) assay

(Thermo Scientific, Rockford, IL) per the manufacturers protocol. Samples containing 20 µg of protein were aliquoted for Western Blotting. 6X sample buffer + β-mercaptoethanol was added at a 1:6 ratio with the LC homogenate and heated at 75°C for 5 minutes. LC homogenate was loaded onto mini-protean gels (Bio-Rad, Hercules, CA), placed into electrophoresis chambers filled with 1X running buffer (Bio-Rad), and run at 135 V for 1 hour. Proteins were transferred from gels onto PVDF membranes in 1X transfer buffer (Bio-Rad) and run at 100 V for 90 minutes. Membranes were blocked in a solution containing a 1:1 ratio of 0.1M PBS:Fluorescent Blocking Buffer (Millipore Sigma, Burlington, MA) for 1 hour prior to incubation in primary antibodies overnight a 4°C. The antibodies for IL-1β (1:200, goat, R & D Systems #AF-501-NA) and the housekeeping protein GapDH (1:1000, mouse, Santa Cruz #sc-365062) were diluted in blocking buffer + 0.2% Tween-20. Membranes were washed in 1X TBS + 1% Tween-20 before incubation in secondary antibodies IR-Dye anti-rabbit 800 nm (LI-Cor #926–32213) and IR-Dye anti-mouse 680 nm (LI-Cor #962–68072) diluted 1:20,000 in blocking buffer + 0.2% Tween-20 + 0.1% sodium dodecyl sulfate (SDS) for 1 hour. Membranes were washed (3x10 minutes in 1X TBS + 1% Tween-20), imaged on a LI-Cor Odyssey scanner (LI-Cor Biotechnology, Lincoln, NE), and protein expression was quantified using LI-Cor Image Studios Software with normalization to GapDH. Full Western Blot images are provided within the supplement (**Supplementary Figure 2**).

Statistical Analysis

All analysis were completed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA). Data were analyzed using either an unpaired t-test, to determine differences between stressed animals treated with either VEH or CNO, or two-way ANOVA with Tukey post-hoc analysis, to compare data in experiments with a 2x2 design assessing CON vs. WS and VEH vs. CNO. Data are presented as mean ± standard error (SEM) with an $\alpha = 0.05$. Significant main effects are reported in the text with significant post hocs reported on the figures.

Results

Characterization of chemogenetic-mediated microglial inhibition

Experiments #1A & B were completed to establish the use of pAAV1 CD68-hM4D(G_i)-mCherry to site-specifically inhibit WS-evoked microglial activity. The CD68 promoter allows selective insertion of G_i receptors onto microglial cells, and this virus has been previously characterized for microglial localization and inhibition in the spinal cord (Grace et al., 2018; Yi et al., 2021). Immunofluorescent labeling of Iba1 was visualized along with mCherry to confirm that virus was solely incorporated into microglial cells at 20x (**Figure 2A**) and 63x 3D magnification (**Figure 2B**). Further analysis of this tissue using the neuronal and astrocytic markers NeuN and GFAP, respectively, revealed a lack of colocalization of mCherry with either neurons or astrocytes (20x, **Figure 2C & D**). Since microglial infection by AAVs has been determined to be brain region specific (Aschauer et al., 2013), we quantified the total number of Iba1 + and Iba1 and mCherry colocalized cells within the LC and established that, on average, around 50% of LC microglia contained mCherry (**Figure 2E**). Subsequently, the ability of this virus to mediate a reduction in neuroimmune signaling that results from WS was assessed as a function of microglia morphology and cytokine release. CNO (1 mg/kg, i.p.) or vehicle was administered 1 hour prior to a single acute session of WS, and microglial activation was assessed through levels of IL-1β and morphological state in tissue collected 6 hours after stress. Chemogenetic-mediated microglial inhibition within the LC reduced levels of the pro-inflammatory cytokine IL-1β evoked by exposure to WS *(***Figure 2F**, t_{10} = 1.928, p = 0.0414*).* Further, when microglial morphology was manually scored based on cell body size and projection complexity, with ramified and rodlike cells counted as “homeostatic” and reactive and ameboid cells counted as “reactive” (**Supplementary Figure 1**), WS increased the presence of ameboid, reactive cells while CNO treatment in these rats prevented this response, as evidenced by an increase in the ramified, homeostatic phenotype (**Supplementary Figure 3**). Further, CNO prior to stress elicited a reduction in the percentage of reactive microglia (**Figure 2G**, t_6 = 7.816, p = 0.0002) and an increase in homeostatic microglia

(**Figure 2H**, t_6 = 7.816, p = 0.0002). A decrease in stress-induced hypervigilant behavior paralleled this shift in microglial morphology. Specifically, CNO-treated females displayed a decreased amount of burying in response to WS (**Figure 2I**, t_{13} = 2.978, p = 0.0053), a behavior that we have previously determined to be linked to LC neuroimmune activity (Finnell et al., 2018; Pate et al., 2023). Further, to ensure there were no off-target effects of CNO, a separate set of surgically naïve rats with no viral injection were treated with either VEH or CNO. While stress alone increased the percentage of reactive microglia and decreased the proportion of homeostatic cells, CNO had no effect in rats lacking the viral infusion (**Figure 2J,** reactive microglia, stress effect; $F_{1,11}$ = 39.09, p < 0.0001, drug effect; $F_{1,11}$ = 0.3312, p = 0.5766, **Figure 2K,** homeostatic microglia, stress effect, $F_{1,12}$ = 8.541, p = 0.0129; drug effect, $F_{1,12}$ = 1.45, p = 0.2517). Similarly, while there was a significant increase in burying behavior during WS when compared to CON, there was no effect of CNO alone on behavior during WS or in home cage behaviors following handling in CON rats (**Figure 2L**, stress effect; $F_{1,17}$ = 45.59, p < 0.0001, drug effect; $F_{1,17}$ = 0.00019, p = 0.9892).

Microglial inhibition prevents the behavioral effects of acute and repeated WS

A separate group of female rats were injected with the CD68-hM4Di-expressing virus selectively within the LC prior to assessment of behavioral tasks following stress. When first analyzing behaviors in response to acute WS, there was a significant effect of stress on burying behavior (**Figure 3A**, main effect of stress, $F_{1,29}$ = 18.3, p = 0.0002; main effect of drug, $F_{1,29}$ = 9.349, p = 0.0048), replicating both our previous studies (Finnell et al., 2018; Pate, 2023; Smiley et al., 2023), additional studies using the WS model (Barbetti et al., 2023), and the results in **Figure 2H**. Additionally, CNO treatment one hour prior to the stressor diminished the burying response typically observed in females exposed to WS (**Figure 3A**, p < 0.005, WS + VEH vs. WS + CNO). Furthermore, WS exposed rats treated with CNO exhibited a longer latency to begin burying (**Figure 3B**, t_{15} = 2.478, p = 0.0128) and an increased duration of rearing (**Figure 3C**, t_{15} = 1.548, p = 0.0712) when compared to WS females that received VEH. These acute effects were

also observed in response to repeated stress. On WS Day #5, burying behavior was heightened in stressed females and significantly attenuated after CNO treatment prior to each stress exposure (**Figure 3D**, interaction, $F_{1,28}$ = 10.06, p = 0.0037; main effect of stress, $F_{1,28}$ = 19.74, p = 0.0001; main effect of drug, $F_{1,28}$ = 12.56, p = 0.0014; p = 0.0002, WS+VEH vs. WS+CNO). While there was no longer a significant increase in the latency to begin burying, due to an increased latency in the VEH treated animals (**Figure 3E**, t_{16} = 0.4082, p = 0.3443), there was still a higher amount of rearing observed in stressed females that were treated with CNO (**Figure 3F**, t_{16} = 1.701, p = 0.0542).

