Repeated activation of pyramidal neurons in the prefrontal cortex alters microglial phenotype in male mice

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Running Title: Neuronal modulation of microglial function

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Text Pages: 27
Tables: 0 primary, 3 supplementary
Figures: 5 primary, 2 supplementary
References: 40

Abstract: 232 words
Introduction: 484 words
Discussion: 1015 words

Abbreviations: CD11b, integrin alpha M; CD68, cluster of differentiation 68; CNO, clozapine N-oxide; CSF1, colony stimulating factor 1; DREADD, designer receptors exclusively activated by designer drugs; FOSB, FBJ murine osteosarcoma viral oncogene homolog B; FST, forced swim test; IBA1, ionized calcium-binding adaptor molecule 1; mPFC, medial prefrontal cortex; NND, nearest neighbor distance; TOR, temporal object recognition task

Section Assignment: Neuropharmacology
Significance Statement: Microglia are highly attuned to fluctuations in neuronal activity. Here, we show that repeated activation of pyramidal neurons in the prefrontal cortex induces broad changes in microglia phenotype; this includes upregulation of pathways associated with microglial proliferation, microglia-neuron interactions, and lysosome induction. Our findings suggest that studies using chemogenetic or optogenetic approaches to manipulate neural circuits should be mindful of indirect effects on non-neuronal cells and their potential contribution to measured outcomes.
Abstract

Aberrant neuronal activity in the cortex alters microglia phenotype and function in several contexts, including chronic psychological stress and neurodegenerative disease. Recent findings even suggest that heightened levels of neuronal activity spur microglia to phagocytose synapses, with potential impacts on cognition and behavior. Thus, the present studies were designed to determine if activation of neurons alone – independent of disease or dysfunction – is sufficient to alter microglial phenotype in the medial prefrontal cortex (mPFC), a brain region critical in emotion regulation and cognition. In these studies, we used both an AAV-mediated and Cre-dependent chemogenetic (DREADD) approach to repeatedly activate excitatory pyramidal neurons (CaMKIIa+) neurons in the mPFC. Various molecular, cytometric, and behavioral endpoints were examined. Recurrent DREADD-induced neuronal activation led to pronounced changes in microglial density, clustering, and morphology in the mPFC, and increased microglia-specific transcripts implicated in synaptic pruning (e.g., Csfr1, Cd11b). Further analyses revealed that the magnitude of DREADD-induced neuronal activation was significantly correlated with measures of microglial morphology in the mPFC. These alterations in microglial phenotype coincided with an increase in microglial lysosome volume in the mPFC and selective deficits in working memory function. Altogether, these findings indicate that repeated neuronal activation alone is sufficient to drive changes in microglial phenotype and function in the mPFC. Future studies using optogenetic and chemogenetic approaches to manipulate neural circuits need to consider microglial- and other non-neuronal contributions to physiological and behavioral outcomes.
Introduction

Microglia form a critical component of the central nervous system’s cellular network, with functions aimed at directing synapse development and maintaining neuronal homeostasis (Woodburn et al., 2021b; Paolicelli et al., 2022). Much of this is done through a highly complex array of branched processes that constantly survey the brain’s microenvironment, making direct contact with neighboring neurons, amongst other cell types (Nimmerjahn et al., 2005). Recent studies show that microglia dynamically respond to changes in neuronal activity; this includes shifts in glutamatergic and GABAergic neurotransmission, amongst other signaling pathways (Dissing-Olesen et al., 2014; Logiacco et al., 2021).

Aberrant increases in neuronal activity lead to alterations in microglial phenotype and function. For instance, acute activation of excitatory pyramidal neurons (CaMKIIα Gq DREADD) is sufficient to induce broad transcriptional changes in microglia, microglial process outgrowth, and microglia-synapse contact (Badimon et al., 2020; Umpierre et al., 2020). These effects are magnified considerably in status epilepticus, a dysfunctional state of heightened excitatory neurotransmission and neuronal CSF1-microglial CSF1R axis activation (Luo et al., 2013; Eyo et al., 2014; Umpierre et al., 2020). In this context, microglia engage with neurons to dampen hyperactive synapses; this is accomplished through the release of various neuromodulators (e.g., ADP/adenosine, cytokines, growth factors) and the phagocytosis of synaptic elements (e.g., dendritic spines) (Stevens et al., 2007; Parkhurst et al., 2013; Badimon et al., 2020; Cheadle et al., 2020). These activity-dependent microglial actions normalize neuronal activity and limit cellular damage associated with neuronal hyperactivity. While these studies demonstrate a critical role for microglia in disease-related models, less is known about the role of microglia in non-disease states and how this shapes behavior or cognitive performance.

