Microglia depletion facilitates the display of maternal behavior and alters activation of the maternal brain network in nulliparous female rats

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Abbreviations used:

colony-stimulating factor 1 receptor (CSF1R)
phosphate buffered saline (PBS)
Normal Donkey Serum (NDS)
prelimbic prefrontal cortex (PFC)
nucleus accumbens (NAc)
preoptic area (POA)
medial amygdala (MeA)
periaqueductal grey (PAG)
anterior olfactory nucleus (AON)
barrel field within the primary somatosensory cortex (S1BF)

Abstract

As a pregnancy progresses, inhibition of aversion circuitry and activation of reward-related pathways is necessary for the onset of maternal care postpartum. We and others have also demonstrated significant neuroimmune changes that emerge during late pregnancy and persist postpartum, most prominently decreased microglia numbers within limbic brain regions. Here we hypothesized that microglial downregulation is important for the onset and display of maternal behavior. To test this, we recapitulated the peripartum neuroimmune profile by depleting microglia in non-mother (i.e., nulliparous) female rats who are typically not maternal but can be induced to behave maternally towards foster pups after repeated exposure, a process called maternal sensitization. BLZ945, a selective colony-stimulating factor 1 receptor (CSF1R) inhibitor, was administered systemically to nulliparous rats, which led to ~75% decrease in microglia number. BLZ- and vehicle-treated females then underwent maternal sensitization and tissue stained for ΔfosB to examine activation across maternally relevant brain regions. We found BLZ-treated females with microglial depletion met criteria for displaying maternal behavior significantly sooner than vehicle-treated females and displayed increased pup-directed behaviors. Microglia depletion also reduced threat appraisal behavior in an open field test. Notably, nulliparous females with microglial depletion had decreased numbers of ΔfosB+ cells in the medial amygdala and periaqueductal gray, and increased numbers in the prefrontal cortex and somatosensory cortex compared to vehicle. Our results demonstrate that microglia regulate maternal behavior in adult females, possibly by shifting patterns of the activity in the maternal brain network.

1. Introduction

Over 200 million people worldwide become pregnant each year [1]. Successful maternal care is necessary for the well-being of both mother and offspring, with life-long consequences when perturbed [2-4]. Across mammalian species, new mothers exhibit coordinated changes in the neural circuits that regulate processes essential for appropriate maternal behavior. When considering maternal care in rodents, this involves inhibition of aversion circuitry and activation of reward-related pathways, resulting in a shift from pup avoidance or attack, to pup-directed behavior [5,6].

Neuroendocrine factors mediating the transition from aversion to reward have been well-elucidated [7-9]. However, pregnancy affects nearly every system in the body [10], and one system that undergoes particularly profound changes is the immune system. In the periphery, pregnancy induces a shift from a pro-inflammatory toward a more anti-inflammatory signaling milieu. This occurs to prevent an attack on non-self cells (i.e., the fetus), and is therefore necessary to support a successful pregnancy and healthy fetal development [11-13]. While the timing and general purpose of these shifts in the peripheral immune milieu are well established, considerably less is known about the status of immune cells and inflammatory signaling *within the central nervous system* during pregnancy. We and others have recently shown that the brain of pregnant and postpartum rats displays significant decreases in the number of innate immune cells, called microglia [14-16]. These decreases are particularly observed in several limbic brain regions that play critical roles in maternal care [16], including the prefrontal cortex, nucleus accumbens, amygdala, and hippocampus. This decrease in microglia emerges during late pregnancy and persists until at least postpartum day 8, the time frame during which maternal behavior is at its highest. Microglia return in number by postpartum day 21, the same time at which pups are ready to be weaned.

Given the concurrent timing of microglia downregulation in the peripartum brain and the onset of maternal behavior, we aimed to investigate whether these microglia changes are involved in the onset and display of maternal behavior. One approach for examining the role of microglia involves global depletion with selective colony-stimulating factor 1 receptor (CSF1R) inhibitors. The CSF1 pathway is essential for microglia survival, and accordingly its inhibition causes rapid apoptosis [17]. In adult male mice, microglia depletion with PLX5622, a CSF1R inhibitor [18] did not have any significant effects on locomotor, anxiety-like, or cognitive behavior. Similarly, there were no effects on sociability in male mice following microglia depletion via systemic administration of another CSF1R inhibitor, PLX3397 [19]. However, since females were not examined, the possibility remains that microglia are important for the display of female-specific social behaviors like maternal caregiving.

Rats that have never been mothers (e.g., nulliparous females) do not spontaneously display maternal behavior, but alloparenting behavior can be induced through continuous or repeated exposure to foster pups. This process, known as maternal sensitization, has been a valuable model for exploring the factors responsible for maternal care [20-27]. Here, we used the maternal sensitization model to investigate

microglia modulation of maternal behavior for the first time. Specifically, we depleted microglia from the brains of nulliparous rats and tested whether recapitulating the decreased microglia tone that we previously observed in the maternal brain would impact their subsequent sensitization to maternal behavior and display of caregiving behaviors. We then examined number of cells expressing ΔfosB across brain regions important for facilitating the shift from aversion to reward in promoting maternal care. As ΔfosB is a marker of neuronal activation and accumulates in neurons after repeated exposure to external stimuli [28], in this case continuous foster pup exposure, this would provide insights into which brain regions across extensive maternal circuitry were differentially active during the sensitization procedure as a result of microglia depletion. Together these studies implicate microglia downregulation in facilitating maternal behavior onset, possibly by shifting patterns of the activity in the maternal brain network.

2. Materials and Methods

2.1 Animals. All procedures were conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by The Ohio State University Institutional Animal Care and Use Committee. Adult female nulliparous Sprague Dawley rats and timed gestational day 8 and gestational day 15 pregnant rats (Harlan/Envigo) were ordered, single housed upon arrival and provided with nesting material. Pregnant rats served as surrogates to provide foster pups for nulliparous animals. Animal cages for all animals were kept in the same temperature- and humidity-controlled room maintained on a 12/12hour light/dark cycle. Animals were given food and water *ad libitum*. Upon study completion, surrogate dams and foster pups were used for other pilot studies within the lab when possible or humanely euthanized with CO_2.