Inhibition of LC neuroimmune signaling recovers behaviors impacted by stress

Rats were tested for novelty-induced hypervigilant behaviors in the marble burying task. When comparing MB-evoked behaviors within subjects between pre- and post-stress testing, rats treated with CNO prior to each stress exposure exhibited a decrease in the number of marbles buried (**Figure 4A**, t_{15} = 1.696, p = 0.0553) with no impact on overall burying duration (**Figure 4B**, t_{15} = 0.8421, p = 0.2065) or marble interaction time (**Figure 4C**, t_{15} = 1.207, p = 0.123). Subsequently, when assessing stress cue-induced hypervigilance in the WS CXT, there was a significant interaction ($F_{1,30}$ = 19.69, p = 0.0001) between stress (main effect of stress, $F_{1,30}$ = 41.58, p < 0.0001) and drug (main effect of drug, $F_{1,30}$ = 20.05, p = 0.0001) in burying duration in response to the stress cues and environment (**Figure 4D**). Stressed females administered CNO displayed an increased latency to begin burying (**Figure 4E**, t_{16} = 1.423, p = 0.08) and no significant differences in the duration of rearing behavior (**Figure 4F**, t_{16} = 0.4285, p = 0.337) when compared to vehicle treated rats in the WS CXT.

Exposure to WS CXT increases LC activity which is prevented by a history of microglial inhibition

Brain tissue was collected two hours following the start of WS/CON CXT for analysis of neuronal activity within the LC in rats with a history of CNO/VEH prior to repeated WS/CON.

There was a significant effect of stress history ($F_{1,29}$ = 20.92, p < 0.0001) and drug treatment ($F_{1,29}$ = 8.126, p = 0.008) in the number of cfos positive TH labeled LC cells in response to the WS CXT (**Figure 4G**). Females exposed to WS+VEH exhibited a significantly higher level of neuronal activation in the LC in response to the stress cues when compared to controls (p = 0.0005) and to WS+CNO (p = 0.0084). Representative images of TH staining in the LC (brown) along with cfos positive cells (black) are shown for all treatment groups in **Figure 4H**. Further, brain slices were collected to confirm that virus was contained within the boundary of the LC (**Supplementary Figure 4**).

Discussion

These studies are among the first to utilize site specific microglial targeted chemogenetics to manipulate neuroimmune activity within the brain and expands our recent evidence that microglia regulate LC-specific hypervigilant behavior resulting from witness stress in females (Pate et al., 2023). These experiments characterized the use of an inhibitory DREADD-expressing virus driven by the CD68 promoter within the LC and determined that this virus selectively colocalized with microglia at a transfection rate of ~50%, confirming published studies using these techniques in the spinal cord (Grace et al., 2016; Grace et al., 2018). Chemogenetic inhibition of microglia reduced WS-evoked inflammatory cytokine release, increased the presence of homeostatic microglial cells, and prevented WS-induced hypervigilant behaviors both during and following stress exposure. Notably, the reduction in neuroimmune signaling during stress prevented the sensitization of hypervigilant behaviors in response to MB and WS CXT and was associated with a lower level of neuronal activity in the LC. Overall, these experiments have characterized the use of a CD68 promoter-driven hM4Di virus for targeting and manipulating microglial activity within the rat brain and established the role of LC neuroimmune signaling in the acute and long-term impact of stress in females.

While chemogenetic techniques have been extensively utilized for their ability to impact neuronal signaling (Keifer et al., 2020; Urban & Roth, 2015), this method has only recently begun to be optimized for use in non-neuronal cell types (Bossuyt et al., 2023; Dheer et al., 2024; Parusel et al., 2023). AAVs, particularly AAV1, have been determined to effectively transduce microglial cells across the brain (Aschauer et al., 2013) and the activity of these cells, along with the resulting pro-inflammatory cytokine release, have also been shown to be regulated through GPCR-mediated mechanisms (Hayashi et al., 2011; Hoffmann et al., 2003). Importantly, viral transduction into microglia has been observed to have regional heterogeneity (Aschauer et al., 2013; Bossuyt et al., 2023), and these are the first experiments to assess the LC and observe a high level of microglial transduction within this region. Further, species specific differences in microglial genetics, primarily the ~6x higher levels of CD68 RNA observed in resting microglia from rat cells versus those from mice (Lam et al., 2017), may influence differential rates of transfection between the species. Additional differences in the immune system have been observed between mice and rats, with species specific neuroimmune responses observed in response to multiple different types of inflammatory insults (Lively et al., 2018; Sroga et al., 2003). These studies identified a rat immune system as one that is more comparable to that of humans (Wildner, 2019) and, taken together, these data highlight the need to differentiate between the murine species when using novel tools to investigate the neuroimmune system.

Recent experiments by Grace et al. have implemented the G_i coupled DREADD virus used in this study to specifically target microglia utilizing a CD68 promoter (Grace et al., 2016; Grace et al., 2018). This virus has been developed to selectively colocalize with the Iba1 marker of microglia and not the astrocytic marker GFAP nor the neuronal NeuN, which was confirmed here for the first time in brain tissue and has a functional impact on the evoked expression of pro-inflammatory mediators IL-6, IL-1β, and TNFα (Grace et al., 2016; Grace et al., 2018). However, these prior published studies utilizing this virus were completed in the spinal cord using the AAV9 serotype, and, thus, the present experiments provide highly novel data regarding the use of

chemogenetics to manipulate microglia within the rat brain to impact neuroimmune signaling and behavior. One further concern when using chemogenetic techniques is the risk for unintended effects of CNO outside of its function at the designed receptors. While higher doses of CNO, such as 10 mg/kg, produce off target effects and yield a high rate of conversion to clozapine, CNO at doses of 1 mg/kg, like those used in the present study, have been shown to have limited, if any, off target effects while plasma levels of clozapine are undetectable in rats (Manvich et al., 2018; Martinez et al., 2019). Importantly, these studies confirmed the lack of effect of CNO on behavior or microglial morphology shifts to alleviate these concerns.

Previous studies by our lab (Finnell et al., 2017; Pate et al., 2023; Wood et al., 2015) and others (Sanguino-Gómez et al., 2022; Schramm & Waisman, 2022; Sugama & Kakinuma, 2020) have highlighted the essential role of neuroimmune signaling in mediating CNS responses to stress (Biltz et al., 2022). While microglia are highly present through the brain, one region in which they are concentrated is the noradrenergic LC in which bidirectional regulation of norepinephrine and microglial cell reactivity plays a key role in the stress response (Iravani et al., 2014). Microglial activity can be regulated by adrenergic receptor signaling (Färber et al., 2005; Johnson et al., 2005) while, conversely, the release of proinflammatory cytokines leads to activation of the norepinephrine-producing LC cells (Borsody & Weiss, 2014). Further, inflammatory mediated regulation of LC activity has been associated with hypervigilant and depressive-like behaviors (Kurosawa et al., 2016; Christoph Naegeli et al., 2018). Therefore, while these experiments determined that microglial activation plays a major role in the regulation of LC neuronal signaling and resulting hypervigilant burying behaviors, there is undoubtably further noradrenergic mechanisms involved in the regulation of this response. Additionally, while these studies focused on microglial signaling within the LC, peripheral immune signaling that occurs in response to stress exposure may also influence central immune activity. Recent studies have determined that there are time-, brain region-, and sex-specific effects on peripheral immune cell trafficking to the brain that may play a role in the stress response, with ~10% of the immune cells

of the hippocampus representing peripheral infiltration in response to acute learned helplessness paradigms (Medina-Rodriguez et al., 2023). Importantly, these results were not generalizable to females, chronic stress conditions, regions including the LC were not assessed, and this infiltration may result from local CNS immune signaling (Medina-Rodriguez et al., 2023). Therefore, while peripheral immune cell infiltration may play a role in microglial responses to stress, further experiments would be required to explore this mechanism in the female LC.