Likewise, the majority of studies examining activity associated shifts in microglia have focused on short-term spikes in neuronal activity, where their actions appear to be beneficial. However, recent reports suggest a contrasting role for microglia in various disorders associated
with chronic or long-term shifts in neuronal activity, including schizophrenia and major depressive disorder (Torres-Platas et al., 2014; Seney et al., 2018; Sellgren et al., 2019; Böttcher et al., 2020). Here, heightened microglial engagement and overactive pruning may impair neuronal function and behavior. For instance, our studies show that chronic stress, a precipitating factor in many neuropsychiatric disorders, initially leads to heightened levels of neuronal activity in the medial prefrontal cortex (mPFC), and this promotes microglia-mediated synaptic remodeling and subsequent deficits in cognition (Bollinger et al., 2020, 2022). To expand on this work, we used both an AAV-mediated and Cre-dependent chemogenetic (DREADD) approach to test if repeated neuronal activation alone alters microglia phenotype in the mPFC. We also tested mice for behavioral and cognitive deficits associated with PFC dysfunction. Our results show that recurrent, DREADD-induced neuronal activation in the PFC increases microglial morphological area and clustering, induces microglial lysosome expression, and selectively impairs aspects of cognition. Altogether, these data indicate that chronic neuronal activation in the PFC shifts microglial phenotype and cognitive function.

Materials and Methods

Animals and experimental design. Wild-type C57BL/6J mice were purchased from Jackson Laboratories (#000664) and transgenic floxed-hM3Dq mice were obtained from in-house breeders backcrossed to C57BL/6J mice (Jackson Laboratories; RC::L-hM3Dq, #026943). Floxed-hM3Dq mice possess a Cre recombinase-responsive Gq-coupled DREADD allele/mCherry fusion protein, allowing for robust and selective somatodendritic hM3Dq expression (Sciolino et al., 2016). All mice (male, 6-8 weeks old at the time of surgery) were group-housed (3-4/cage) in 11.5×7.5×6-inch polypropylene cages under a 12-hour light-dark cycle with ad libitum access to food and water. For experiments in C57BL/6J mice, animals underwent either behavioral testing with no subsequent tissue analysis, brain extraction followed by microglial isolation and gene expression analysis, or brain extraction followed by
histological analysis. For experiments in floxed-hM3Dq mice, animals underwent either behavioral testing followed by histological analysis or brain extraction followed by microglial isolation and gene expression analysis. Animal use and procedures were in accordance with the National Institutes of Health guidelines and approved by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC).

**Stereotactic surgery.** Mice were deeply anesthetized with ketamine:xylazine (80 mg/kg:10 mg/kg) followed by stereotactic surgery. One of two viruses was bilaterally infused (0.5 µl; 0.1 µl/min) into the mPFC (AP: +2.0mm, ML: ±0.2mm, DV: -2.8mm). AAV5-CaMKIIα-hM3d-mCherry (Addgene, 50476-AAV5) was infused in C57BL/6J mice, whereas AAV5-CaMKII-mCherry-Cre (UNC Vector Core, Chapel Hill, NC) was infused in floxed-hM3Dq mice. Syringes were left in place for 5 min after infusion to limit viral spreading in the needle tract. Mice received daily injection of meloxicam (5 mg/kg, i.p.) over 3 days for pain relief. Mice were handled intermittently over three weeks to allow for surgical recovery and viral infection/expression.

**Drug administration.** Clozapine-n-oxide (CNO; National Institutes of Health, Bethesda, Maryland) was diluted to 0.6 mg/mL in 0.9% sodium chloride/0.5% dimethyl sulfoxide (DMSO). Vehicle or CNO (1 mg/kg) was administered daily via intraperitoneal (i.p.) injection for 14 consecutive days. All injections were performed in the morning or after behavioral testing in specified cohorts. Animal body weight was measured at the start and end of injections, with percent weight gain calculated.

**Behavior and cognitive testing.** Forced swim test (FST) and temporal object recognition testing (TOR) were conducted as previously described (Yuen *et al.*, 2012; Wohleb *et al.*, 2018; Horchar and Wohleb, 2019). The mPFC contributes to both stress coping behavior in the FST and discrimination in the TOR, making these behavioral assays particularly relevant to the
neurobiological approaches used in this study (Barker et al., 2007; Warden et al., 2012). All behavioral tests were performed in a normally lit room (white light), between 0800 and 1000 hours. Mice were allowed to habituate to this room for 30 min prior to testing. All mice that underwent behavioral testing were exposed to both the FST (day 14 of CNO administration) and the TOR (day 15). For the FST, mice were placed in a 2-liter beaker of water (24°C+/- 1°C) for 10 min and immobility was measured. In the first trial phase of the TOR, mice were placed in a plastic arena with two plastic Lego™ trees secured to the bottom of the arena. For each phase of the TOR, mice were given 5 min to explore the arena and objects. After a 30 min latency, mice were placed in the same arena with Lego™ blocks (second trial phase). An hour after the second trial phase, mice were placed in the arena with one block and one tree, counterbalanced to prevent bias (test phase). The time spent exploring each of these objects was measured and a discrimination index was calculated as follows: time spent exploring the tree - time spent exploring the block / total time spent exploring the tree and the block.