2.2 Microglia Depletion*.* In this study, we utilized a CSF1R antagonist, BLZ945, that works via a similar mechanism to PLX5622 used by many other groups to deplete microglia in rodents. BLZ945 is equally effective at microglia depletion, [29] but unlike PLX drugs, was widely commercially available at the time we began these experiments. A 10 mg/ml solution of BLZ945 (Selleckchem; #S7725) was prepared 1-2 days before use in vehicle of 20% 2-hydroxy-propyl-β-cyclodextrin (Sigma; H107-5G), (powder dissolved in molecular H_20). Adult female rats were injected intraperitoneally with BLZ945 (N=4) at a dosage of 60 mg/kg or an equivalent volume of vehicle (N= 4) followed by a second administration 48 hours later. A pilot study for dose effectiveness revealed that a larger dose (100mg/kg) achieved the same depletion level as the dose (60mg/kg) used for sensitization experiments. Twenty-four hours following the second injection, rats were euthanized to verify effectiveness of BLZ945 at depleting microglia. Additional groups of adult female rats were randomly assigned to receive vehicle (N = 12) or BLZ945 (N = 12) according to the same administration paradigm, but 24 hours after the second injection, the maternal sensitization procedure began.

2.3 Maternal Sensitization. Each day between 8:30-10:30am, a home cage observation was performed. Nulliparous females were given three same-aged foster pups (1-10 days old) scattered in the home cage opposite to where the nulliparous female was positioned [23, 24]. The youngest available foster pups were used each day,

and age of pups was balanced across conditions. Incidences of maternal and non-maternal behaviors were tabulated every 30 sec for a 30-minute period via scan sampling, for a total of 60 scans (2 per minute). Maternal behaviors included: huddling (laying on or in physical contact with pups), pup-licking (licking the pup's body/anogenital region), pup-sniffing, retrieving (grasping of the pup with the mouth and returning it to the nest), mouthing (grasping the pup with the mouth, carrying it around, but not to the nest), and nest-building (collecting and/or handing nesting material). Non-maternal behaviors included: self-grooming, eating, rearing (two front paws off the ground), sniffing air (with head/neck extended), and resting (no general motor activity, not in contact with pups). Each morning, the pups were removed from the nulliparous female's home cage and replaced by milk-replete foster pups that had remained with a lactating surrogate for at least 24 hours. Continuous pup exposure was chosen as it allows for more rapid sensitization as has been previously reported [21, 30, 31]. Pups can remain healthy for that time frame, and as such there were zero instances of pup death due to poor thermoregulation or malnutrition in the current study. The daily observation began when the replacement pups were added to the cage. All observations took place in the animal colony room and were performed by a single observer. Nulliparous females were considered maternal ("sensitized") when they licked, retrieved, and grouped all three pups within the 30-minute observation period on two consecutive days. The first of these two consecutive days was operationalized as the day the animal become maternal (e.g., the latency to maternal behavior onset).

2.4 Open Field Test The day after maternal criteria were met, rats were subjected to an open field test (60cm x 60cm arena) for 10 minutes under red light following a 10 minute habituation period to the behavioral testing room. The task was video recorded and scored by a rater blind to experimental conditions. Videos were scored for time spent in the center of the arena, number of entries into the center of the arena, and number of grid crosses that occurred as a proxy measure of overall locomotor behavior. One hour following the completion of the open field test, animals were euthanized.

2.5 Tissue processing, histology, and cell counting. Animals were deeply anesthetized with Euthasol and then transcardially perfused with 0.01M phosphate buffered saline (PBS) and 4% paraformaldehyde. Brains were extracted, post-fixed in 4% paraformaldehyde for 24 hours followed by 30% sucrose solution. Brains were sectioned coronally at 40µm into cryoprotectant. Tissue sections were subsequently mounted on charged slides and microglia immunofluorescent staining according to the following procedure. Tissue was washed in 0.01M PBS, followed by 30 minutes in 50% methanol to quench background fluorescence. Antigen retrieval was performed using a tris-EDTA buffer for 10 minutes in a 90°C water bath followed by 10 minutes in the buffer at room temperature. Tissue was permeabilized for 1 hour with 0.4% Triton-x100 in 0.01M PBS and blocked for 1 hour in 5% Normal Donkey Serum (NDS; Lampire Biological Laboratories, #7332100) in 0.4% Triton in 0.01M PBS. To label Iba1+ microglia (Wako [#019-19741]) or ΔfosB (Abcam [ab184938]), tissue incubated in primary antibody (1:500) with 2.5% NDS in 0.4% Triton in 0.01M PBS for 24 hours at 4°C. Slides were well rinsed and secondary antibody (donkey anti-rabbit Alexa Fluor+

highly cross-adsorbed 647+ [Invitrogen, #A32795]; 1:200) added with 2.5% NDS in 0.4% Triton in 0.01M PBS for 2 hours. Slides were cover-slipped with Prolong Diamond Antifade mountant (Invitrogen, #P36970). Four to six representative images per animal for all regions of interest (ROI), counterbalanced across hemispheres, were obtained in StereoInvestigator (MBF Bioscience) on a Zeiss Axioimager M2 microscope and a CX9000 Digital Camera. ROIs analyzed included (with stereotaxic coordinates in reference to bregma [32]: prelimbic prefrontal cortex (PFC; +3.20mm), nucleus accumbens (NAc; +1.20mm), preoptic area (POA; -1.30mm), medial amygdala (MeA; -2.80mm), periaqueductal grey (PAG; -5.60mm), anterior olfactory nucleus (AON; +3.70mm), and the barrel field within the primary somatosensory cortex (S1BF; -1.30mm). Iba1 was captured at 10x and ΔfosB was imaged at 20x. Images were then analyzed by a rater blinded to conditions in ImageJ.