These experiments determined that microglial inhibition impacted behavioral responses to acute and repeated psychosocial stress, and this decrease in hypervigilant behavioral responding also occurred in response to subsequent exposure to the stress cues and environment. The inflammatory consequences of repeated stress have been shown to be sensitized with repeated stress exposure, a phenomenon often referred to as a “dual hit” hypothesis (Biltz et al., 2022; Smiley & Wood, 2022). The neuroimmune signaling and microglial reactivity that occurs in the brain as a result of social stress exposure leads to sensitization, whereby subthreshold stressor exposure in the future, i.e. exposure to the stress context, would therefore lead to an exaggerated inflammatory response (Biltz et al., 2022). Therefore, by preventing the inflammatory cascade that typically occurs in response to social stress (Biltz et al., 2022; Finnell et al., 2018; Liu et al., 2017; B. S. Pate et al., 2023; Wood et al., 2015) with chemogenetic inhibition of microglial activation, these results suggest that microglial priming and stress-induced sensitization of neuroimmune responding to subsequent stress and stress context exposures was blocked. Ultimately, we hypothesized that neuroimmune activity within the LC regulates hypervigilant behaviors through its effects on neuronal signaling, which was confirmed with cfos staining. Blocking microglial activation during stress was associated with lower levels of LC neuronal activation and reduced behavioral responding to stress cues. Future experiments utilizing *in vivo* electrophysiology to directly measure neuronal activity in response to microglial blockade will allow us to further probe the relationship between microglial and neuronal activity in the LC in the female stress response.

In conclusion, these experiments are among the first to site-specifically target microglia within the rat brain for chemogenetic manipulation of neuroimmune signaling in response to stress. These studies confirmed our previous findings establishing LC microglia as a key player in the female stress response (Pate et al., 2023) and expanded the role of neuroimmune signaling to subsequent behavioral and neuronal responses to stress cues. Taken together, these experiments characterized the use of chemogenetics to alter microglial activity and support the role of LC neuroimmune signaling as a key mechanism in the female stress response. Subsequent experiments will further examine the functional relationship between immune activity, neuronal signaling and resulting NE output from the LC, as well as assessing sex differences, but these results highlight the potential for targeting neuroimmune signaling in the treatment of stress-related comorbidities in females.

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Conflicts of Interest

The authors have no conflicts of interest to report.

References

Aschauer, D. F., Kreuz, S., & Rumpel, S. (2013). Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. *PloS one*, *8*(9), e76310. https://doi.org/10.1371/journal.pone.0076310

Bangasser, D. A., & Valentino, R. J. (2014). Sex differences in stress-related psychiatric disorders: neurobiological perspectives. *Front Neuroendocrinol*, *35*(3), 303-319. https://doi.org/10.1016/j.yfrne.2014.03.008

Barbetti, M., Vilella, R., Naponelli, V., Bilotti, I., Magistrati, M., Dallabona, C., . . . Carnevali, L. (2023). Repeated witness social stress causes cardiomyocyte contractile impairment and intracellular Ca2+ derangement in female rats. *Physiology & Behavior*, *271*, 114339. https://doi.org/https://doi.org/10.1016/j.physbeh.2023.114339

Bekhbat, M., & Neigh, G. N. (2018). Sex differences in the neuro-immune consequences of stress: Focus on depression and anxiety. *Brain, behavior, and immunity*, *67*, 1-12. https://doi.org/10.1016/j.bbi.2017.02.006

Benjet, C., Bromet, E., Karam, E. G., Kessler, R. C., McLaughlin, K. A., Ruscio, A. M., . . . Koenen, K. C. (2016). The epidemiology of traumatic event exposure worldwide: results from the World Mental Health Survey Consortium. *Psychol Med*, *46*(2), 327-343. https://doi.org/10.1017/s0033291715001981

Biltz, R. G., Sawicki, C. M., Sheridan, J. F., & Godbout, J. P. (2022). The neuroimmunology of social-stress-induced sensitization. *Nat Immunol*, *23*(11), 1527-1535. https://doi.org/10.1038/s41590-022-01321-z

Bisson, J. I., Cosgrove, S., Lewis, C., & Robert, N. P. (2015). Post-traumatic stress disorder. *Bmj*, *351*, h6161. https://doi.org/10.1136/bmj.h6161

Borodovitsyna, O., Duffy, B. C., Pickering, A. E., & Chandler, D. J. (2020). Anatomically and functionally distinct locus coeruleus efferents mediate opposing effects on anxiety-like behavior. *Neurobiol Stress*, *13*, 100284. https://doi.org/10.1016/j.ynstr.2020.100284

Borsody, M. K., & Weiss, J. M. (2002). Alteration of locus coeruleus neuronal activity by interleukin-1 and the involvement of endogenous corticotropin-releasing hormone. *Neuroimmunomodulation*, *10*(2), 101-121. https://doi.org/10.1159/000065186

Borsody, M. K., & Weiss, J. M. (2004). The effects of endogenous interleukin-1 bioactivity on locus coeruleus neurons in response to bacterial and viral substances. *Brain Res*, *1007*(1), 39-56. https://doi.org/https://doi.org/10.1016/j.brainres.2004.02.011

Borsody, M. K., & Weiss, J. M. (2014). Peripheral endotoxin causes long-lasting changes in locus coeruleus activity via IL-1 in the brain. *Acta Neuropsychiatrica*, *14*(6), 303-321. https://doi.org/10.1034/j.1601-5215.2002.140605.x

Bossuyt, J., Van Den Herrewegen, Y., Nestor, L., Buckinx, A., De Bundel, D., & Smolders, I. (2023). Chemogenetic modulation of astrocytes and microglia: State-of-the-art and implications in neuroscience. *Glia*, *71*(9), 2071-2095. https://doi.org/https://doi.org/10.1002/glia.24390

Cohen, S., Janicki-Deverts, D., & Miller, G. E. (2007). Psychological Stress and Disease. *JAMA*, *298*(14), 1685-1687. https://doi.org/10.1001/jama.298.14.1685

Davidson, J. R. (2000). Trauma: the impact of post-traumatic stress disorder. *J Psychopharmacol*, *14*(2 Suppl 1), S5-12. https://doi.org/10.1177/02698811000142s102

Dheer, A., Bosco, D. B., Zheng, J., Wang, L., Zhao, S., Haruwaka, K., . . . Wu, L. J. (2024). Chemogenetic approaches reveal dual functions of microglia in seizures. *Brain, behavior, and immunity*, *115*, 406-418. https://doi.org/10.1016/j.bbi.2023.11.002

Engler, H., Benson, S., Wegner, A., Spreitzer, I., Schedlowski, M., & Elsenbruch, S. (2016). Men and women differ in inflammatory and neuroendocrine responses to endotoxin but not in the severity of sickness symptoms. *Brain, Behavior, and Immunity*, *52*, 18-26. https://doi.org/https://doi.org/10.1016/j.bbi.2015.08.013

Fan, Y., Chen, P., Li, Y., Cui, K., Noel, D. M., Cummins, E. D., . . . Zhu, M. Y. (2014). Corticosterone administration up-regulated expression of norepinephrine transporter and dopamine β-hydroxylase in rat locus coeruleus and its terminal regions. *J Neurochem*, *128*(3), 445-458. https://doi.org/10.1111/jnc.12459

Färber, K., Pannasch, U., & Kettenmann, H. (2005). Dopamine and noradrenaline control distinct functions in rodent microglial cells. *Molecular and Cellular Neuroscience*, *29*(1), 128-138. https://doi.org/https://doi.org/10.1016/j.mcn.2005.01.003

Finnell, Muniz, B. L., Padi, A. R., Lombard, C. M., Moffitt, C. M., Wood, C. S., . . . Wood, S. K. (2018). Essential Role of Ovarian Hormones in Susceptibility to the Consequences of Witnessing Social Defeat in Female Rats. *Biological Psychiatry*, *84*(5), 372-382. https://doi.org/10.1016/j.biopsych.2018.01.013

Finnell, J. E., Lombard, C. M., Melson, M. N., Singh, N. P., Nagarkatti, M., Nagarkatti, P., . . . Wood, S. K. (2017). The protective effects of resveratrol on social stress-induced cytokine release and depressive-like behavior. *Brain Behav Immun*, *59*, 147-157. https://doi.org/10.1016/j.bbi.2016.08.019

Finnell, J. E., Muniz, B. L., Padi, A. R., Lombard, C. M., Moffitt, C. M., Wood, C. S., . . . Wood, S. K. (2018). Essential Role of Ovarian Hormones in Susceptibility to the Consequences of Witnessing Social Defeat in Female Rats. *Biol Psychiatry*, *84*(5), 372-382. https://doi.org/10.1016/j.biopsych.2018.01.013