Percoll gradient enrichment of microglia. For these studies, mice were euthanized by rapid cervical dislocation followed immediately by brain extraction. Whole brains were then split by making a transverse cut (ventral-to-dorsal) at the optic chiasm. Cortex rostral to this split was enriched for the PFC by dissecting out all structures below the forceps minor/corpus callosum followed by removal of ventral cortex. Tissue samples were immediately stored on ice, with all subsequent procedures performed at 4°C. Dissected frontal cortex was passed through a 70 µm cell strainer. Both mechanical tissue homogenization and reduced temperatures (4°C) have been shown to limit isolation-associated shifts in microglial gene expression (Ocañas et al., 2021). Homogenates were centrifuged at 800g for 8 min. Supernatant was then removed and cells were enriched using one of two Percoll-based approaches (GE Healthcare, Uppsala, Sweden, #17089102). For experiments using AAV5-CaMKIIα-hM3d-mCherry, cell pellets were re-suspended in 70% isotonic Percoll and a discontinuous Percoll density gradient was layered.
as follows: 30% and 0% isotonic Percoll (phosphate buffered saline, PBS). For experiments using AAV5-CaMKII-mCherry-Cre, cell pellets were re-suspended in 30% isotonic Percoll and layered with 0% isotonic Percoll. Gradients were then centrifuged for 20 min at 2000g and cells were collected from either the interphase between the 70% and 30% Percoll layers, or below the 30% Percoll layer. Enriched microglia were then labeled with antibodies for flow cytometry and fluorescence-activated cell sorting (FACS).

**Fluorescence-activated cell sorting (FACS).** Staining of cell surface antigens was performed as previously described (Horchar and Wohleb, 2019). In brief, Fc receptors were blocked with anti-CD16/CD32 antibody (BioLegend, San Diego, CA, U.S.A., #553141). Cells were washed and then incubated with conjugated antibodies (PerCp-Cy5-CD11b, #550993; PE-CD45, #103105 or PE-P2Y12, #848004; BioLegend) for 45 min at 4°C. Cells were washed and re-suspended in fluorescence activated cell sorting (FACS) buffer. Approximately 30,000 microglia were sorted based on CD11b/CD45 or CD11b/P2Y12 expression using a BioRad S3e four-color cytometer/cell sorter (Hercules, CA, U.S.A.). Data were analyzed using FlowJo software (Ashland, OR, U.S.A.).

**RNA isolation and quantitative real-time PCR.** RNA was extracted from whole brain regions (PFC) using TRizol Reagent according to manufacturer’s protocol (Invitrogen). RNA was extracted from microglia using a Single Cell RNA purification kit (Norgen Biotek Corp., Thorold, Canada, #51800). Samples were reverse transcribed, and quantitative real-time PCR was conducted as previously described (Wohleb et al., 2018; Horchar and Wohleb, 2019). Primer sequences are listed in Table S1.

**Immunohistology.** Approximately 24 h after the final injection of CNO or vehicle, mice were administered a lethal dose of Fatal-Plus (Vortech Pharmaceuticals, Dearborn, MI, U.S.A.)
followed by intracardiac perfusion with PBS and 4% paraformaldehyde (PFA). Brains were post-fixed in 4% PFA for 24 h and incubated in 30% sucrose until cryoprotected. Brains were then rapidly frozen and sectioned on a Leica CM2050 S cryostat (40 µm). Free-floating sections containing the mPFC were selected for analysis. Sections were washed, blocked in 1% bovine serum albumin (BSA; Fisher Scientific, Waltham, MA, U.S.A; #BP9703) with 5% normal donkey serum (NDS; EMD Milipore, Billerica, MA; #S30-100ML) for 1 h at room temperature, washed, and then incubated with primary antibody: rabbit anti-IBA1 (1:1000, Wako; 019-19741), rat anti-CD68 (1:500, Bio-Rad; MCA1957), or rabbit anti-FosB (1:500, Abcam; ab184938) overnight at 4°C. Sections were then washed and incubated with conjugated secondary antibody overnight at 4°C (1:1000, Invitrogen; Alexa Fluor 488; Alexa Fluor 647). Sections were subsequently washed, mounted on gel-coated slides, and coverslipped with Fluoromount-G (ThermoFisher Scientific; 00-4958-02).