2.6 Statistical analysis. One-way ANOVAs with Tukey's post hoc comparisons were used to analyze efficacy of the two different drug doses on microglia depletion. Unpaired two-tailed t-tests were conducted to compare vehicle vs BLZ treatment outcomes on behavior, and Welch's corrections used when between group variances were unequal. Statistical significance for these experiments was set at $\alpha = 0.05$. Pearson's correlations were conducted between: open field behavior and latency to reach maternal criteria, ΔfosB counts and select maternal behaviors (latency to reach maternal criteria, licking pups, and sniffing pups), and ΔfosB counts between brain regions within both experimental groups. Fisher r-to-z transformations were performed followed by Fisher Z-tests on transformed data [33] to assess any potential differences in correlations between experimental groups. Bonferroni correction was applied to these analyses ($\alpha = 0.05$/# comparisons) to control for multiple comparisons. Data points were considered statistical outliers when greater than ±2 standard deviations away from the mean and removed when appropriate. Effect size (eta squared [R^2]) calculations were reported for significant effects, where a value of 0.01 is considered a small effect, 0.06 a medium effect, and 0.14 (or higher) a large effect. Statistics were conducted in Prism 9 Software (GraphPad Software; San Diego, CA) or R (version 4.2).

3. Results

3.1 BLZ945 treatment successfully and rapidly depleted microglia

BLZ945 treatment led to a significant decrease in microglia number relative to vehicle treatment across all brain regions examined. The average reduction in microglia number across region was ~75%, and there were no differences in the level of depletion produced between the two doses of BLZ945. Full statistical results are shown in Table 1. Only the data for vehicle vs 60mg/kg are shown in Figure 1 because the 60 mg/kg dose was chosen for use in subsequent experiments. Data for 100mg/kg dosing is depicted in Supplemental Figure 1.

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240 **Table 1.** One-way ANOVA results with Tukey's multiple comparisons between vehicle, 60mg/kg of BLZ945, and 100mg/kg of BLZ945 demonstrating effects on microglia cell numbers across brain regions.

	One-way ANOVA			Tukey's HSD		
	F(df)	p	R^2	Vehicle vs 60mg/kg BLZ	Vehicle vs 100mg/kg BLZ	60mg/kg vs 100mg/kg BLZ
PFC	112.4 (2,9)	$p < 0.0001$	0.962	$p < 0.0001$	$p < 0.0001$	$p = 0.512$
NAc	256.9 (2,9)	$p < 0.0001$	0.983	$p < 0.0001$	$p < 0.0001$	$p = 0.912$
POA	83.44 (2,9)	$p < 0.0001$	0.949	$p < 0.0001$	$p < 0.0001$	$p = 0.635$
AMY	109.3 (2,9)	$p < 0.0001$	0.961	$p < 0.0001$	$p < 0.0001$	$p = 0.496$
PAG	161.0 (2,9)	$p < 0.0001$	0.973	$p < 0.0001$	$p < 0.0001$	$p = 0.374$
AON	71.68 (2,8)	$p < 0.0001$	0.947	$p < 0.0001$	$p < 0.0001$	$p = 0.806$
S1BF	106.6 (2,9)	$p < 0.0001$	0.960	$p < 0.0001$	$p < 0.0001$	$p = 0.381$