Frans, Ö., Rimmö, P.-A., Åberg, L., & Fredrikson, M. (2005). Trauma exposure and post-traumatic stress disorder in the general population. *Acta Psychiatrica Scandinavica*, *111*(4), 291-290. https://doi.org/https://doi.org/10.1111/j.1600-0447.2004.00463.x

Giustino, T. F., Ramanathan, K. R., Totty, M. S., Miles, O. W., & Maren, S. (2020). Locus Coeruleus Norepinephrine Drives Stress-Induced Increases in Basolateral Amygdala Firing and Impairs Extinction Learning. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *40*(4), 907-916. https://doi.org/10.1523/jneurosci.1092-19.2019

Grace, P. M., Strand, K. A., Galer, E. L., Urban, D. J., Wang, X., Baratta, M. V., . . . Watkins, L. R. (2016). Morphine paradoxically prolongs neuropathic pain in rats by amplifying spinal NLRP3 inflammasome activation. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(24), E3441-3450. https://doi.org/10.1073/pnas.1602070113

Grace, P. M., Wang, X., Strand, K. A., Baratta, M. V., Zhang, Y., Galer, E. L., . . . Watkins, L. R. (2018). DREADDed microglia in pain: Implications for spinal inflammatory signaling in male rats. *Experimental neurology*, *304*, 125-131. https://doi.org/10.1016/j.expneurol.2018.03.005

Gu, C., Chen, Y., Chen, Y., Liu, C. F., Zhu, Z., & Wang, M. (2021). Role of G Protein-Coupled Receptors in Microglial Activation: Implication in Parkinson's Disease. *Front Aging Neurosci*, *13*, 768156. https://doi.org/10.3389/fnagi.2021.768156

Hänsel, A., Hong, S., Cámara, R. J. A., & von Känel, R. (2010). Inflammation as a psychophysiological biomarker in chronic psychosocial stress. *Neuroscience & Biobehavioral Reviews*, *35*(1), 115-121. https://doi.org/https://doi.org/10.1016/j.neubiorev.2009.12.012

Haque, M. E., Kim, I.-S., Jakaria, M., Akther, M., & Choi, D.-K. (2018). Importance of GPCR-Mediated Microglial Activation in Alzheimer's Disease [Review]. *Frontiers in cellular neuroscience*, *12*. https://doi.org/10.3389/fncel.2018.00258

Hayashi, Y., Kawaji, K., Sun, L., Zhang, X., Koyano, K., Yokoyama, T., . . . Nakanishi, H. (2011). Microglial Ca(2+)-activated K(+) channels are possible molecular targets for the analgesic effects of S-ketamine on neuropathic pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *31*(48), 17370-17382. https://doi.org/10.1523/jneurosci.4152-11.2011

Hoffmann, A., Kann, O., Ohlemeyer, C., Hanisch, U. K., & Kettenmann, H. (2003). Elevation of basal intracellular calcium as a central element in the activation of brain macrophages (microglia): suppression of receptor-evoked calcium signaling and control of release function. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *23*(11), 4410-4419. https://doi.org/10.1523/jneurosci.23-11-04410.2003

Iravani, M. M., Sadeghian, M., Rose, S., & Jenner, P. (2014). Loss of locus coeruleus noradrenergic neurons alters the inflammatory response to LPS in substantia nigra but does not affect nigral cell loss. *Journal of Neural Transmission*, *121*(12), 1493-1505. https://doi.org/10.1007/s00702-014-1223-1

Irish, L. A., Fischer, B., Fallon, W., Spoonster, E., Sledjeski, E. M., & Delahanty, D. L. (2011). Gender differences in PTSD symptoms: an exploration of peritraumatic mechanisms. *J Anxiety Disord*, *25*(2), 209-216. https://doi.org/10.1016/j.janxdis.2010.09.004

Johnson, J. D., Campisi, J., Sharkey, C. M., Kennedy, S. L., Nickerson, M., Greenwood, B. N., & Fleshner, M. (2005). Catecholamines mediate stress-induced increases in peripheral and central inflammatory cytokines. *Neuroscience*, *135*(4), 1295-1307. https://doi.org/10.1016/j.neuroscience.2005.06.090

Karperien, A., Ahammer, H., & Jelinek, H. F. (2013). Quantitating the subtleties of microglial morphology with fractal analysis. *Frontiers in cellular neuroscience*, *7*, 3. https://doi.org/10.3389/fncel.2013.00003

Kedia, S., & Chattarji, S. (2014). Marble burying as a test of the delayed anxiogenic effects of acute immobilisation stress in mice. *Journal of Neuroscience Methods*, *233*, 150-154. https://doi.org/https://doi.org/10.1016/j.jneumeth.2014.06.012

Keifer, O., Kambara, K., Lau, A., Makinson, S., & Bertrand, D. (2020). Chemogenetics a robust approach to pharmacology and gene therapy. *Biochemical Pharmacology*, *175*, 113889. https://doi.org/https://doi.org/10.1016/j.bcp.2020.113889

Kettenmann, H., Hanisch, U. K., Noda, M., & Verkhratsky, A. (2011). Physiology of microglia. *Physiol Rev*, *91*(2), 461-553. https://doi.org/10.1152/physrev.00011.2010

Krystal, J. H., Abdallah, C. G., Pietrzak, R. H., Averill, L. A., Harpaz-Rotem, I., Levy, I., . . . Southwick, S. M. (2018). Locus Coeruleus Hyperactivity in Posttraumatic Stress Disorder: Answers and Questions. *Biological Psychiatry*, *83*(3), 197-199. https://doi.org/https://doi.org/10.1016/j.biopsych.2017.09.027

Kurosawa, N., Shimizu, K., & Seki, K. (2016). The development of depression-like behavior is consolidated by IL-6-induced activation of locus coeruleus neurons and IL-1β-induced elevated leptin levels in mice. *Psychopharmacology*, *233*(9), 1725-1737. https://doi.org/10.1007/s00213-015-4084-x

Lam, D., Lively, S., & Schlichter, L. C. (2017). Responses of rat and mouse primary microglia to pro- and anti-inflammatory stimuli: molecular profiles, K(+) channels and migration. *Journal of neuroinflammation*, *14*(1), 166. https://doi.org/10.1186/s12974-017-0941-3

Levesque, P., Desmeules, C., Béchard, L., Huot-Lavoie, M., Demers, M.-F., Roy, M.-A., & Deslauriers, J. (2023). Sex-specific immune mechanisms in PTSD symptomatology and risk: A translational overview and perspectives. *Brain Research Bulletin*, *195*, 120-129. https://doi.org/https://doi.org/10.1016/j.brainresbull.2023.02.013

Liu, Y. Z., Wang, Y. X., & Jiang, C. L. (2017). Inflammation: The Common Pathway of Stress-Related Diseases. *Front Hum Neurosci*, *11*, 316. https://doi.org/10.3389/fnhum.2017.00316

Lively, S., Lam, D., Wong, R., & Schlichter, L. C. (2018). Comparing Effects of Transforming Growth Factor β1 on Microglia From Rat and Mouse: Transcriptional Profiles and Potassium Channels. *Frontiers in cellular neuroscience*, *12*, 115. https://doi.org/10.3389/fncel.2018.00115

Manvich, D. F., Webster, K. A., Foster, S. L., Farrell, M. S., Ritchie, J. C., Porter, J. H., & Weinshenker, D. (2018). The DREADD agonist clozapine N-oxide (CNO) is reverse-

metabolized to clozapine and produces clozapine-like interoceptive stimulus effects in rats and mice. *Sci Rep*, *8*(1), 3840. https://doi.org/10.1038/s41598-018-22116-z

Marshall, G. N., Schell, T. L., Glynn, S. M., & Shetty, V. (2006). The role of hyperarousal in the manifestation of posttraumatic psychological distress following injury. *J Abnorm Psychol*, *115*(3), 624-628. https://doi.org/10.1037/0021-843x.115.3.624

Martinez, V. K., Saldana-Morales, F., Sun, J. J., Zhu, P. J., Costa-Mattioli, M., & Ray, R. S. (2019). Off-Target Effects of Clozapine-N-Oxide on the Chemosensory Reflex Are Masked by High Stress Levels. *Front Physiol*, *10*, 521. https://doi.org/10.3389/fphys.2019.00521