**Quantitative immunofluorescence.** Confocal images were captured from adjacent brain sections in both hemispheres of the mPFC (3-4 sections/sample spanning the rostral-caudal axis, 2.10 – 1.34 mm Bregma; Nikon C2+ microscope). FosB immunolabeled tissue sections were imaged using a 10× objective (NA: 0.95, z-stack: 0.8 µm, image size: 1024×1024). To quantify FosB+ cells, bright immunolabeled cell bodies were thresholded and then counted using the ImageJ (NIH) Analyze Particles function (circularity: 0.2-1, size: 30-300). FosB+ cell density was calculated as cells per mm². For analysis of IBA1+ material, tissue sections were imaged using a 20× objective (NA: 0.95, z-stack: 0.8 µm, image size: 1024×1024). Individual IBA1+ cell counts were obtained by hand and cell density was calculated (cells per mm²). For microglial area, images were thresholded, total area was recorded (µm²), and area per microglial cell was calculated (µm²/cell). To assess microglial clustering, the distance between each individual microglia and their closest neighboring microglia was measured using the Nearest Neighbor Distance (NDD) plugin. To examine microglial morphology and the microglial lysosome marker
CD68, images from layer I of the mPFC were captured using a 40x objective (NA: 0.95, zoom: 2x, z-stack: 0.2 µm, image size: 1024×1024). Microglia were morphologically reconstructed in 3-dimensional space using Imaris 10 (Oxford Instruments, Abingdon, United Kingdom), with microglia soma volume (µm$^3$/cell), total branch volume (µm$^3$/cell), total branch length (µm/cell), and branch points (#/cell) quantified (3+ microglia reconstructed/animal, 41-44 microglia/group). To assess process distribution, a Sholl analysis was performed on 2-dimensional renderings of reconstructed microglia using concentric circles advancing 1 µm in step from the cell soma (encompassing 0-40 µm in space) (Sholl, 1953). CD68+ lysosomes were observed and measured in 3-dimensional space using volume view mode in Nikon NIS-Elements analysis software. The total volume of microglial CD68 was calculated in these images, with data expressed as CD68 volume (µm$^3$)/total number of IBA1+ cells counted per image (µm$^3$/IBA1+ cell).

Statistical analysis. Data were analyzed using GraphPad Prism 9.1.2 (La Jolla, California). Differences between group means were evaluated using Student's t-test for the majority of analyses. Sholl data were evaluated using a two-way repeated measures ANOVA (distance from soma × neuronal activation) followed by F-protected comparisons of each Sholl step using Fisher's LSD test. Pearson correlation coefficients were computed and analyzed for select variables. The number of animals examined in each analysis and full statistical details are compiled in Table S2-S3.

Results

Recurrent prefrontal activation driven by an AAV-mediated approach shifts microglial morphology and phenotype in the mPFC without affecting behavior.

In these studies, mice received bilateral infusion of AAV5-CaMKIIα-hM3d-mCherry into the mPFC followed by three weeks of recovery. Mice were then treated daily with vehicle or
CNO for 14 days (Fig 1A). Cognition and behavior were assessed via FST immobility, object exploration, and performance in the TOR task (n=7-8/group). Additional histological (n=6-8/group), cytometric (n=4/group), and molecular (n=4/group) measures were collected. As expected, administration of CNO led to a robust increase in the number of FosB+ neurons – a putative marker of neuronal activation – in the mPFC (t_{(12)}=4.357, p=0.0009, Fig 1B-C). In a separate cohort of mice, behavioral and cognitive testing was carried out. Repeated activation of neurons in the mPFC had no effect on animal weight gain (n=11-12/group, t_{(21)}=0.774, p=0.448, Fig 1D), time spent immobile in the FST (t_{(13)}=1.342, p=0.203, Fig 1E), time spent exploring objects in the TOR (t_{(14)}=0.167, p=0.870, Fig 1F), or discrimination index in the TOR (t_{(14)}=0.667, p=0.516, Fig 1G). It is worthwhile to note that 14 days of CNO treatment alone (no DREADD) had no effect on behavior in the FST, TOR, or a number of other common assays (Fig S1). Likewise, treatment with CNO alone did not disrupt the mPFC neuroimmune microenvironment (Fig S1), thus off-target effects of CNO are likely not impacting the behavioral and molecular measures examined here.

Additional immunohistology showed that activation of pyramidal neurons via AAV5-CaMKIIα-hM3d-mCherry did not affect the density of microglia (t_{(13)}=0.678, p=0.510) or microglial clustering as assessed using nearest neighbor analysis (t_{(13)}=0.546, p=0.594) in the mPFC (Fig 1H-I). However, treatment with CNO significantly increased the amount of IBA1+ area per microglia in this region (IBA1+ area/cell; t_{(13)}=4.811, p=0.001; Fig 1J). The degree of neuronal activation (FosB+ cell density) was marginally correlated with the level of IBA1 immunolabeling in the mPFC (r_{(12)}=0.446, p=0.110; Fig 1K), suggesting a link between the magnitude of DREADD-induced neuronal activation in the mPFC and markers of microglial morphology.

