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Archival Report

The Role of Microglia in Sex- and Region-Specific Blood-Brain Barrier Integrity During Nicotine Withdrawal

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ABSTRACT

BACKGROUND: Smoking is the largest preventable cause of death and disease in the United States, with <5% of quit attempts being successful. Microglia activation and proinflammatory neuroimmune signaling in reward neurocircuitry are implicated in nicotine withdrawal symptomology. Microglia are integral regulators of blood-brain barrier (BBB) functionality as well; however, whether the effects of nicotine withdrawal on microglia function impact BBB integrity is unknown.

METHODS: Mice were treated with chronic nicotine (12 mg/kg/day) and subjected to 48 hours nicotine withdrawal. Regional BBB permeability, together with messenger RNA and protein expression of tight junction proteins, were assessed. PLX5622 chow was used to deplete microglia to evaluate the role of microglia in regulating BBB integrity and nicotine withdrawal symptomology.

RESULTS: Female mice had higher baseline BBB permeability in the prefrontal cortex and hippocampus than males. Nicotine withdrawal further exacerbated the BBB permeability selectively in the prefrontal cortex of females. These effects were concurrent with prefrontal cortex alterations in a subset of tight junction proteins with increased proinflammatory responses following nicotine withdrawal in females. Depletion of microglia via PLX5622 treatment prevented all these molecular effects and attenuated withdrawal-induced anxiety-like behavior in female mice.

CONCLUSIONS: These results are the first to show sex differences in regional BBB permeability during nicotine withdrawal. This represents a possible link to both the reduced smoking cessation success seen in women and women's increased risk for smoking-related neurovascular disorders. Furthermore, these findings open an avenue for sex-specific therapeutics that target microglia and BBB dysfunction during nicotine withdrawal in women.

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Cigarette smoking is associated with the development and progression of cancer (1), stroke (2), and cardiovascular (3) and cerebrovascular diseases (4) and is the leading cause of preventable morbidity and mortality worldwide (5). While smoking cessation decreases the risk of these consequences, nearly 80% of the people who attempt to quit still fail (6). Nicotine, the main psychostimulant in cigarettes, induces its rewarding effects by acting on nicotinic acetylcholine receptors (nAChRs) in the brain. These neuronal nAChRs are the primary target for 2 of the 3 Food and Drug Administration (FDA)–approved nicotine cessation pharmacotherapies currently available (varenicline and nicotine replacement therapy) (7–9).

Sex differences in tobacco use disorder endophenotypes are well-described [for review, see (10)]. Previous studies have shown that while males are more likely to smoke than females (11), females are more susceptible to smoking relapse (12) due to more severe withdrawal symptoms (13) and cravings (14). Regarding symptomology specifically, a clinical study showed that female smokers reported higher levels of anxiety than male smokers after 16 hours of withdrawal (15). Further exacerbating this problem, nicotine replacement therapy is less beneficial in females, likely underlying the higher relapse rates in females than males (16).

The blood-brain barrier (BBB) is the physical barrier between the blood and the brain parenchyma, which regulates the transport of biomolecules and xenobiotic compounds in and out of the brain (17). BBB integrity is tightly regulated by a family of junctional proteins including tight junction proteins (TJPs) (claudins, occludin, zonula occludens), gap junction proteins (connexins), and adherens junction proteins (actin, myosin, microtubules, catenin) (18). Altered expression and aberrant organization of any of these proteins at the BBB leads to compromised BBB integrity and leakiness (19,20). A compromised BBB leads to the passage of unwanted proteins and biomolecules into the brain parenchyma, which induces neuroinflammation and leads to behavioral deficits (21). For example, increased BBB permeability and altered BBB function are associated with neurobehavioral deficits including anxiety (22), depression (22,23), mood disorders (24), and cognitive deficits (25), all of which are also endophenotypes of

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nicotine withdrawal symptomology. Furthermore, recent studies have shown sex differences in the development of smoking-associated cerebrovascular diseases like intracerebral hemorrhage (26), subarachnoid hemorrhage (27), and stroke (28), with higher susceptibility in female smokers. However, while these studies have strongly linked smoking, sex, and cerebrovascular diseases, the mechanistic basis of these observed sex differences is not understood. The current study begins to address this gap. Our studies are the first to find sex and regional differences in BBB permeability and underlying molecular mechanisms during early nicotine withdrawal. Furthermore, we showed evidence that microglia are critical mediators of BBB integrity and anxiety-like behaviors in female mice during early nicotine withdrawal.

METHODS AND MATERIALS

The full methodology can be found in the Supplement.

Animals

Male and female B6/129S F1 mice that were 6 to 8 weeks of age were used for this study. Animals were maintained on a 12-hour light/dark cycle with food and water ad libitum per the University of Kentucky Animal Care and Use Committee.

Treatment, Molecular, and Behavioral Methods

See the Supplement.

Statistical Analyses

All statistical analyses were done in GraphPad Prism 8.1.2 (GraphPad Software). An unpaired *t* test was used for comparisons of differences between 2 independent experimental groups. Statistical significance was calculated by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests for groupwise comparisons unless stated otherwise. When comparing multiple groups, statistical differences between groups were determined using two-way ANOVA with sex and treatment as the primary variables followed by Bonferroni post hoc tests. Outliers were checked for all the treatment groups during the development of graphs.

RESULTS

Nicotine Withdrawal Exacerbates BBB Permeability in the Prefrontal Cortex of Female, but Not Male, Mice

We used a clinically relevant preclinical model of nicotine dependence with intermittent nicotine treatment for our experiments. First, mice were administered intermittent saline or nicotine (12 mg/kg/day) over 2 weeks via subcutaneous minipump implantation. Following exposure, 48-hour withdrawal was initiated via pump removal in mice earmarked for nicotine withdrawal and their saline surgical controls, while the nicotine group and their saline controls underwent sham surgery. BBB permeability in the prefrontal cortex (PFC), hippocampus, and occipital cortex of male and female mice were assessed by using Evans blue (EB) (albumin-bound EB molecular weight: ~69,000 Da). Overall, sex differences were observed in region-specific BBB permeability, with female mice having higher levels of EB dye in the PFC (Figure 1B) ($F_{1,21}$ = 55.20, $p < .0001$) and hippocampus (Figure 1C) ($F_{1,21}$ = 28.56, $p < .0001$), but lower levels of EB in the occipital cortex (Figure 1D) ($F_{1,21}$ = 7.781, p = .0110) than males irrespective of the treatment. Nicotine withdrawal further exacerbated BBB permeability selectively in the PFC of female mice compared with their saline counterparts, suggesting sex- and region-specific effects of nicotine and withdrawal on BBB integrity (Figure 1B) (drug × sex interaction: $F_{2,21}$ = 3.710, p = .0417).