McCall, J. G., Al-Hasani, R., Siuda, E. R., Hong, D. Y., Norris, A. J., Ford, C. P., & Bruchas, M. R. (2015). CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety. *Neuron*, *87*(3), 605-620. https://doi.org/10.1016/j.neuron.2015.07.002

McCall, J. G., Siuda, E. R., Bhatti, D. L., Lawson, L. A., McElligott, Z. A., Stuber, G. D., & Bruchas, M. R. (2017). Locus coeruleus to basolateral amygdala noradrenergic projections promote anxiety-like behavior. *Elife*, *6*. https://doi.org/10.7554/eLife.18247

Medina-Rodriguez, E. M., Han, D., Lowell, J., & Beurel, E. (2023). Stress promotes the infiltration of peripheral immune cells to the brain. *Brain, behavior, and immunity*, *111*, 412-423. https://doi.org/https://doi.org/10.1016/j.bbi.2023.05.003

Miao, X. R., Chen, Q. B., Wei, K., Tao, K. M., & Lu, Z. J. (2018). Posttraumatic stress disorder: from diagnosis to prevention. *Mil Med Res*, *5*(1), 32. https://doi.org/10.1186/s40779-018-0179-0

Mikics, E., Baranyi, J., & Haller, J. (2008). Rats exposed to traumatic stress bury unfamiliar objects — A novel measure of hyper-vigilance in PTSD models? *Physiol Behav*, *94*(3), 341-348. https://doi.org/https://doi.org/10.1016/j.physbeh.2008.01.023

Mizoguchi, Y., & Monji, A. (2017). Microglial Intracellular Ca(2+) Signaling in Synaptic Development and its Alterations in Neurodevelopmental Disorders. *Frontiers in cellular neuroscience*, *11*, 69. https://doi.org/10.3389/fncel.2017.00069

Moieni, M., Irwin, M. R., Jevtic, I., Olmstead, R., Breen, E. C., & Eisenberger, N. I. (2015). Sex differences in depressive and socioemotional responses to an inflammatory challenge: implications for sex differences in depression. *Neuropsychopharmacology*, *40*(7), 1709-1716. https://doi.org/10.1038/npp.2015.17

Naegeli, C., Zeffiro, T., Piccirelli, M., Jaillard, A., Weilenmann, A., Hassanpour, K., . . . Mueller-Pfeiffer, C. (2018). Locus Coeruleus Activity Mediates Hyperresponsiveness in Posttraumatic Stress Disorder. *Biol Psychiatry*, *83*(3), 254-262. https://doi.org/10.1016/j.biopsych.2017.08.021

Naegeli, C., Zeffiro, T., Piccirelli, M., Jaillard, A., Weilenmann, A., Hassanpour, K., . . . Mueller-Pfeiffer, C. (2018). Locus Coeruleus Activity Mediates Hyperresponsiveness in Posttraumatic Stress Disorder. *Biological Psychiatry*, *83*(3), 254-262. https://doi.org/10.1016/j.biopsych.2017.08.021

O'Donnell, T., Hegadoren, K. M., & Coupland, N. C. (2004). Noradrenergic mechanisms in the pathophysiology of post-traumatic stress disorder. *Neuropsychobiology*, *50*(4), 273-283. https://doi.org/10.1159/000080952

Paolicelli, R. C., Sierra, A., Stevens, B., Tremblay, M.-E., Aguzzi, A., Ajami, B., . . . Wyss-Coray, T. (2022). Microglia states and nomenclature: A field at its crossroads. *Neuron*, *110*(21), 3458-3483. https://doi.org/https://doi.org/10.1016/j.neuron.2022.10.020

Parusel, S., Yi, M. H., Hunt, C. L., & Wu, L. J. (2023). Chemogenetic and Optogenetic Manipulations of Microglia in Chronic Pain. *Neurosci Bull*, *39*(3), 368-378. https://doi.org/10.1007/s12264-022-00937-3

Pate, Bouknight, S. J., Harrington, E. N., Mott, S. E., Augenblick, L. M., Smiley, C. E., . . . Wood, S. K. (2023). Site-Specific knockdown of microglia in the locus coeruleus regulates

hypervigilant responses to social stress in female rats. *Brain, behavior, and immunity*, *109*, 190-203. https://doi.org/10.1016/j.bbi.2023.01.011

Pate, B. S. (2023). Site-Specific knockdown of microglia in the locus coeruleus regulates hypervigilant responses to social stress in female rats. *Brain, behavior, and immunity*, *109*, 190-203. https://doi.org/https://doi.org/10.1016/j.bbi.2023.01.011

Pate, B. S., Bouknight, S. J., Harrington, E. N., Mott, S. E., Augenblick, L. M., Smiley, C. E., . . . Wood, S. K. (2023). Site-Specific Knockdown of Microglia in the Locus Coeruleus Regulates Hypervigilant Responses to Social Stress in Female Rats. *Brain, Behavior, and Immunity*. https://doi.org/https://doi.org/10.1016/j.bbi.2023.01.011

Piña, R., Santos-Díaz, A. I., Orta-Salazar, E., Aguilar-Vazquez, A. R., Mantellero, C. A., Acosta-Galeana, I., . . . Rosas-Arellano, A. (2022). Ten Approaches That Improve Immunostaining: A Review of the Latest Advances for the Optimization of Immunofluorescence. *Int J Mol Sci*, *23*(3). https://doi.org/10.3390/ijms23031426

Ravi, M., Miller, A. H., & Michopoulos, V. (2021). The immunology of stress and the impact of inflammation on the brain and behaviour. *BJPsych Advances*, *27*(3), 158-165. https://doi.org/10.1192/bja.2020.82

Sanguino-Gómez, J., Buurstede, J. C., Abiega, O., Fitzsimons, C. P., Lucassen, P. J., Eggen, B. J. L., . . . Krugers, H. J. (2022). An emerging role for microglia in stress-effects on memory. *Eur J Neurosci*, *55*(9-10), 2491-2518. https://doi.org/10.1111/ejn.15188

Sarkar, D. (2020). A Review of Behavioral Tests to Evaluate Different Types of Anxiety and Anti-anxiety Effects. *Clin Psychopharmacol Neurosci*, *18*(3), 341-351. https://doi.org/10.9758/cpn.2020.18.3.341

Schell, T. L., Marshall, G. N., & Jaycox, L. H. (2004). All Symptoms Are Not Created Equal: The Prominent Role of Hyperarousal in the Natural Course of Posttraumatic Psychological Distress. *Journal of Abnormal Psychology*, *113*, 189-197. https://doi.org/10.1037/0021-843X.113.2.189

Schramm, E., & Waisman, A. (2022). Microglia as Central Protagonists in the Chronic Stress Response. *Neurol Neuroimmunol Neuroinflamm*, *9*(6). https://doi.org/10.1212/nxi.0000000000200023

Smiley, C. E., Pate, B. S., Bouknight, S. J., Francis, M. J., Nowicki, A. V., Harrington, E. N., & Wood, S. K. (2023). Estrogen receptor beta in the central amygdala regulates the deleterious behavioral and neuronal consequences of repeated social stress in female rats. *Neurobiol Stress*, *23*, 100531. https://doi.org/https://doi.org/10.1016/j.ynstr.2023.100531

Smiley, C. E., & Wood, S. K. (2022). Stress- and drug-induced neuroimmune signaling as a therapeutic target for comorbid anxiety and substance use disorders. *Pharmacology & Therapeutics*, *239*, 108212. https://doi.org/https://doi.org/10.1016/j.pharmthera.2022.108212

Sroga, J. M., Jones, T. B., Kigerl, K. A., McGaughy, V. M., & Popovich, P. G. (2003). Rats and mice exhibit distinct inflammatory reactions after spinal cord injury. *J Comp Neurol*, *462*(2), 223-240. https://doi.org/10.1002/cne.10736

Sugama, S., & Kakinuma, Y. (2020). Stress and brain immunity: Microglial homeostasis through hypothalamus-pituitary-adrenal gland axis and sympathetic nervous system. *Brain Behav Immun Health*, *7*, 100111. https://doi.org/10.1016/j.bbih.2020.100111