To further examine neuronal activation-associated alterations in microglia, microglia from the frontal cortex were isolated from mice that received infusions of AAV5-CaMKIIα-hM3d-mCherry into the mPFC (Fig 2A). Microglia were enriched via Percoll gradient isolation and
purified by FACS. Representative dot plots show the distribution of microglia (CD11b<sup>+</sup>/CD45<sup>lo</sup>) and brain macrophages (CD11b<sup>+</sup>/CD45<sup>hi</sup>) from the frontal cortex (Fig 2B). The proportion of microglia and brain macrophages were not significantly altered by DREADD-induced neuronal activation (microglia: t<sub>(12)</sub>=0.743, p=0.472; macrophages: t<sub>(12)</sub>=0.093, p=0.927). Subsequent gene expression analyses revealed an increase in microglial Csf1r transcript following DREADD-induced neuronal activation (t<sub>(6)</sub>=2.946, p=0.026; Fig 2C). Repeated neuronal activation also altered cytokine expression in sorted microglia, with analyses indicating increased Il1b transcript (t<sub>(6)</sub>=2.527, p=0.045) and decreased Tnfa transcript (t<sub>(6)</sub>=2.642, p=0.039). Neuronal activity had no effect on microglial Cx3cr1 (t<sub>(6)</sub>=0.081, p=0.938), Tgfbr1 (t<sub>(6)</sub>=1.690, p=0.142), or Cd11b (t<sub>(6)</sub>=0.622, p=0.557).

Repeated neuronal activation induced by a Cre-dependent DREADD leads to microglial dysregulation in the mPFC and impairs working memory function.

Bilateral infusion of AAV5-CamkIIα-mCherry-Cre in floxed-hM3Dq mice enabled selective expression of activating DREADDs in PFC pyramidal neurons. After 3 weeks of recovery, mice were injected daily with vehicle or CNO (Fig 3A). Cognition, behavior, and microglial phenotype were examined (behavior: n=10/group, histology: n=9-10/group, molecular: n=6-8/group). Similar to our prior findings, chronic treatment with CNO increased the number of FosB+ cells/mm<sup>2</sup> in the mPFC (t<sub>(17)</sub>=4.012, p=0.0009; Fig 3B-C). This had no effect on animal weight gain (n=19-20/group, t<sub>(37)</sub>=0.998, p=0.325, Fig 3D), time spent immobile in the FST (t<sub>(18)</sub>=1.199, p=0.246, Fig 3E), or object exploration in the TOR (t<sub>(18)</sub>=0.273, p=0.788, Fig 3F). However, chronic treatment with CNO disrupted object discrimination in the TOR in floxed-hM3Dq mice (t<sub>(18)</sub>=2.781, p=0.012; Fig 3G). This suggests that recurrent neuronal activation in the PFC, as driven by a Cre-dependent DREADD, is sufficient to disrupt prefrontal function and influence cognitive performance.
Further immunohistology showed that administration of CNO increased microglial density (IBA1+ cells/mm$^2$; $t_{(18)}=2.655$, $p=0.016$), microglial clustering ($t_{(18)}=2.663$, $p=0.016$), and microglial area (IBA1+ area/cell; $t_{(18)}=3.272$, $p=0.004$) in the mPFC (Fig 4A-D, Fig S2). Further analysis of microglial morphology revealed a neuronal activity-induced increase in microglia soma volume in the mPFC (μm$^3$/cell; $t_{(19)}=4.067$, $p=0.001$; Fig 4E). Interestingly, there was no change in the volume of microglial processes (μm$^3$/cell; $t_{(19)}=0.439$, $p=0.666$; Fig 4F) or the number of process branch points ($t_{(19)}=1.074$, $p=0.595$, data not shown). However, repeated neuronal activation reduced the overall length of microglial branches (μm/cell; $t_{(19)}=2.165$, $p=0.043$; Fig 4G) and led to a significant redistribution of microglial processes (distance from soma × neuronal activation interaction, $F_{(40, 760)}=2.706$, $p<0.001$; Fig 4H). Follow-up comparisons indicate that microglia in mice treated with CNO had fewer process intersections at 16-17 μm ($p=0.039-0.042$) and 20-28 μm ($p=0.004-0.029$) from the soma. In addition to shifts in density and morphology, CNO increased the volume of CD68+ lysosomes per microglia in the mPFC ($t_{(18)}=2.372$, $p=0.029$; Fig 4I). Correlational analyses revealed an association between FosB induction in the mPFC with increased microglial area ($r_{(17)}=0.425$, $p=0.052$; Fig 4J), soma volume ($r_{(17)}=0.639$, $p=0.003$; Fig 4J), and dysfunction in the TOR ($r_{(17)}=-0.625$, $p=0.004$; Fig 4K). Likewise, increases in microglial clustering ($r_{(18)}=0.557$, $p=0.011$) and CD68+ lysosome volume ($r_{(18)}=-0.503$, $p=0.024$) were associated with greater deficits in the TOR (Fig 4K). Together these data indicate an activity-dependent relationship between neuronal activity in the mPFC, microglia phenotype, and behavior.