Next, we tested the hypothesis that nicotine treatment and withdrawal also increase BBB permeability in the PFC of male mice but only for smaller biomolecules. To determine this, a much smaller molecular weight tracer dye, sodium fluorescein (molecular weight: 376 Da), was used to assess possible BBB damage in treated male mice (Figure S2B). Similar to our EB data, no change in the brain uptake of sodium fluorescein dye was observed in the PFC of male mice ($F_{2,12}$ = 0.6168, p = .5559), suggesting that nicotine treatment and withdrawal do not affect BBB permeability in male mice.

Nicotine Treatment and 48-Hour Nicotine Withdrawal Do Not Affect the Brain Vascular Volume and Density in the PFC of Male and Female Mice

Next, we evaluated whether baseline sex differences or nicotine treatment impacted vascularization in the PFC as a possible mechanism for sex-specific differences in BBB integrity. To investigate this, we fluorescently labeled the vasculature in treated male and female mice. Brains were subsequently extracted and subjected to tissue clearing to allow optical penetration throughout the sample. We used light sheet imaging on this cleared tissue to measure the volume and density of vessels in the PFC. We did not observe any effects of nicotine treatment on the density or volume of the vasculature. However, we did observe an impact of sex on vasculature volume, with females having lower volume than males (main effect sex: $F_{1,24}$ = 5.211, p = .0316), but this sex difference was not observed in density (Figure 1E–G).

Nicotine Treatment and 48-Hour Nicotine Withdrawal Modulate the Expression of BBB- and Neuroinflammation-Related Genes in the PFC of Female Mice

Because we observed an elevated BBB permeability in the PFC of female mice selectively during 48-hour nicotine withdrawal, we next asked whether the observed functional BBB changes in female mice were due to expression changes in TJPs. We analyzed TJP messenger RNA (mRNA) and protein expression changes (i.e., claudin-5 [*Cldn5*], zonula occludens-1 [*Tjp1*/ZO-1], and occludin [*Ocln*]) in the PFC of treated female mice. We found no changes in the relative mRNA expression of *Cldn5* or *Tjp1* genes; however, mRNA expression of *Ocln* was found to be decreased in the PFC of treated female mice (Figure 2C) ($F_{2,18}$ = 6.644, p = .0069). Similar to mRNA expression, the protein levels of OCLN were also decreased in the PFC of females exposed to 48-hour nicotine withdrawal (Figure 2G) ($F_{2,32}$ = 3.442, p = .0443), while protein levels of CLDN5 and ZO-1 were unchanged.

Next, we assessed whether neuroinflammatory processes are also altered in the female PFC following nicotine treatment and withdrawal. To do this, we analyzed mRNA expression of

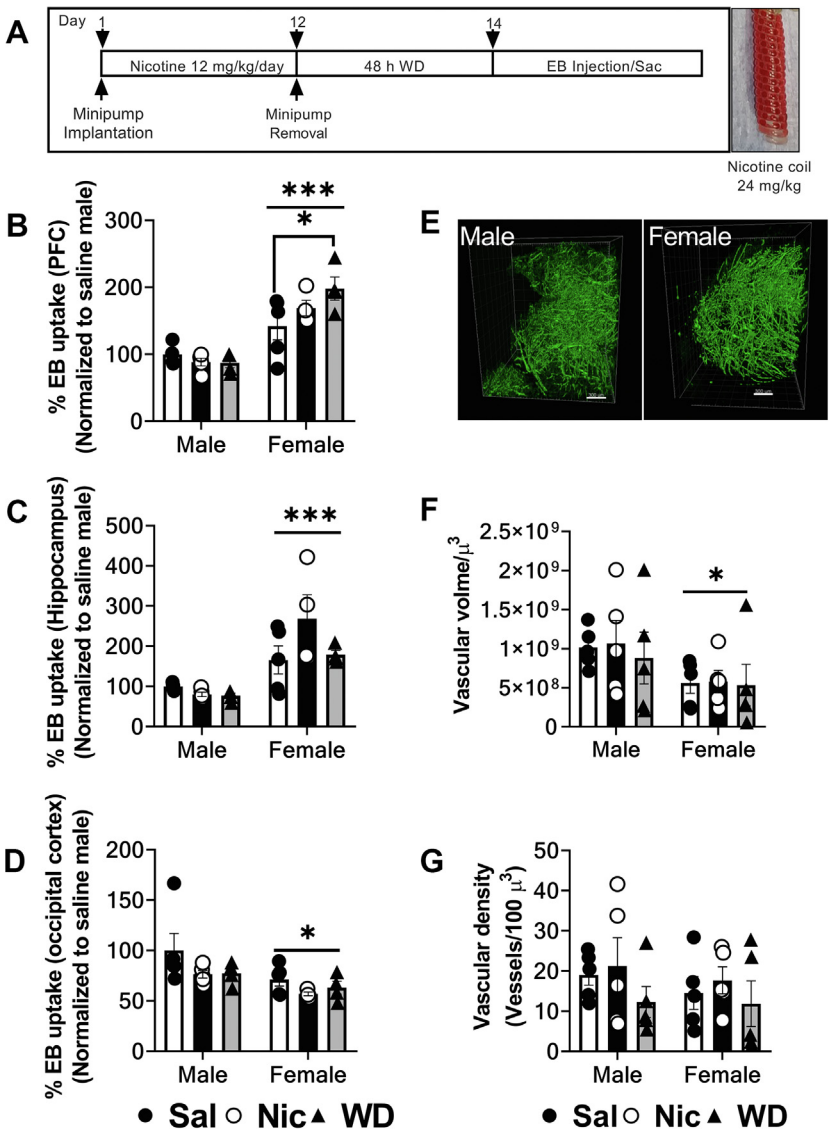

Figure 1. Nicotine withdrawal exacerbates blood-brain barrier permeability in the PFC of female mice. Treatment paradigm followed for the study **(A)**; After 14 days of nicotine treatment and 48-hour nicotine withdrawal, male and female mice were injected with 2% (wt/vol) EB dye intravenously through tail vein injection. Quantification of diffused EB dye in the PFC **(B)**; hippocampus **(C)**, and occipital cortex **(D)** of male and female mice. Representative images of surface-rendered 3D reconstruction of the vasculature from a cleared section containing the PFC of the male and female mouse after nicotine treatment and 48-hour nicotine withdrawal labeled with albumin-Alexa 488 and imaged using light sheet microscopy **(E)**; Scale bar = 300 μm. The total vasculature volume in the PFC compared across different conditions in male and female mice was calculated from 3D reconstructions of light sheet images of cleared tissue **(F)**. The vascular density (vessel segments per 100 μm^3) in the PFC compared across different conditions in male and female mice was calculated from 3D reconstructions of light sheet images of cleared tissue **(G)**. Data were analyzed using two-way analysis of variance with Bonferroni post hoc tests. (Compared with saline male: $^*p < .05$, $^{***}p < .001$; n = 4–5 per treatment group. Bars show mean ± SEM.) 3D, 3-dimensional; EB, Evans blue; Nic, nicotine; PFC, prefrontal cortex; Sal, saline; WD, withdrawal.