Urban, D. J., & Roth, B. L. (2015). DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility. *Annual Review of Pharmacology and Toxicology*, *55*(1), 399-417. https://doi.org/10.1146/annurev-pharmtox-010814-124803

Wildner, G. (2019). Are rats more human than mice? *Immunobiology*, *224*(1), 172-176. https://doi.org/https://doi.org/10.1016/j.imbio.2018.09.002

Wood, S. K., Wood, C. S., Lombard, C. M., Lee, C. S., Zhang, X. Y., Finnell, J. E., & Valentino, R. J. (2015). Inflammatory Factors Mediate Vulnerability to a Social Stress-Induced Depressive-like Phenotype in Passive Coping Rats. *Biol Psychiatry*, *78*(1), 38-48. https://doi.org/10.1016/j.biopsych.2014.10.026

Figure Legends

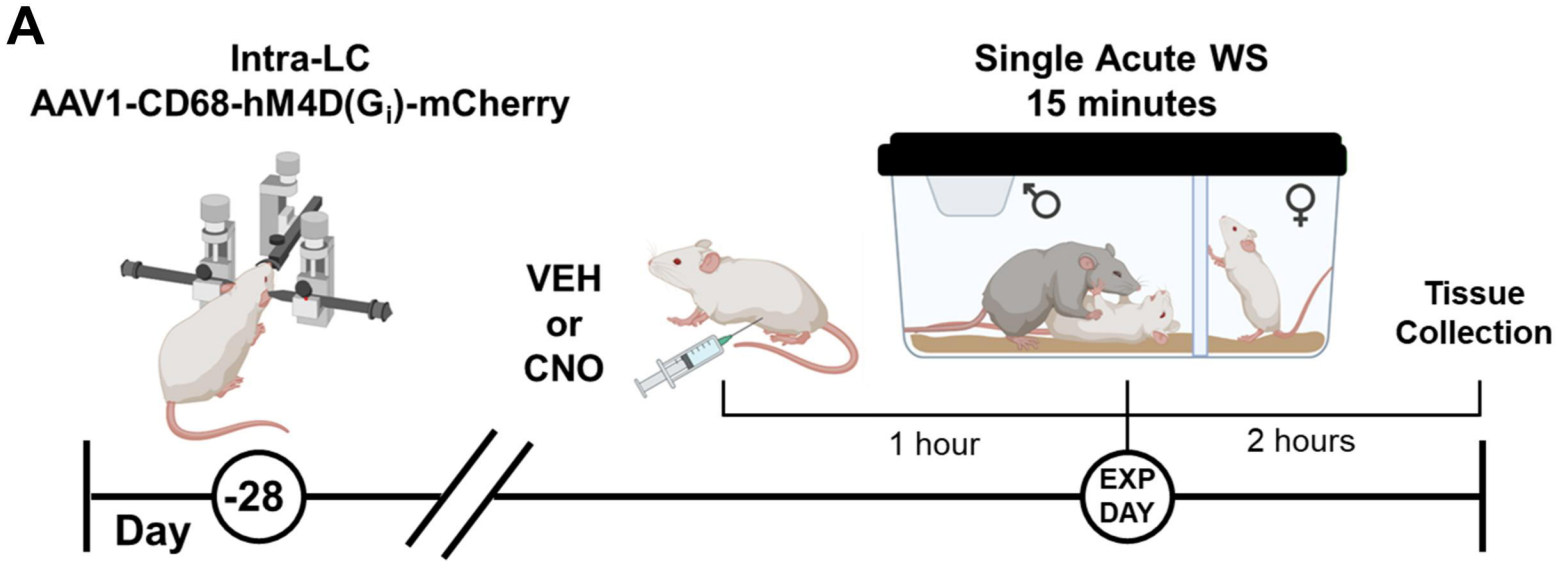
Figure 1. Timeline and experimental design. A) Female rats in Experiment 1A underwent stereotaxic surgery with electrophysiological assistance to locate and inject an inhibitory DREADDs virus targeted to microglia into the locus coeruleus (LC). A separate subset of animals remained surgically naïve with no viral injection (1B). Following four weeks for surgical recovery, rats were subjected to a single experimental day that consisted of intraperitoneal injections of saline (0.9%, vehicle) or 1 mg/kg CNO. One hour later, females experienced a single session of witness stress (WS) for 15 minutes and brain tissue was collected two hours following the start of stress. **B)** Experiment 2 commenced with the same surgery as described above along with the four week recovery time, during which rats underwent pre-stress behavioral testing in the marble burying (MB) paradigm. Subsequently, females were exposed to repeated WS for 15 minutes/day for five days with either vehicle or CNO injections one hour prior to each session. The MB test was repeated post-stress and rats underwent a WS context challenge with brain tissue collected two hours after the start of context.

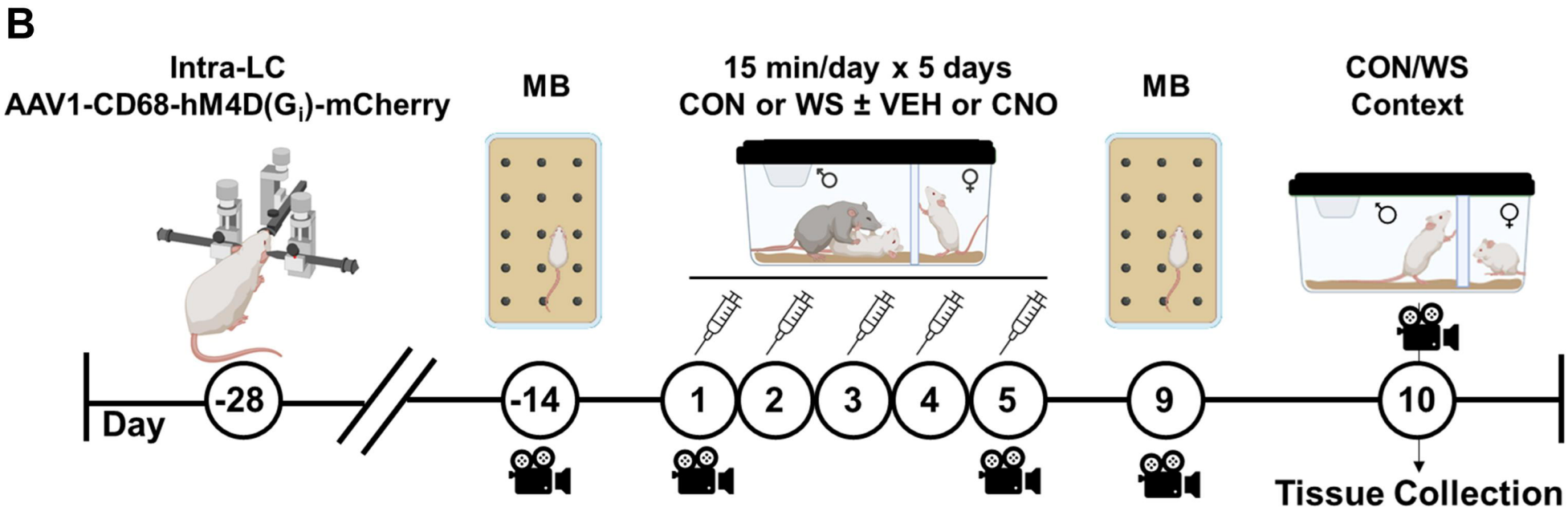
Figure 2. Characterization of microglial targeting DREADDs for the reduction of neuroimmune activity. A) Representative image of mCherry (red) and Iba1 positive cells (green) colocalized within the LC (yellow) at 20X magnification. **B)** 3D rendering of 63x magnified image displaying mCherry (red) integrated within Iba1 cells (green). mCherry (red) does not colocalize with neurons (**C**, NeuN, cyan, 20X) or astrocytes (**D**, GFAP, cyan, 20X). **E)** Microglial inhibition during stress prevents the accumulation of the proinflammatory cytokine IL-1β in the LC, an effect that is typically observed in females in response to WS (*$p < 0.05$, $n = 6$/group). In females previously injected with the CD68 DREADD virus in the LC, CNO treatment prior to stress led to a significant reduction in the percentage of microglial cells that were reactive in response to WS (**F**, ***$p = 0.0002$), an increase in homeostatic microglial cells (**G**, ***$p = 0.0002$), and a reduction in the duration of burying behavior displayed in response to acute WS (**H**, **$p < 0.005$). Conversely, in surgically naïve females without viral injection into the LC, there was an overall impact of stress with WS leading to increased microglial reactivity (**I**, ***$p < 0.0001$) and decreased homeostatic microglial cells (**J**, * $p < 0.05$) and augmented burying behavior (**K**, *$p < 0.05$) with no effect of CNO on either microglial morphology or WS-evoked behavioral changes.