In a separate cohort, microglia were isolated from the frontal cortex using FACS and microglial gene expression was analyzed (Fig 5A-B). Repeated administration of CNO increased levels of $Csf1r$ ($t_{(14)}=2.382$, $p=0.032$) and $Cd11b$ ($t_{(14)}=2.162$, $p=0.048$) expression in frontal cortex microglia, with little effect on levels of $Cx3cr1$ ($t_{(13)}=1.087$, $p=0.297$), $Tgfbr1$ ($t_{(13)}=0.165$, $p=0.872$), $P2ry12$ ($t_{(13)}=1.509$, $p=0.155$), $Cd68$ ($t_{(14)}=0.116$, $p=0.909$), $Il1b$ ($t_{(10)}=1.126$, $p=0.287$), or $Tnfa$ ($t_{(13)}=0.623$, $p=0.544$) transcript (Fig 5C). This suggests that
repeated neuronal activation modulates specific microglial pathways including those relevant to microglia-mediated synaptic remodeling (i.e., Csf1r, Cd11b).

Discussion

In these studies, we used two chemogenetic approaches to repeatedly activate pyramidal neurons in the mPFC. This is relevant because clinical studies and preclinical models suggest that altered neuronal activity and connectivity, particularly in the PFC, is observed in psychiatric disorders and neurological disease (Fleisher et al., 2009; Walter et al., 2009; Tian et al., 2014). Overall, we found that repeated neuronal activation led to an increase in the density of microglia, restructuring of microglial processes, and soma hypertrophy in the mPFC. This is comparable to the morphological remodeling seen in microglia following acute neuronal activation (Umpierre et al., 2020; Nebeling et al., 2023), and suggests that microglial responses may be both rapid and sustained across recurrent bouts of activity. Repeated activation of pyramidal neurons also upregulated microglial expression of Csf1r and Cd11b in the frontal cortex. These pathways are critical in microglial proliferation and homeostasis, and have been implicated in activity-dependent, microglia-mediated synaptic remodeling (Chitu et al., 2016, 2020; Arreola et al., 2021). For instance, neuronal activity increases CSF1 expression in the cortex, with CSF1-CSF1R signaling shown to guide microglia-synapse engagement, phagocytosis of dendritic material, and dendritic spine loss in the mPFC, amongst other brain regions (Luo et al., 2013; Iaccarino et al., 2016; Wohleb et al., 2018; Zhou et al., 2019). Likewise, CD11b – a component of complement receptor 3 (CR3) – contributes to microglia-mediated synaptic pruning with downstream consequences on neuronal function and behavior (Stevens et al., 2007; Schafer et al., 2012; Hong et al., 2016). Engagement of these pathways implicated in microglial phagocytosis dovetails with our finding of increased soma- and lysosome volume in microglia in the mPFC, suggesting heightened microglial phagocytosis or debris clearance in this region. Though not examined directly and speculative, these convergent
findings may indicate that repeated activation of pyramidal neurons drives microglia-neuron engagement and subsequent remodeling of synapses in the mPFC. In support of this, studies show that acute doses of CNO increase glutamatergic signaling in hM3Dq expressing pyramidal neurons (Wang et al., 2018; Pati et al., 2019). This leads to heightened levels of microglial process motility and microglia-synapse contact (Badimon et al., 2020; Nebeling et al., 2023), with additional studies showing that glutamatergic neurotransmission increases microglial process extension and phagocytosis of neuronal material (Dissing-Olesen et al., 2014; Eyo et al., 2014; Sipe et al., 2016). Future work will be needed to determine whether repeated neuronal activation is sufficient to drive microglia-mediated synaptic remodeling in the PFC and subsequent alterations in behavior.

While both AAV-mediated and Cre-dependent DREADDs led to similar changes in microglial area and Csf1r upregulation in the mPFC, several surprising differences were observed. Mice with Cre-dependent DREADD expression showed neuronal activity-associated increases in microglial density and clustering, whereas AAV-mediated DREADD mice did not. Likewise, activation of pyramidal neurons in the mPFC selectively impaired TOR discrimination in mice with Cre-dependent—but not AAV-driven—DREADD expression. This could be due to potential differences in the location, pattern, or amount of hM3Dq receptors inserted at the plasma membrane and, subsequently, differences in the magnitude of neuronal activation elicited by these two unique chemogenetic approaches (Sciolino et al., 2016). For instance, the floxed-hM3Dq mice used here were designed to concentrate Gq-coupled DREADD receptors on the soma and dendrites of neurons, with the goal of eliciting robust ligand-mediated cellular activation. It is possible that DREADD receptors may be more diffuse or inconsistently expressed using an AAV-driven approach (Atasoy et al., 2012). Indeed, mice with Cre-dependent DREADD expression showed a nearly 382% increases in the number of FosB+ cells in the mPFC following chronic CNO administration, as compared to only a 185% increase in mice with AAV-driven DREADD expression. This would be in line with our data linking FosB+
cell density in cre-dependent DREADD mice with microglial morphological remodeling in the mPFC and the severity of impairment in the TOR. Additional studies examining both the neurobiological mechanisms underlying- and behavioral outcomes following- AAV-driven and Cre-dependent DREADD expression may further contextualize our results.