genes associated with oxidative stress (*Nox2*), microglia (*CD68*, *Iba1*, *CD11b*), proinflammatory cytokines (*Tnfα*, *Il1β*), and the extracellular matrix (*Mmp2*, *Mmp9*, *Timp1*, *Timp2*) in the PFC of female mice undergoing 48-hour nicotine withdrawal (Figure 2I). *Tnfα* mRNA increased during nicotine treatment ($F_{2,18}$ = 4.998, p = .0188), while *Nox2* expression increased during nicotine withdrawal compared with their counterparts in the saline and nicotine groups ($F_{2,17}$ = 14.61, p = .0002). In contrast, *Mmp9* mRNA expression decreased in the PFC of females as a result of chronic nicotine exposure but returned to baseline after 48-hour nicotine withdrawal ($F_{2,18}$ = 7.701, p = .0038).

Brain Vasculature Is the Primary Site Expressing Genes for TJPs

TJPs are primarily expressed by endothelial cells (29). However, recent single-cell RNA sequencing data (30,31) show that TJP genes can also be expressed by microglia and astrocytes in addition to endothelial cells. This led us to directly assess cell-specific mRNA expression of primary TJPs (*Cldn5*, *Ocln*, *Tjp1*) in microglia, astrocytes, and isolated brain vasculature. We utilized magnetic-activated cell sorting to isolate and purify these cell types from the PFC tissue of control female mice and validated our approach based on cell-specific markers (i.e., *CD11b*, *Iba1*, and *CD68* gene expression compared with total brain homogenate or other isolated brain cell types [Figure 3A, i–iii]). Using these validated samples, we next assessed the mRNA expression of TJPs in total brain homogenate, microglia, astrocytes, and brain vessels isolated from the PFC. The mRNA expression of *Cldn5*, *Tjp1*, and *Ocln* were found to be highly enriched in total homogenate and isolated brain vessels as expected (Figure 3B, i–iii). However, a residual mRNA expression of all 3 TJPs was also observed in microglia and