Figure 3. Microglial inhibition during stress prevents the acute responses to witness stress. A) Exposure to acute WS increases burying when compared to controls (***$p < 0.0002$) and is prevented with inhibition of microglial activation using CNO ($^{f}p < 0.005$). CNO treated stressed females also exhibited a longer latency to begin burying (**B**, *$p < 0.05$) and displayed an increased amount of rearing behavior (**C**, $^{\#}p < 0.075$). These trends continued with repeated stress where, on the fifth day of WS, stressed animals exhibited heightened burying (***$p < 0.0002$) that was prevented with CNO (**D**, $^{f}p < 0.005$). Further, there were no significant differences in the latency to begin burying **(E)** while a trend remained with regards to increased rearing behavior in stressed females that received CNO (**F**, $^{\#}p < 0.055$).

Figure 4. A history of microglial blockade during stress prevents the long-term behavioral and neuronal effects of repeated stress. In post-stress behavioral testing in the marble burying task, stressed females that received CNO prior to each WS exposure exhibited a trend towards a reduced number of marbles buried (**A**, $^{\#}p < 0.065$) with no significant alteration in the total burying duration **(B)** or time spent interacting with the marbles **(C)**. When presented with the cues and environment in which they originally received stress, WS exposed females exhibited an increase in burying behavior that is not evident in controls and was prevented with a history of CNO treatment prior to stress (**D**, ****$p < 0.0001$). CNO treated stressed females also displayed

a trend towards a longer latency to begin burying when compared to those that received vehicle prior to stress (**E**, $^{\#}p < 0.087$) with no differences in rearing behavior **(F)**. Brain tissue collected from these stressed females displayed a significant increase in LC activity when compared to controls (**G**, $^{***}p < 0.0004$) and to those that had previously received CNO prior to each WS exposure (**G**, $^{**}p < 0.0065$). Cfos and TH staining is displayed in representative images from rats from each treatment group along with visual criteria for what was considered cfos, TH, and colocalized cells (**H**).


A
Intra-LC
AAV1-CD68-hM4D(Gi)-mCherry
VEH
or
CNO
Single Acute WS
15 minutes
Tissue
Collection
1 hour
2 hours
Day
-28
EXP
DAY


B
Intra-LC
AAV1-CD68-hM4D(Gi)-mCherry
MB
15 min/day x 5 days
CON or WS ± VEH or CNO
MB
CON/WS
Context
Day
-28
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1
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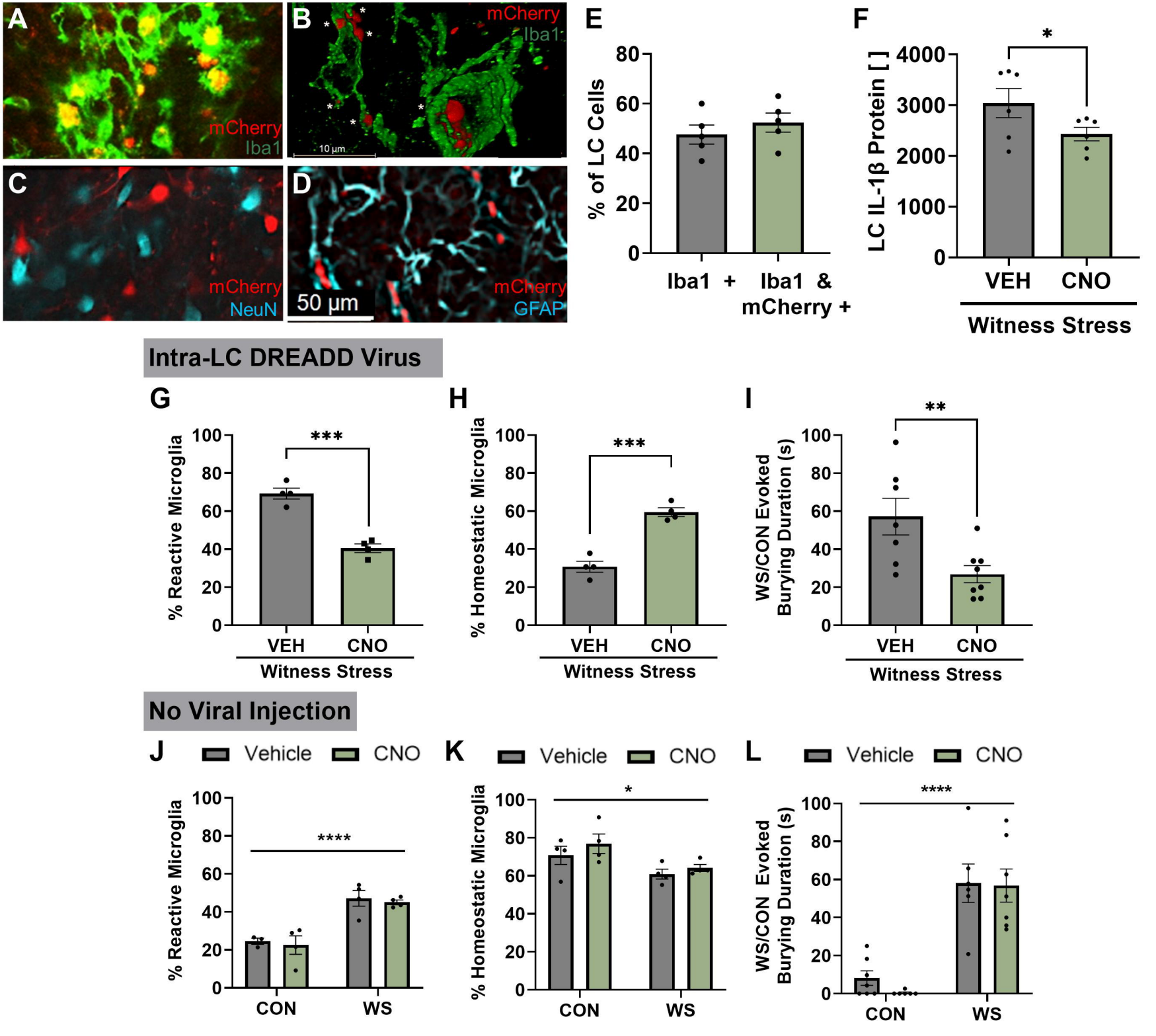
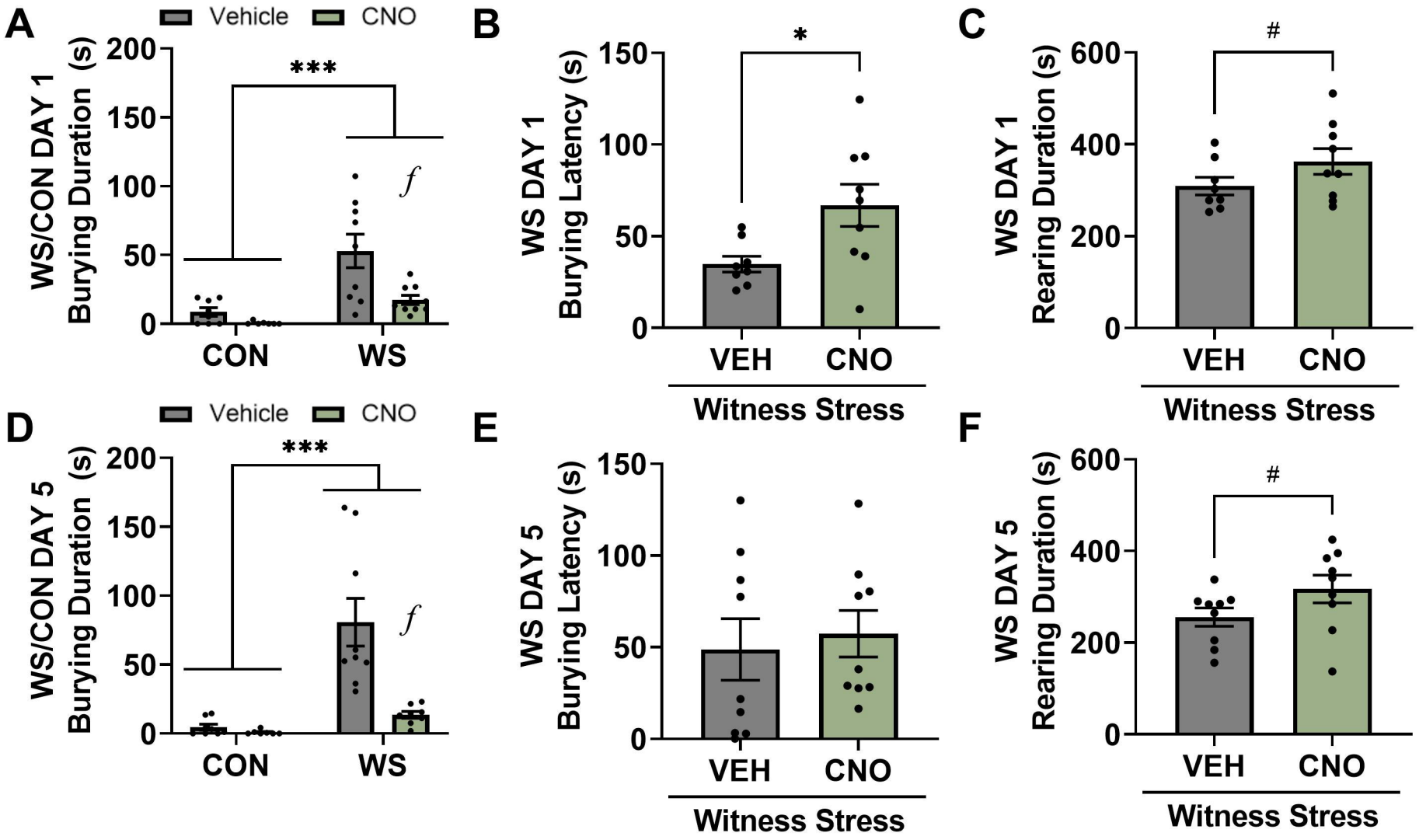
Tissue Collection