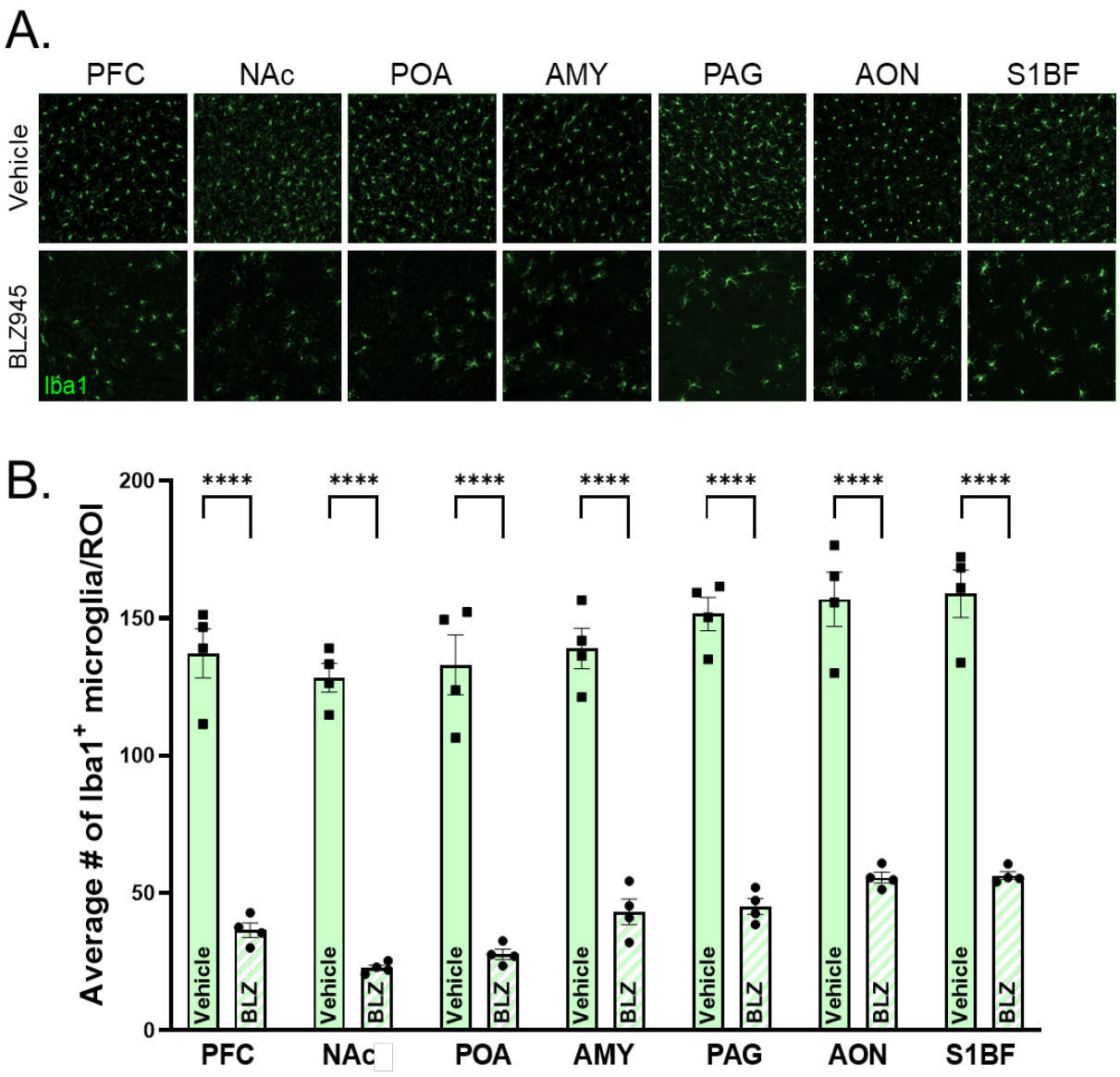

Figure 1. *A.)* Representative images (10x) from the prefrontal cortex (PFC), nucleus accumbens (NAc), preoptic area (POA), amygdala (AMY), periaqueductal gray (PAG), anterior olfactory nucleus (AON), and barrel field within primary somatosensory cortex (S1BF). and *B.)* quantification of Iba1+ microglia of vehicle- and BLZ945-treated (60mg/kg) adult females 24hrs following treatment administration. Individual data points represent animal average of 4 images. Error bars represent mean ± SEM. One-way ANOVA, Tukey's HSD: **** $P < 0.0001$. Data from BLZ945 100mg/kg treatment group not depicted.

3.2 Microglia depletion promoted onset of maternal behavior and reduced threat appraisal behavior

After verifying that the BLZ945 treatment strategy led to substantial and rapid microglia depletion, we applied the maternal sensitization procedure to another cohort of nulliparous females one day following the second administration of BLZ945. Nulliparous females undergoing maternal sensitization must display retrieval, grouping,

and huddling to be considered fully maternal, but the full criteria for maternal behavior is typically preceded by exhibiting sporadic maternal behaviors as well as other non-maternal behaviors [25, 26].

BLZ945- and vehicle-treated animals showed several notable differences in behavior in the days leading up to reaching maternal sensitization criterion. Prior to reaching the threshold for sensitization, BLZ-treated females showed increased pup-directed behaviors that included more licking, $t(11.09) = 2.54$, $p = 0.027$, $R^2 = 0.369$, and sniffing of foster pups relative to vehicle-treated females, $t(13.98) = 3.35$, $p = 0.005$, $R^2 = 0.445$, while vehicle-treated females had higher rates of 'other' behaviors $t(9.20) = 2.46$, $p = 0.036$, $R^2 = 0.260$ (Fig. 2A). All other observed behaviors did not vary by experimental treatment, *p's* > 0.1. With regard to the days needed to reach sensitization threshold, BLZ-treated females had a significantly shorter latency to display full maternal behavior than vehicle-treated animals, $t(22) = 2.23$, $p = 0.0365$, $R^2 = 0.184$ (Fig. 2B).

The day after an animal was designated as maternal, it was tested in the open field to assess threat appraisal behavior. BLZ-treated animals showed decreased threat appraisal behavior compared to vehicle-treated animals, as measured by increased time spent in the center of an open field, $t(21) = 4.67$, $p < 0.001$, $R^2 = 0.509$, as well as increased number of entries into the center of the open field, $t(21) = 3.21$, $p = 0.004$, $R^2 = 0.329$. These differences were not due to changes in locomotor activity, as indicated by similar numbers of grid crosses between groups, $t(21) = 0.26$, $p = 0.780$. (Fig. 2C). When correlating time spent in the center of the open field with latency to maternal criteria (Fig. 2D), there was only a significant correlation in the vehicle-treated animals, $r = -0.658$, $p = 0.027$, $R^2 = 0.433$, and not the BLZ-treated group, $r = -0.044$, $p = 0.891$.

3.3 Microglia depletion prior to maternal sensitization altered ΔfosB expression across maternally relevant brain regions

Next, we examined the extent to which there was a difference in activity across brain regions governing these behaviors resulting from microglia depletion, using $\Delta fosB^+$ cells as a proxy for chronic neuronal activation. In the sensitized animals who previously had microglia depleted with BLZ945, there was increased number of $\Delta fosB^+$ cells in both the PFC, $t(22) = 2.346$, $p = 0.028$, $R^2 = 0.20$ and the S1BF, $t(21) = 2.248$, $p = 0.035$, $R^2 = 0.194$, when compared to those that were treated with vehicle (Fig. 3A). Furthermore, there was a significant decrease in number of $\Delta fosB^+$ cells in both fear appraisal regions assessed, the MeA, $t(20) = 2.947$, $p = 0.008$, $R^2 = 0.303$, and the PAG, $t(19) = 5.363$, $p < 0.0001$, $R^2 = 0.602$, in BLZ-treated animals compared to vehicle-treated. BLZ-treated animals trended towards increased numbers of $\Delta fosB^+$ cells in both the NAc, $t(22) = 1.808$, $p = 0.084$, and the POA, $t(22) = 1.824$, $p = 0.082$, compared to those treated with vehicle, although both failed to reach significance. There was no difference in the AON, $t(21) = 0.1099$, $p = 0.914$, between treatment conditions.