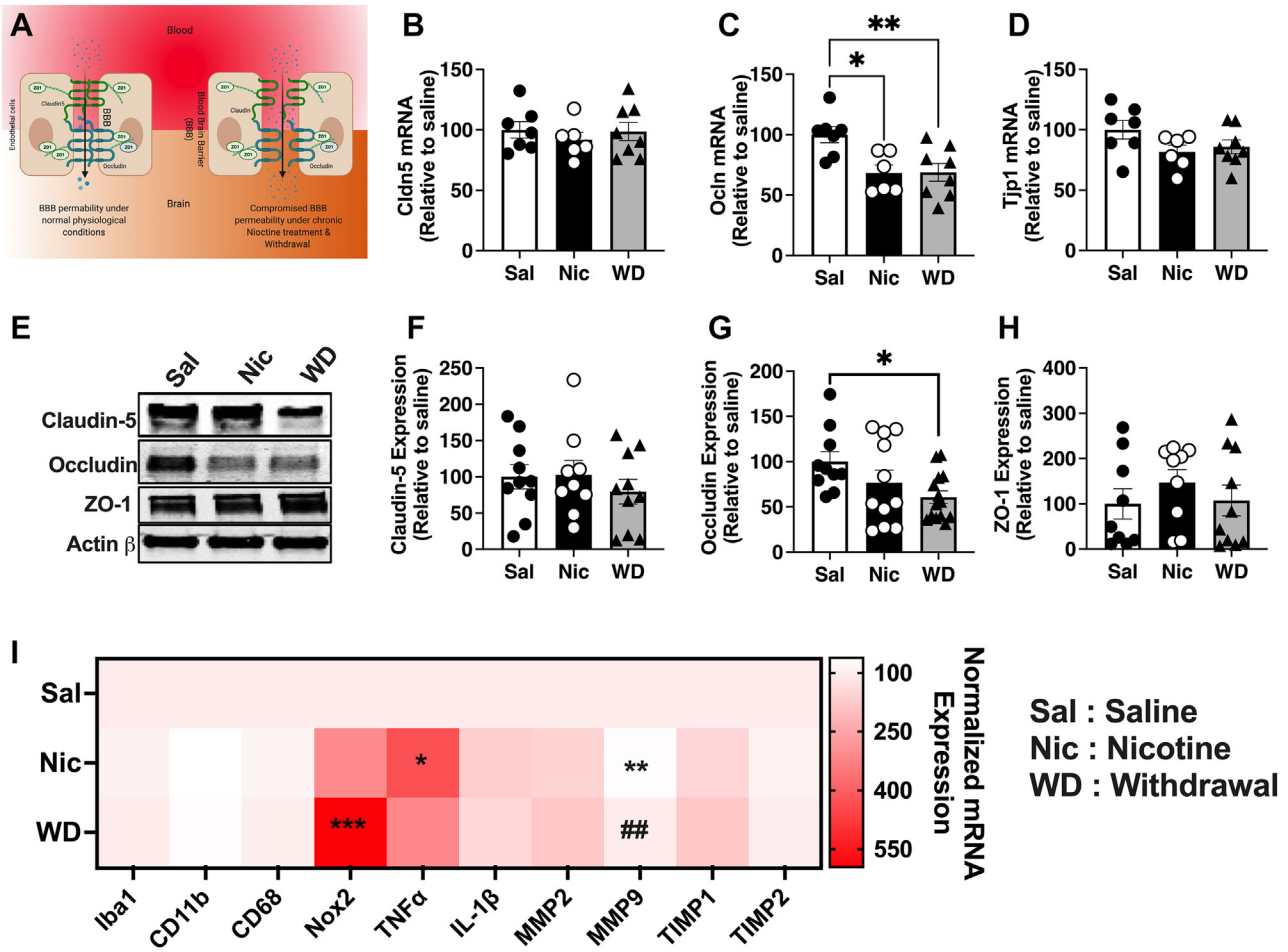

Figure 2. Nicotine treatment and nicotine withdrawal (48 hours) reduces the levels of tight junction proteins and modulates the expression of genes associated with oxidative stress, neuroinflammation, and extracellular matrix in the PFC of female mice. Pictorial illustration showing the cellular distribution of tight junction proteins on endothelial cells providing integrity to the BBB in normal physiological conditions **(A)**. Relative mRNA expression of *Cldn5* **(B)**; *Ocln* **(C)**; *Tjp1* **(D)** in the PFC of female mice. Representative images of Western blots **(E)**; relative protein expression of Claudin-5 **(F)**; Occludin **(G)**; tight junction protein-1/Zonula occludens-1 **(H)** in the PFC of female mice. Heat map showing normalized mRNA expression of oxidative stress (*Nox2*), microglia (*Iba1, CD11b, CD68*), neuroinflammation (*Il1β*, *Tnfα*), and extracellular matrix (*Mmp2*, *Mmp9*, *Timp1*, *Timp2*)–specific genes in the PFC of female mice **(I)**. Data were analyzed using one-way analysis of variance with Bonferroni post hoc tests. (Compared with saline: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$; compared with nicotine: $^{\#\#}p < .01$; n = 6–7 per treatment group for reverse transcriptase–polymerase chain reaction and n = 10–14 per treatment group for Western blotting. Bars show mean ± SEM.) BBB, blood-brain barrier; mRNA, messenger RNA; Nic, nicotine; PFC, prefrontal cortex; Sal, saline; WD, withdrawal.

astrocytes, but the expression was significantly lower than that in total homogenate and brain vessels. These data demonstrate that while brain vasculature is the primary site of TJP mRNA expression, glial cells that are not physically associated with brain vessels express these genes as well but at significantly lower levels.

Nicotine Treatment and 48-Hour Withdrawal Induce Molecular Adaptations in the Brain Vasculature Isolated From the PFC of Female Mice

Given that the highest expression of TJPs occurs in brain vasculature, we examined whether chronic nicotine treatment and nicotine withdrawal affect the expression of TJPs in isolated brain vessels. Microvessels represent the BBB in total on a minute scale and are comprised not only of endothelial cells but also astrocytic end feet and microglial processes (32). To test whether TJP expression was altered in this minute BBB unit following chronic nicotine treatment or 48-hour nicotine withdrawal, we isolated brain microvessels from the PFC of female mice treated with saline, nicotine, and 48-hour nicotine withdrawal. To ensure the purity of the isolated microvessels, we 1) assessed the morphology of the isolated brain vessels under a light microscope (Figure S3A), 2) examined mRNA expression of endothelial cell-specific genetic markers (Figure S3B), and 3) evaluated protein expression of endothelial cell-specific marker (CD31) and pan-neuronal marker (TUJ1) (Figure S3C–E). Following validation of our approach, brain microvessels were labeled with anti-CD31, anti-CLDN5, anti-ZO1, and anti-OCLN antibodies and imaged for quantification (Figure 4A).

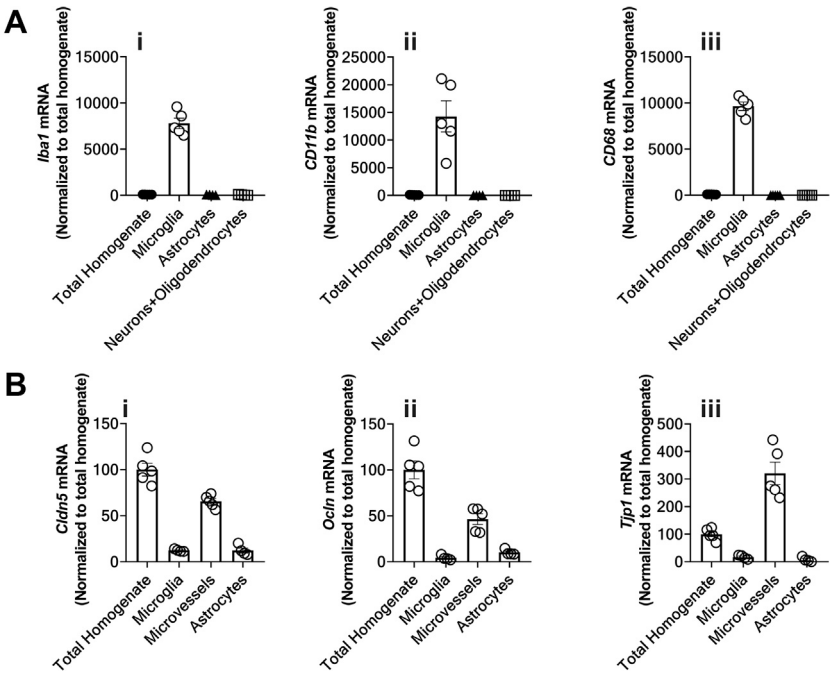

Figure 3. Brain vasculature is the primary site expressing genes for tight junction proteins. Bar graph showing quantitative polymerase chain reaction analysis of microglia markers: **(A)**: *Iba1* **(i)**, *CD11b* **(ii)**, and *CD68* **(iii)** in the total brain homogenate and specific brain cell types. Bar graph showing mRNA expression of tight junction proteins **(B)**: *Cldn5* **(i)**, *Ocln* **(ii)**, and *Tjp1* **(iii)** in the total brain homogenate, microglia, astrocytes, and isolated brain microvessels. Data were analyzed using one-way analysis of variance with Bonferroni post hoc tests. (n = 4–5 per treatment group. Bars show mean ± SEM.) mRNA, messenger RNA.

PFC microvessels from nicotine-treated female mice displayed significantly higher levels of CLDN5 than saline-treated subjects, with 48-hour nicotine withdrawal samples being significantly different from neither saline- nor nicotine-treated groups (Figure 4B) ($F_{2,33}$ = 4.016, p = .0275). Conversely, ZO-1 was found to be decreased in microvessels isolated from 48-hour nicotine withdrawal-treated females compared with mice in the saline- and nicotine-treated groups (Figure 4D) ($F_{2,46}$ = 4.397, p = .0179). No changes were observed in OCLN levels (Figure 4C) or microvessel diameter (Figure 4E). Collectively, these data show that chronic nicotine treatment and 48-hour nicotine withdrawal altered TJP levels at the functional BBB site in the PFC of female mice.