Despite these compelling findings, several points warrant discussion. Our studies examined microglial responses to repeated neuronal activation in male mice only and are thus limited in their interpretation. Reports indicate marked sex differences in microglial morphology, transcription, and developmental profile across numerous brain regions including the mPFC (Schwarz et al., 2012; Bollinger et al., 2017; Barko et al., 2022), with sex-specific microglial responses observed following early-life opioid exposure, cannabis use, and psychological stress (Bollinger et al., 2016; Woodburn et al., 2021a; Smith et al., 2022; Freels et al., 2023).

Considering this, it is likely that microglia mount a unique response to repeated neuronal activation in the mPFC in females. Another limitation of our studies is that microglial responses to repeated neuronal activation were examined following activation of primarily excitatory CaMKIIα+ pyramidal neurons in one brain region. Microglia are extremely heterogenous both within- and across- brain regions, and respond differentially to activation of excitatory versus inhibitory neuronal subtypes (Lawson et al., 1990; Cantaut-Belarif et al., 2017; De Biase et al., 2017; Masuda et al., 2019; Badimon et al., 2020; Lyu et al., 2023). Thus, it is conceivable that repeated activation of pyramidal neurons may evoke unique changes in microglia based on brain region or associated neural circuits, and that activation of specific neuronal populations (e.g., parvalbumin interneurons) may induce distinct alterations in microglia phenotype and function. Future studies examining sex-, region-, and neuronal subtype- specific microglial responses to changes in brain activity are needed.

In summary, our data indicate that repeated activation of excitatory pyramidal neurons in the mPFC is sufficient to drive robust changes in microglia, including morphological remodeling, lysosome induction, and the expression of various microglia-neuron interaction factors (e.g.,
Csf1r, Cd11b). Our results further suggest that the degree of neuronal activation may contribute to both the magnitude of microglial remodeling and unique patterns of microglial clustering and transcription in the mPFC. These findings represent an important consideration for studies using chemogenetic or optogenetic manipulation of neuronal circuits as these approaches have indirect effects on microglia (and other glia), which likely influence physiological and behavioral outcomes.
Author Contributions

Participated in research design: Bollinger and Wohleb.

Conducted experiments: Bollinger, Horchar, and Wohleb.

Performed data analysis: Bollinger and Wohleb

Wrote or contributed to the writing of the manuscript: Bollinger and Wohleb
Data Availability Statement

All data presented in this manuscript is available upon reasonable request. Please contact Dr. Eric Wohleb (eric.wohleb@uc.edu) with any data inquiries.
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Footnotes

This work was supported by the Brain and Behavior Research Foundation (E.S.W.) and the National Institutes of Health National Institute of Mental Health (F32MH123051).

No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

**Figure 1. Repeated neuronal activation in the mPFC as driven by an AAV-mediated DREADD is not sufficient to alter behavior or cognition but increases microglial area. A)** Male C57BL/6J mice received bilateral infusion of AAV5-CaMKIIα-hM3d-mCherry (DREADD) in the mPFC. After recovery, mice were injected with vehicle or clozapine-n-oxide (CNO; 1 mg/kg/day, i.p.) for 14 days. One cohort was assessed in the forced swim test (FST) and temporal object recognition (TOR) test on subsequent days (n = 7-8/group). A separate cohort was used for immunohistology (n = 6-8/group). **B)** Representative images showing AAV expression (mCherry) and FosB immunolabeling (top), alongside confocal images of microglial IBA1 immunolabeling (bottom) in the mPFC. White scale bar indicates 100 µm. **C)** Average number of FosB+ cells/mm² is shown. **D)** Percent weight change following 14 days of treatment with either vehicle or CNO (n = 10-12/group, includes behavioral and histology cohorts). **E)** Time spent immobile in the forced swim test (FST), **F)** time spent exploring objects in the temporal object recognition (TOR) test, and **G)** discrimination index in the TOR is shown. Various aspects of microglia phenotype were analyzed in the same brains used for FosB immunolabeling (1B-C), this included **H)** IBA1+ cell density, **I)** IBA1+ cell clustering as assessed using nearest neighbor distance, and **J)** IBA1+ cell area. **K)** Association between FosB+ cell density and IBA1+ cell area is noted (Pearson's r). Bars represent mean ± S.E.M. * denotes p < 0.05, Student’s T-test.