We then assessed whether there was a pattern to changes in ΔfosB cell numbers between brain regions. Network analyses of each treatment group (Fig. 4A) show within group correlations (see Supplement Table 1 for full statistical results). We examined whether these correlations varied by treatment condition and found significantly different patterns of activation in vehicle-treated rats compared to rats that had microglia

depleted prior to maternal sensitization (Fig. 4B). Finally, we examined whether ΔfosB numbers correlated with the display of maternal behaviors. Statistical results are displayed in Supplemental Table 2. There were no significant correlations with overall latency to reach maternal criteria, or pup-directed licking or sniffing with ΔfosB numbers across brain regions in either vehicle or BLZ treated animals.

Overall, microglia depletion prior to maternal sensitization led to shifts in $\Delta fosB^+$ staining throughout the maternal brain network of sensitized animals. Moreover, there are distinct differences between treatment conditions in the pattern of $\Delta fosB^+$ between brain regions, though no relationship was found with the display of maternal behaviors.

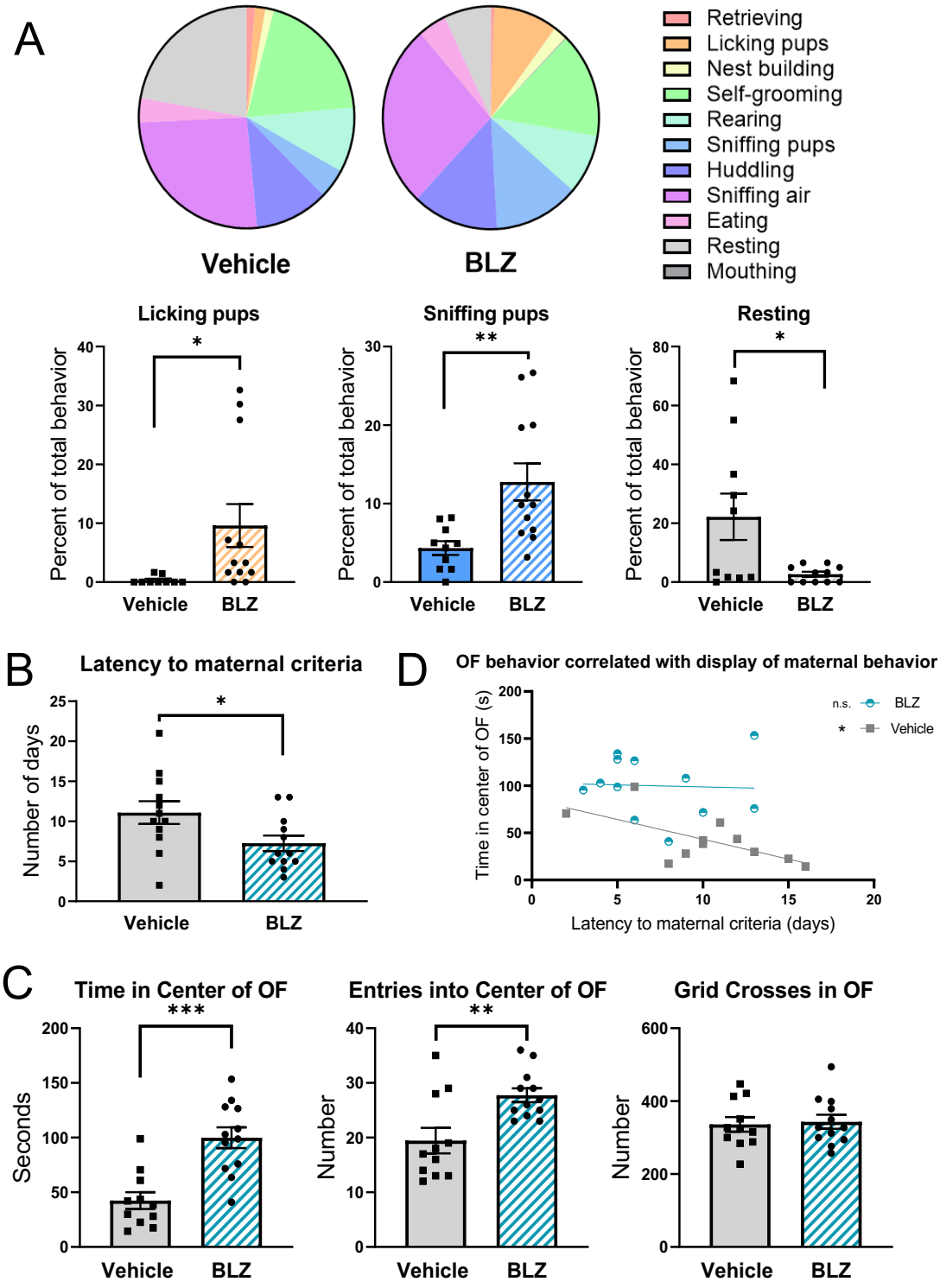

Figure 2. *A.)* Behaviors quantified during maternal sensitization observation the day prior to maternal criteria being met, represented as percentage of total behaviors recorded. Bar graphs show individual data points for behaviors that were significantly different between experimental treatments. *B.)* Latency corresponding to the first day of two consecutive displays of maternal behavior. *C.)* Time in and entries into center of the Open Field (OF) arena, and number of grid crosses made during 10min recording. *D.)* Latency to maternal criteria correlated to time spent in the center of the Open Field for each experimental treatment (same result when correlated to number of entries into center). Individual data points for each animal are included. Error bars represent mean ± SEM. Unpaired two-tailed t-tests: * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, ns – not significant.