Next, isolated microvessels from the same animals were assessed for gene expression changes linked to nicotine effects on endothelial function (33), arterial stiffness, susceptibility to hypertension (34), and dysregulation of TJPs (35,36). Collectively, these targets represent potential mechanisms for sex-specific BBB disruption during nicotine exposure and withdrawal. Genes associated with angiotensin signaling (*Agtr1*: $F_{2,12}$ = 5.550, p = .0197; *Ace1*: $F_{2,12}$ = 4.464, p = .0355), oxidative stress (*eNOS*: $F_{2,12}$ = 4.722, p = .0309; *Edn1*: $F_{2,12}$ = 8.383, p = .0053), and TJPs (*Cldn5*: $F_{2,12}$ = 8.812, p = .0044; *Tjp1*: $F_{2,12}$ = 6.179, p = .0143; *Ocln*: $F_{2,12}$ = 6.491, p = .0123) were significantly increased in microvessels isolated from the PFC of female mice exposed to chronic nicotine and/or 48-hour nicotine withdrawal (Figure 4F). Taken together, these findings suggest that brain vasculature undergoes extensive molecular remodeling during nicotine exposure and early abstinence, inducing mRNA expression of oxidative stress, arterial stiffness, and endothelial tight junction signature protein marker genes in the PFC of females.

Pharmacological Depletion of Microglia Preserves BBB Integrity, Reverses Neuroinflammation in the PFC, and Attenuates Nicotine Withdrawal Symptoms in Female Mice

We have previously shown that microglial *Nox2* expression is the primary source of reactive oxygen species (ROS) in the nucleus accumbens during nicotine withdrawal (37). Next, we examined whether microglial generation of ROS is a critical intermediary for our PFC BBB observations following nicotine withdrawal as well. To do this, we utilized the selective colony-stimulating factor-1 receptor (CSF1R) inhibitor PLX5622 to deplete microglia in the brain, thereby disrupting both microglial and Nox2 functions. Following a week of eating either control or PLX5622 chow, female mice were treated with saline or nicotine for 2 weeks, after which the minipumps were removed from a subset of animals to initiate spontaneous withdrawal (experimental design shown in Figure S4A).

Our previous work (37) demonstrated that this protocol resulted in a 70% to 80% reduction in $Iba1^+$ cells/mm^2 in the nucleus accumbens compared with control-chow treated controls, which corresponded to significantly reduced expression of the pan-microglial markers *Tmem119* and *P2ry12*. Similarly, our current data replicated this significant reduction in these markers following PLX5622 treatment (*Iba1*: $F_{1,35}$ = 38.19, $p < .0001$; *Tmem19*: $F_{1,26}$ = 10.05, p = .0039; *P2ry12*: $F_{1,36}$ = 45.80, $p < .0001$) (Figure 5A–C). Interestingly, we also observed a main effect of the drug ($F_{2,36}$ = 5.244, p = .0100) and a drug × diet interaction ($F_{2,36}$ = 4.419, p = .0192) in the expression of *P2ry12*, with both nicotine and nicotine withdrawal causing expression decreases compared with the control chow mice treated with nicotine and/or withdrawal.