A
mCherry
Iba1
B
mCherry
Iba1
10 µm
C
mCherry
NeuN
D
mCherry
GFAP
50 µm
E
% of LC Cells
Iba1 +
Iba1 & mCherry +
F
LC IL-1β Protein [ ]
VEH
CNO
Witness Stress
Intra-LC DREADD Virus
G
% Reactive Microglia
H
% Homeostatic Microglia
I
WS/CON Evoked Burying Duration (s)
No Viral Injection
J
Vehicle
CNO
% Reactive Microglia
CON
WS
K
% Homeostatic Microglia
L
WS/CON Evoked Burying Duration (s)


A
Vehicle
CNO
WS/CON DAY 1
Burying Duration (s)
200
150
100
50
0
***
f
CON
WS
B
WS DAY 1
Burying Latency (s)
150
100
50
0
*
VEH
CNO
Witness Stress
C
WS DAY 1
Rearing Duration (s)
600
400
200
0
#
VEH
CNO
Witness Stress
D
Vehicle
CNO
WS/CON DAY 5
Burying Duration (s)
200
150
100
50
0
***
f
CON
WS
E
WS DAY 5
Burying Latency (s)
150
100
50
0
VEH
CNO
Witness Stress
F
WS DAY 5
Rearing Duration (s)
600
400
200
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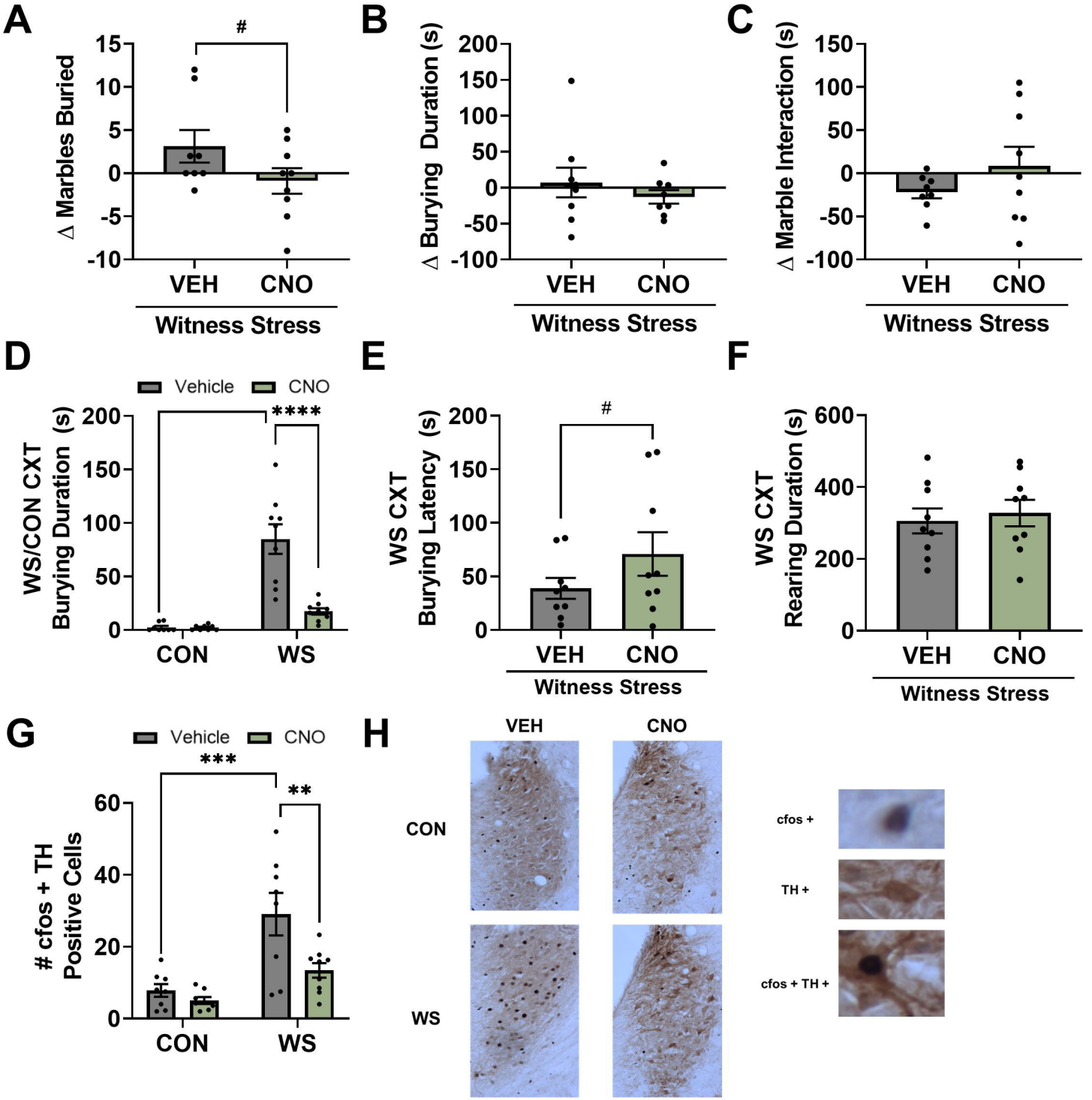
VEH
CNO
Witness Stress


A
Δ Marbles Buried
#
VEH
CNO
Witness Stress
B
Δ Burying Duration (s)
VEH
CNO
Witness Stress
C
Δ Marble Interaction (s)
VEH
CNO
Witness Stress
D
Vehicle
CNO
WS/CON CXT Burying Duration (s)
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CON
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CON
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