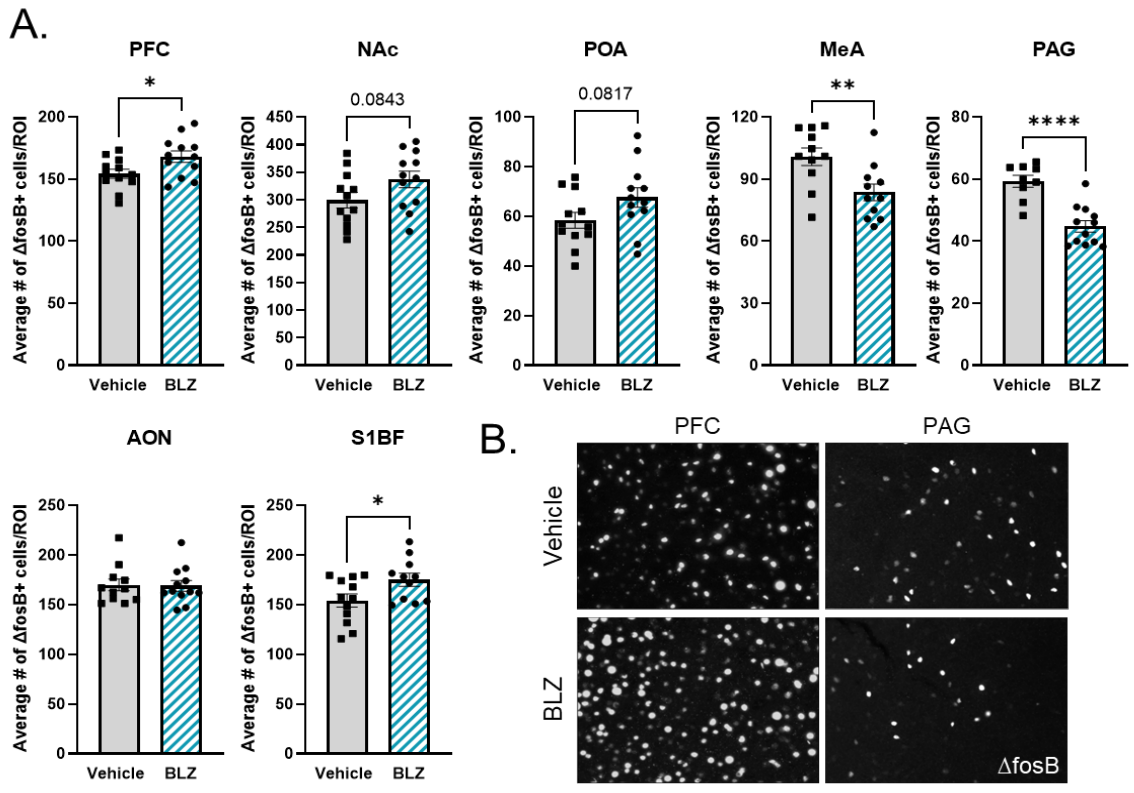

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Figure 3. *A.)* Quantification of ΔfosB positive cells in maternally-sensitized animals across regions of interest: prefrontal cortex (PFC), nucleus accumbens (NAc), preoptic area (POA), medial amygdala (MeA), periaqueductal gray (PAG), anterior olfactory nucleus (AON), and barrel field within primary somatosensory cortex (S1BF). *B.)* Representative images (20x) of ΔfosB staining in Vehicle- vs BLZ-treated animals taken in the PFC and PAG. Individual data points for each animal are included. Error bars represent mean ± SEM. Unpaired two-tailed t-tests: * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

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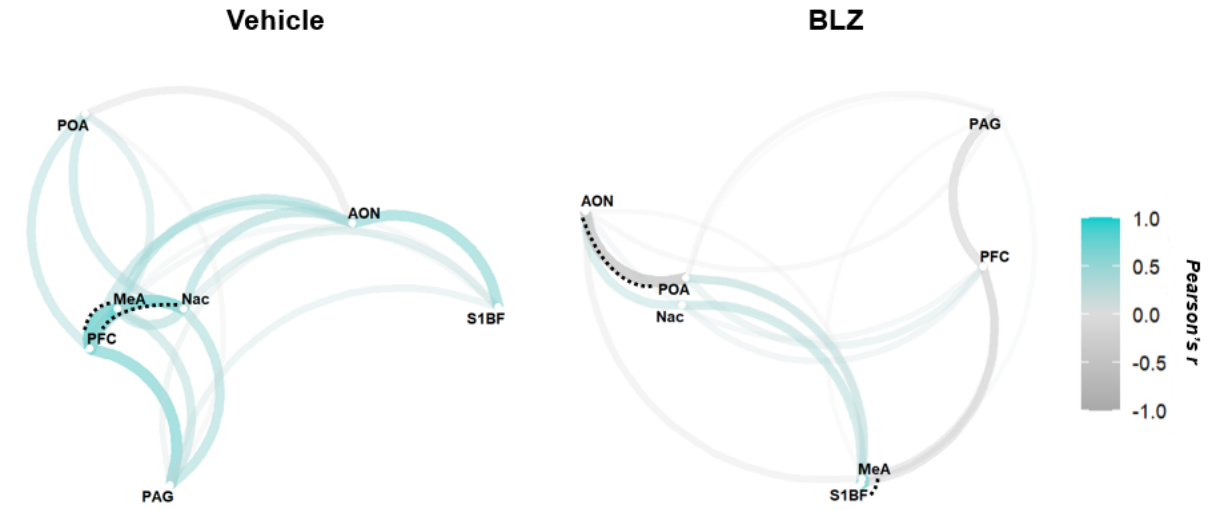
A.



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B.