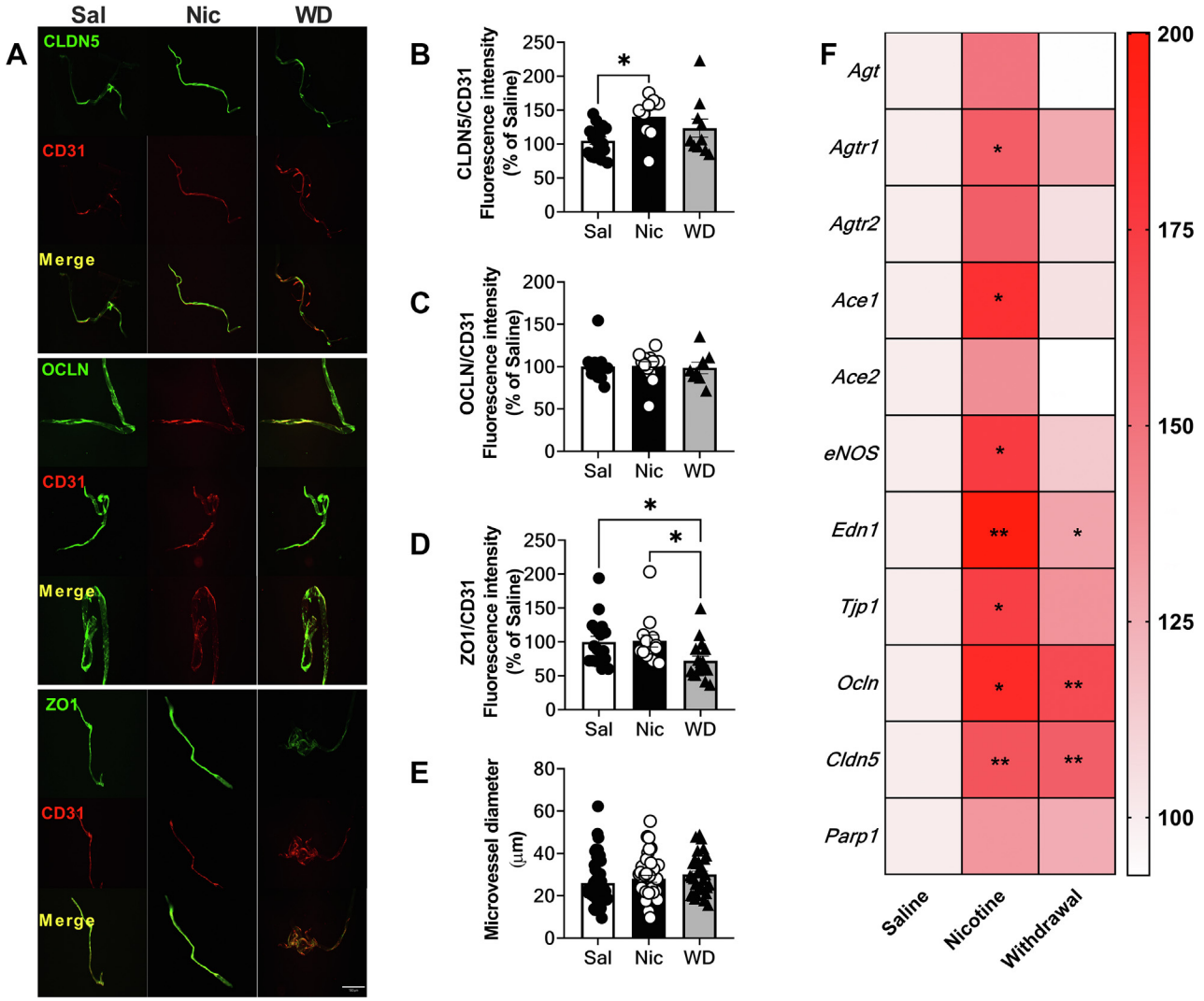

Figure 4. Nicotine treatment and nicotine withdrawal (48 hours) modulates the expression of tight junction proteins and induces molecular adaptations in brain vessels isolated from the prefrontal cortex of female mice. Representative fluorescence microscopic images of Claudin-5, Occludin, and Zonula occludens-1 **(A)**. Bar graph showing quantitative fluorescence intensities of Claudin-5 **(B)**, Occludin **(C)**, and Zonula occludens-1 **(D)** normalized to CD31 fluorescence intensity. Bar graph showing the diameter of microvessels **(E)** isolated from the prefrontal cortex of saline-, nicotine-, and withdrawal (48 hours)–treated female mice. Heat map showing normalized mRNA expression of angiotensin signaling (*Agt*, *Agtr1*, *Agtr2*, *Ace1*, *Ace2*), oxidative stress (*eNOS*, *End1*, *Parp1*), and junctional protein (*Cldn5*, *Ocln*, *Tjp1*)–specific genes in the brain vessels isolated from the prefrontal cortex of female mice. Data were analyzed using one-way analysis of variance with Bonferroni post hoc tests. [Compared with saline: $^{*}p < .05$, $^{**}p < .01$; **(B–C)** n = 8–18; **(E)** n = 34–49; **(F)** n = 5 per treatment group. Bars show mean ± SEM.] mRNA, messenger RNA; Nic, nicotine; Sal, saline; WD, withdrawal.

We also observed a significant increase in the mRNA expression of C-C-chemokine receptor 2 (*Ccr2*) (a pan myeloid cells marker) in the PFC of female mice exposed to nicotine treatment and 48-hour nicotine withdrawal, suggesting a role for infiltrating monocytes in this process. Similar to microglial markers following PLX5622 treatment, mRNA expression of *Ccr2* was nearly undetectable in the PFC of female mice fed with PLX5622 chow (Figure 5D) (main effect of diet: $F_{1,36}$ = 22.36, $p < .0001$; main effect of drug: $F_{2,36}$ = 2.798, p = .0742; interaction: $F_{2,36}$ = 3.139, p = .0554).

Next, we evaluated the effect of microglia depletion on BBB permeability in our model (Figure 6). Post hoc multiple comparison analysis confirmed our previous finding that female mice on control chow had higher PFC EB uptake following nicotine treatment and withdrawal than saline controls (Figure 6A). Microglia depletion via PLX5622 supplementation prevented the withdrawal-induced increase in EB levels in the PFC (main effect of diet: $F_{1,61}$ = 8.629, p = .0047; diet × drug interaction: $F_{2,61}$ = 3.333, p = .0423) with no significant changes in blood concentration (Figure 6B). PLX5622 treatment had primary effects on *Cldn5* (main effect of diet: $F_{1,39}$ = 4.340, p = .0438) and *Ocln* (main effect of diet: $F_{1,39}$ = 70.83, $p < .0001$) mRNA expression, suggesting that microglia actively influenced BBB functioning in the PFC of female subjects (Figure 6C–D). Furthermore, microglial depletion also prevented reductions in the *Ocln* mRNA resulting from chronic

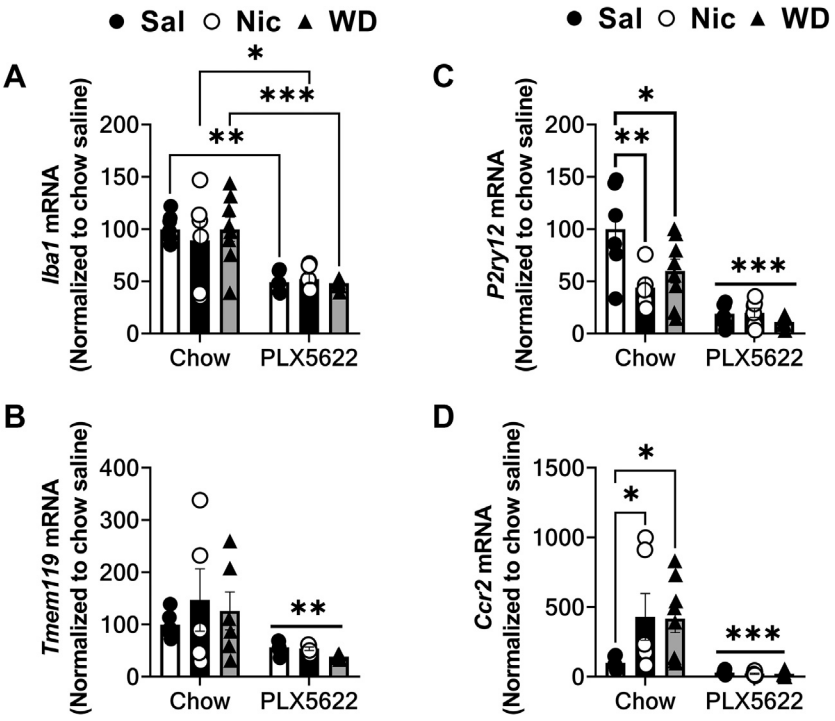

Figure 5. PLX5622 supplementation depletes microglia in the prefrontal cortex of female mice as assessed by real-time reverse transcriptase–polymerase chain reaction using pan microglia and monocyte marker genes (*Iba1*, *Tmem119*, *P2ry12*, *Ccr2*): Bar graph showing relative mRNA expression of *Iba1* **(A),** *Tmem119* **(B),** *P2ry12* **(C),** and *Ccr2* **(D)** in the prefrontal cortex after 14 days of nicotine treatment and 48-hour nicotine withdrawal in normal chow– and PLX5622 mixed chow–supplemented female mice. Data were analyzed using two-way analysis of variance with Bonferroni post hoc tests. (Compared with chow saline: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$; n = 5–8 per treatment group. Bars show mean ± SEM.) mRNA, messenger RNA; Nic, nicotine; Sal, saline; WD, withdrawal.

nicotine and 48-hour nicotine withdrawal (Figure 6D) (main effect of drug: $F_{2,39} = 4.604$, $p = .0160$). These effects were concurrent with reduced mRNA expression of proinflammatory cytokines, i.e., *Tnfα* (main effect of diet: $F_{2,37} = 15.14$, $p = .0004$) and *Il1β* (main effect of diet: $F_{2,38} = 15.04$, $p = .0004$) (Figure 6E, F).