**Figure 2. Repeated DREADD-induced neuronal activation shifts the molecular phenotype of microglia in mice expressing AAV5-CamKIIα-hM3dq. A)** Male C57BL/6 mice received bilateral infusion of AAV5-CaMKIIα-hM3d-mCherry (DREADD) in the mPFC. After recovery, mice were injected with vehicle or clozapine-n-oxide (CNO) (1 mg/kg/day, i.p.) for 14 days. 24 hours after the final CNO injection, frontal cortex was dissected out and brain myeloid cells were enriched by Percoll gradient. Fluorescence activated cell sorting (FACS) was used to isolate
microglia based on CD11b⁺/CD45lo expression. B) Representative dot plots indicating FSC-H/CD11b-H and CD45-H/CD11b-H, with both microglial (CD11b⁺/CD45lo) and brain macrophage (CD11b⁺/CD45hi) populations noted. Following FACS, mRNA was collected from microglia. C) Relative fold change in mRNA levels in sorted frontal cortex microglia is shown (n = 4/group). Bars represent mean ± S.E.M. * denotes p < 0.05, Student’s T-test.

**Figure 3. Recurrent activation of prefrontal neurons in mice expressing a Cre-dependent DREADD induces cognitive dysfunction.** A) Male hM3dq floxed mice received bilateral infusion of AAV5-CaMKIIα-mCherry-Cre in the mPFC. After recovery, mice were injected with vehicle or clozapine-n-oxide (CNO; 1 mg/kg/day, i.p.) for 14 days. Animals were assessed in the forced swim test (FST) and temporal object recognition (TOR) test on subsequent days (n = 10/group). Approximately 4-hours after TOR testing, mice were euthanized and brains were extracted for immunohistology. B) Representative images showing AAV expression (mCherry) and FosB immunolabeling in the mPFC. White scale bar indicates 100 µm. C) Average number of FosB+ cells/ mm² is shown. D) Percent weight change following 14 days of treatment with either vehicle or CNO (n = 19-20/group, includes all experimental cohorts). E) Time spent immobile in the forced swim test (FST), F) time spent exploring objects in the temporal object recognition (TOR) test, and G) discrimination index in the TOR is shown.

**Figure 4. Repeated activation of prefrontal neurons leads to profound changes in microglial density, morphology, and lysosome induction.** A) Representative images showing IBA1 and CD68 immunolabeling in the mPFC (top) alongside 3-dimensional reconstructions of each respective microglia (bottom). White- and black scale bars represent 10 µm. See Fig S2 for additional images. Various aspects of microglia phenotype were analyzed in the same brains as previously used for FosB immunolabeling (Fig 3), this included B) IBA1+ cell density, C) IBA1+ cell clustering as assessed using nearest neighbor distance, D) IBA1+ cell
area, **E)** microglial soma volume, **F)** microglial process volume, **G)** overall process length per microglia, **H)** microglial process intersections in relation to soma proximity, and **I)** microglial CD68 volume per cell. **J)** Association between FosB+ cell density, IBA1+ cell area (left), and microglial soma volume (right) is noted (Pearson’s r). **K)** Associations between discrimination in the TOR and FosB+ cell density (left), microglial clustering (middle), and microglial CD68 volume (right) are noted (Pearson’s r). Bars represent mean ± S.E.M. * denotes p < 0.05, Student’s T-test or Fisher’s LSD test.

**Figure 5. Repeated, Cre-dependent DREADD-induced neuronal activation in the mPFC alters microglial transcription.** **A)** Male hM3dq floxed mice received bilateral infusion of AAV5-CaMKIIα-mCherry-Cre in the mPFC. After recovery, mice were injected with vehicle or clozapine-n-oxide (CNO) (1 mg/kg/day, i.p.) for 14 days. 24 hours after the final CNO injection, frontal cortex was dissected out and brain myeloid cells were enriched by Percoll gradient. Fluorescence activated cell sorting (FACS) was used to isolate microglia based on CD11b+/P2Y12+ expression. **B)** Representative dot plots indicating FSC-H/CD11b-H and CD11b-H/P2Y12-H, with P2Y12+ microglia noted. Following FACS, mRNA was collected from microglia. **C)** Relative fold change in mRNA levels in sorted frontal cortex microglia is shown (n = 6-8/group). Bars represent mean ± S.E.M. * denotes p < 0.05, Student’s T-test.
Figure 2

A. Male C57BL/6J mice (5-6 weeks old)

mPFC infusion:
AAV5CamKII-hM3D-mCherry

Treatment (14 days)

Vehicle, CNO \(^{(1 \text{ mg/kg/d})}\)  

Frontal cortex dissection → Percoll enrichment → FACS + qPCR

B.  

- FSC-H vs CD45-H (FL2)
- CD11b-H (FL4) vs CD45-H (FL2)

Brain Mφ 0.42%
Microglia 98.5%
77.1%

C. 

Microglia mRNA (fold change)

- Csf1r
- Cx3cr1
- Tgfr1
- Cd11b
- Il1b
- Tnfa

* indicates significance
Figure 3

A. Treatment (14 days)

- Vehicle, CNO (1 mg/kg/d)
- FST TOR (d14, d15)

B. Histology

- Vehicle
- CNO

C. mPFC infusion: AAV5 CamKII-mCherry-Cre

D. Weight change (%)

E. FST immobility, s

F. TOR (exploration, s)

G. TOR (disc. index)

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