Fisher's Z-test for differences between correlations in Vehicle vs BLZ-treated animals

	Vehicle *r*	BLZ *r*	*z*	*p*
PFC-MeA	0.97	-0.28	4.999	< 0.0001
PFC-Nac	0.95	0.15	3.573	< 0.001
PFC-PAG	0.76	-0.44	2.768	0.006
MeA-S1BF	0.09	0.87	-2.380	0.017
AON-S1BF	0.65	-0.06	1.711	0.087
POA-AON	-0.24	-0.74	1.448	0.148
MeA-AON	0.51	-0.17	1.422	0.155
Nac-POA	0.40	-0.08	1.069	0.285
PFC-S1BF	0.11	-0.38	1.044	0.296
Nac-PAG	0.50	0.03	0.978	0.328
Nac-MeA	0.46	0.02	0.954	0.340
PFC-AON	0.44	0.11	0.732	0.464
MeA-PAG	0.40	0.03	0.691	0.490
POA-S1BF	0.01	0.29	-0.589	0.556
PAG-AON	0.17	-0.08	0.455	0.649
PFC-POA	0.40	0.21	0.440	0.660
Nac-AON	0.48	0.37	0.300	0.764
Nac-S1BF	0.29	0.41	-0.292	0.771
PAG-S1BF	0.21	0.06	0.289	0.772
POA-PAG	-0.09	-0.11	0.039	0.969
POA-MeA	0.29	0.29	0.023	0.981

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Figure 4. *A.)* Correlation networks of ΔfosB counts between brain regions within Vehicle and BLZ-treated animals. Transparency and width of lines represents strength of correlation. Regions that have similar correlation patterns cluster together. Black dashed lines represent within group correlations between regions that have p-values < 0.05, but do not remain significant following Bonferroni correction (significance set at $p < 0.002$). *B.)* Statistical comparison of ΔfosB correlations in Vehicle-treated animals vs correlations in BLZ-treated animals. Between treatment group comparisons that reached statistical significance ($p < 0.002$) are denoted above dashed line for ease of presentation.

4. Discussion

We and others have previously reported decreased microglia number in the maternal brain that emerges during late pregnancy and persists for at least one week postpartum [14, 15]. Here, we showed that experimentally inducing decreased microglia tone in nulliparous female rats led to an accelerated onset of maternal behavior in a pup sensitization paradigm. Thus, even in the absence of hormonal manipulations, recapitulating the neuroimmune profile seen in the peripartum brain is sufficient to promote the display of maternal behaviors in nulliparous females. Moreover, we found that microglia depleted females showed increased investigative behaviors specifically directed toward pups, and evidence of decreased threat appraisal behavior on the open field test without impacting overall locomotor activity levels. The novel finding that microglia manipulations facilitate maternal caregiving behavior in maternally-unexperienced animals points to an unappreciated role for neuroimmune cells in adaptive function in the adult female brain.

Pups are initially aversive stimuli to non-sensitized female rats [30], and avoidance behavior is partially mitigated by inhibition of brain regions involved in fear and anxiety-like behaviors [31, 34-36]. Thus, we sought to determine whether a more rapid display of maternal behavior could be attributed to a reduction in threat appraisal. There were significant changes in ΔfosB staining in the MeA and PAG, two regions critical for the aversion behavior that non-maternal rodents exhibit in response to pup exposure. Specifically, there was a decrease in activation of these regions if the animals were treated with BLZ prior to being sensitized, compared to those treated with vehicle. While the loss of microglia did also reduce threat appraisal behavior in the open field, this effect did not correlate with a faster onset of maternal care. Thus, changes in threat appraisal alone are likely insufficient to account entirely for the accelerated onset of maternal care after microglial depletion. This is consistent with previous work showing that only inhibiting the fear response was inadequate in promoting a quicker onset of maternal behavior in nulliparous females [37].

Somatosensory and chemosensory input from pups is also critical in facilitating the reinforcing properties of maternal-pup interactions and reducing neophobic responses [7, 38]. The somatosensory cortex in mothers is responsible for governing tactile interactions with pup, both through whisker stimulation that occurs during sniffing, and in maternal animals, suckling during lactation [8, 39, 40]. Increased ΔfosB cell numbers in S1BF following microglia depletion is consistent with the increased display of pup-directed sniffing exhibited by the BLZ-treated animals. The olfactory system also mediates maternal caregiving, with pup odor being a well-established aversive cue in nulliparous females that normally acts to inhibit maternal care. There is some evidence that microglia depletion leads to olfactory deficits, which if present in the current study, could have led to accelerated maternal sensitization [35, 37]. However, studies showing olfactory deficits examined developmental microglia depletion and weeks-long depletion in adult animals [41, 42]. Here, using acute microglia depletion, we did not see any difference in activation of the AON, and behaviorally the BLZ-treated animals spent *more* time engaging in sniffing the foster pups. Thus, olfactory impairments are unlikely to be responsible for the maternal phenotype displayed after microglia depletion.

Increased activation of reward-related regions is necessary for maternal care to be displayed [5]. Retrieval and pup-directed licking and sniffing are behaviors often linked with increased dopaminergic activity [43] in mesolimbic reward pathways [9]. ΔfosB staining, though trending higher following microglia depletion, did not reach significance in the NAc. There may have been a ceiling effect in this measure, as we collected tissue after animals were fully sensitized, and pup interactions should be rewarding to animals across both treatment groups. Examining immediate early gene expression earlier in the sensitization process may be more likely to capture differences in reward-related regions. The POA serves as an important central integrator to regulate approach and avoidance responses [9] and as all sensitized animals completed the shift from avoidance to approach, it is possible that microglia loss would also significantly impact activation of this area earlier in the sensitization procedure than we examined here.

We found increased activation in the PFC in microglia depleted animals compared to those treated with vehicle. The PFC, especially the medial prelimbic subregion where our analyses were focused, is important for modulating maternal care behaviors. Via output projections to the nucleus accumbens, the prelimbic PFC regulates dopaminergic activity and guides motivated maternal behavior [44]. Moreover, it has been shown that lesions of the PFC impair pup retrieval and pup licking [45], two behaviors that in the current study were promoted in microglia-depleted females. Increases in dendritic architecture in the PFC have also been linked to improved attention and behavioral flexibility in maternal rats, as tested in an attentional set shifting task [46]. Rats that perform worse in an attentional set shifting task also have been found to be less attentive to pups and spend less time licking pups [47]. Behavioral flexibility is necessary in sensitization paradigms [48], therefore it would be valuable to determine the consequence of PFC microglia loss on cognitive and executive function, and the extent to which it affects the display of maternal behaviors. Determining the consequences of both PFC and widespread microglia loss on cognition in adult females could also reveal important sex-specific roles for adult microglia function, as previous studies of microglia depletion in male rodents reported no major changes in cognitive outcomes [49], but may be missing female-specific effects.