Finally, we assessed microglial depletion effects on nicotine withdrawal–induced weight gain and affective-related behaviors in female mice. Female mice that were on the control diet gained more weight after 48-hour nicotine withdrawal than their saline control counterparts ($F_{1,93} = 17.40$, $p < .0001$); however, no differences were observed in the body weight of saline, nicotine, or 48-hour withdrawal treatment animal groups fed with PLX5622 chow (Figure S4C–D). In the marble burying test, 48-hour nicotine withdrawal-treated female mice on the control diet buried more marbles than their saline controls (main effect of drug: $F_{2,69} = 8.280$, $p = .0006$), indicating an anxiogenic-like response. In contrast, no differences were observed in the number of marbles buried in saline, nicotine, or 48-hour withdrawal treatment animal groups that were fed with PLX5622 chow (Figure 7A). Similarly, an open field test further demonstrated that control chow 48-hour withdrawal-treated female mice spent less time in the center zone of the arena compared with their saline controls (main effect drug: $F_{2,105} = 4.441$, $p = .0141$), also indicating an anxiogenic-like response; no differences were detected between any groups that were fed the PLX5622 diet (Figure 7B, D). The observed changes were not due to alterations in locomotor activity because no differences were detected in the total distance traveled between treatment groups that were fed the control or the PLX5622 diet (Figure 7C). In contrast to our models for anxiety, we found no effect of nicotine treatment or diet on behaviors in the splash test, which is a model for anhedonia (Figure 7E–F). Overall, our data indicate that microglia depletion by PLX5622 supplementation attenuated nicotine withdrawal–induced weight gain and anxiety-like behavior, but not anhedonia-like behavior, in female mice.

DISCUSSION

Cigarette smoking is strongly associated with elevated neuroinflammation (38), BBB dysfunction (39), high risk of stroke (2), and development of other cerebrovascular diseases (40). While earlier studies have demonstrated the effects of nicotine on BBB functions in brain health and diseases (35,41–43), the pathophysiology is unknown. To address this gap, our key findings include the following: 1) female subjects had higher BBB permeability in the PFC and hippocampus than male subjects, with nicotine withdrawal exacerbating this selectively in the female PFC; 2) these effects were concurrent with increased oxidative stress, proinflammatory responding, and TJP expression changes in the PFC; and 3) depleting microglia via PLX5622 treatment rescued these molecular effects and attenuated withdrawal-induced weight gain and anxiety-like behavior in female mice. These observations point to the therapeutic value of targeting microglia and the BBB during early cessation specifically in female smokers.

Robust sex differences have been reported in smoking cessation end points. For example, while men are more likely to smoke than women, women are more susceptible to

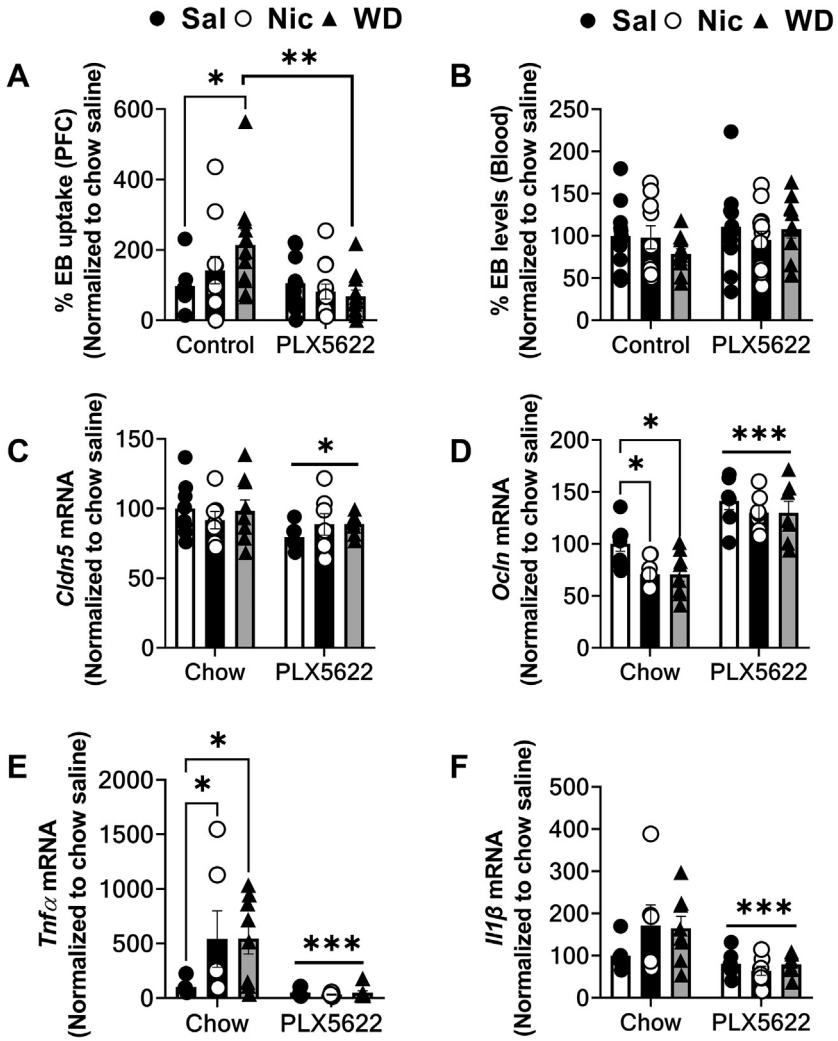

Figure 6. Pharmacological depletion of microglia with PLX5622 treatment preserves blood-brain barrier integrity and prevents neuroinflammation in the PFC of female mice exposed to nicotine and nicotine withdrawal. After 14 days of nicotine treatment and 48-hour withdrawal, control chow– and PLX5622-treated female mice were injected with 2% (wt/vol) EB dye intravenously through tail vein injection. Quantification of diffused EB dye in the PFC **(A)** and blood serum **(B)** of female mice. Bar graph showing normalized mRNA expression of *Cldn5* **(C)**, *Ocln* **(D)**, *Tnfα* **(E)**, and *Il1β* **(F)** in the PFC of female mice exposed to saline, nicotine, and withdrawal fed with either control chow or PLX5622-supplemented chow. Data were analyzed using two-way analysis of variance with Bonferroni post hoc tests. (Compared with chow saline: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$; n = 7–13 per treatment group. Bars show mean ± SEM.) EB, Evans blue; mRNA, messenger RNA; Nic, nicotine; PFC, prefrontal cortex; Sal, saline; WD, withdrawal.