These studies provide an important foundation for future work determining which regions of the maternal brain are most impacted by the peripartum downregulation of microglia. Microglia-neuronal crosstalk is important for maintaining homeostatic conditions [50, 51]. Microglia shape neural circuitry, and consequently behavioral outputs, by regulating myelination, neurogenesis, and synaptic patterning [29, 52, 53]. This can be accomplished either through directly phagocytosing synaptic elements, or through the release of diffusible factors, such as cytokines, chemokines, complement components, and growth factors, leading to activation of downstream signaling pathways [51, 53, 54]. During late pregnancy, the brain rapidly adapts a widespread neuroplastic state to allow the mother to successfully provide care for her offspring. Absence of microglia has been linked to increased dendritic spine density [55], while increases in dendritic spines have also been shown in the postpartum brain [46, 56, 57].

Given that maternal microglia alterations are occurring at this same time [14], there may be a mechanistic link between microglia downregulation and circuit remodeling necessary to permit the onset of maternal behavior [58].

Here, we examined ΔfosB expression as an indicator of which regions of the maternal circuitry might be implicated in the behavioral phenotype displayed following microglia depletion. Correlations of ΔfosB expression between brain regions showed different patterns of activation between vehicle-treated animals and those who had microglia depleted prior to sensitization, which suggests circuit level differences in neuronal activity. Additional studies examining co-localization of specific neuronal markers with ΔfosB to determine which populations are being activated would be a useful first step in understanding how microglia downregulation led to changes in the patterns of activity of the maternal brain network. For example, in the POA, ΔfosB expression co-localizes with oxytocin neurons following parturition in maternal animals [59], and reactivity in this region can increase following pup exposure [60]. However, ΔfosB can be expressed by diverse cell populations across brain regions [59, 61, 62]. The breadth of regions examined here allows for the possible combination of excitatory and inhibitory populations experiencing changes in fosB expression, which would have varying implications for circuitry output. Another possibility is that transcription of fosB itself is an essential component for promoting maternal sensitization, as mice lacking the fosB gene have been found to be unable to care for or nurture pups [63]. Elucidating whether microglia depletion promoted an adaptive neuronal response to drive behavioral changes, or whether behavior changes promoted neuronal responses following microglia depletion could provide valuable mechanistic insight into the phenotype demonstrated here.

Correlations of fosB cell numbers across brain regions with the display of specific maternal behaviors (latency to reach maternal criteria, pup-directed sniffing, and pup-directed licking) yielded no significant results in both vehicle and BLZ-treated animals. This strengthens the idea that circuit levels differences in neuronal activity might be more relevant to consider when further exploring the mechanism governing behavioral changes following microglia depletion. It is also important to again consider that fosB measurements were taken after all animals were fully sensitized. It is possible that group-based relationships between fosB expression in individual brain regions with the display of a specific behaviors might be detectable earlier in the sensitization process, when brain remodeling to permit caregiving behaviors is underway.

One caveat related to our experimental approach is that certain aspects of our microglia depletion strategy did not exactly recapitulate the downregulation of microglia seen in the maternal brain. We previously documented that microglia numbers decrease significantly across limbic regions in the maternal brain, but that the decrease does not occur in the motor cortex [14]. In contrast, peripheral administration of the CSF1R inhibitor BLZ945 led to decreased microglia in both limbic and non-limbic regions in adult female rats in this study. Moreover, global CSF1R inhibition has been shown to deplete peripheral monocytes and macrophages in addition to microglia [64]. Trafficking of peripheral immune cells into the brain can impact affective behavior [65, 66], so it is possible that the loss of peripheral innate immune cells could contribute to the social

behavior phenotype we observed. However, the ability of peripheral immune cells to impact affiliative behavior in homeostatic conditions or in maternal animals remains to be established. CSF1R inhibitors are useful tools in examining how simultaneous microglia downregulation across many brain regions in a functional circuit is important in modulating behavior, but it is relevant to consider that a global depletion may have different outcomes than those occurring more specifically in a particular subset of brain regions.

In our current study using the maternal sensitization paradigm, we did not directly assess how pup characteristics may be influencing the development of maternal behavior of the nulliparous animal after microglia loss. For example, some previous work has shown that male and female pups can elicit varying caregiving responses due to sex differences in urine odor [67] or pup ultrasonic vocalizations [68]. Whether the effect of microglia manipulations on maternal behavior is influenced or moderated by pup characteristics would be an important future question as we consider a broader role for microglia as mediators of social behavior.

In conclusion, our results show BLZ945 was effective at rapidly and non-invasively depleting microglia in the adult female rat brain. Further, we demonstrate a strong pro-social and anxiolytic behavioral phenotype following microglia depletion in the healthy adult brain of nulliparous female rats. These behavioral changes were accompanied by alterations in ΔfosB staining across brain regions critical for maternal care. There are two major implications of these findings. First, when considered with prior work suggesting little behavioral impact of microglial depletion in adult males [18, 49] the current data suggest that there may in fact be key functions for microglia in modulating behavior in adult females. Second, these data give insight into the parts of the maternal brain network that are most impacted by microglia loss, and thereby may lead to advancements in our understanding of neuroimmune function in the maternal brain. Overall, this work suggests that the suppression of microglia seen in the brain during pregnancy may serve an adaptive role, permitting the onset of maternal behavior. Together, these data are transformative in that they provide evidence that microglia functional changes are relevant to study across a variety of research areas in neuroscience and behavior.

Author contributions

CND performed experiments, analyzed the data, and wrote the manuscript. DF contributed to behavior analysis. BL and KML conceptualized and supervised the experiments and edited the manuscript.

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Competing interests

The authors have nothing to disclose.

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