developing severe withdrawal symptoms (13), increased craving for nicotine (14), and a higher relapse rate than men (12). Moreover, sex differences have also been observed in the development of smoking-associated cerebrovascular disorders, with female smokers having a higher risk of stroke (27). Our observations suggest a possible mechanism for this heightened risk of stroke in women. Our findings corroborate previous findings that chronic nicotine treatment leads to compromised BBB functionality (41,44,45) and broadly increases BBB permeability in vivo (35,43). However, these findings have been described at a whole-brain level (35,41,43–45) and often only with single-sex samples (35,43). Our work clarifies these observations and demonstrates that regiospecificity is an important aspect of this sex-specific BBB permeability.

TJPs regulate the homeostasis and integrity of the BBB (18,46) and thus represent a potential mechanism underlying this regiospecificity. We found decreased mRNA and protein expression of Ocln, but no changes in Cldn5 or Tjp1/ZO-1 in PFC samples from female mice that were exposed to nicotine and 48-hour withdrawal. Furthermore, while we detected very low transcript levels of these TJPs in microglia and astrocytes, nicotine treatment and withdrawal increased expression of Cldn5 while reducing ZO-1 expression in purified PFC microvessels, respectively, which is the primary expression site of these proteins as well as a representative BBB unit in miniature. A recent study found increased BBB permeability and lower expression of *Cldn5* in the PFC of female mice that were susceptible to social defeat stress (47). In the same study, a decrease in *CLDN5* expression was also observed in postmortem brain samples from women diagnosed with major depressive disorder, and suppression of *Cldn5* expression in the PFC of female mice was sufficient to induce anxiety-like and depression-like behaviors. However, while BBB permeability was related to affective dysfunction in both studies, the Cldn5 findings suggest divergent mechanistic targets of stress and nicotine in the female PFC that both result in BBB dysfunction and affective disruption. However, additional

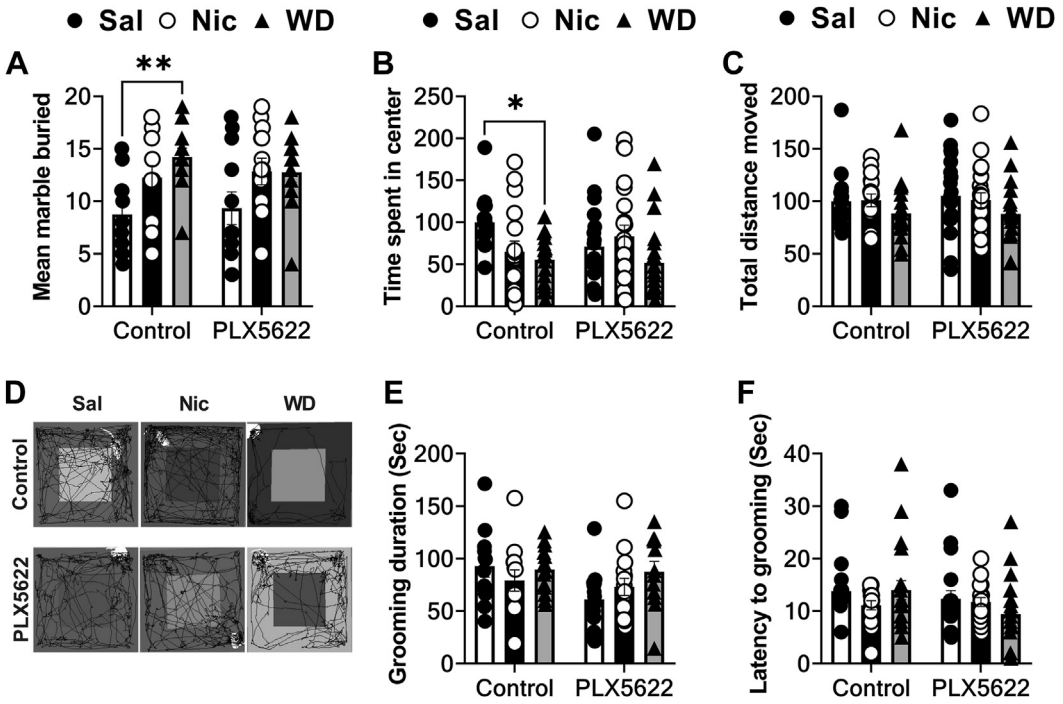

Figure 7. Pharmacological depletion of microglia with PLX5622 prevents nicotine withdrawal–induced anxiety-like behavior in female mice. After 14 days of nicotine treatment and 48-hour nicotine withdrawal, control chow– and PLX5622-treated female mice were subjected to different behavioral tests to assess nicotine withdrawal phenotype-like anxiety. Bar graph showing the number of marbles buried **(A)** in the marble burying test. Bar graph showing time spent in the center zone **(B)** and total distance moved **(C)** in the open field test. Track plots of animals in the open field test **(D)**. Bar graph showing grooming duration **(E)** and latency to groom **(F)** in the splash test. Data were analyzed using two-way analysis of variance with Bonferroni post hoc tests. (Compared with chow saline: $^{*}p < .05$, $^{**}p < .01$; n = 13–22 per treatment group. Bars show mean ± SEM.) Nic, nicotine; Sal, saline; WD, withdrawal.

research is required to assess the direct causal relationship between BBB dysfunction and nicotine withdrawal–induced anxiety because the current study only mechanistically probed the role of microglia.

We recently demonstrated that increased oxidative stress and inflammatory processes are strongly implicated in nicotine withdrawal symptomology (37). NADPH oxidase systems are key molecular mechanisms implicated in disease-related aberrant ROS production (48). Among the Nox isoforms that are expressed in the brain (49), we have shown Nox2 to be the primary source of excessive ROS in the nucleus accumbens during nicotine withdrawal (37). Consistent with these findings, PFC *Nox2* mRNA expression was increased following 48-hour nicotine withdrawal. We also observed that *Mmp9* expression was decreased after nicotine treatment but returned to baseline levels during nicotine withdrawal, suggesting that 1) it may be involved in the restoration of BBB integrity following nicotine exposure, or 2) its rebound expression may partially underlie nicotine withdrawal endophenotypes. Previous findings have demonstrated that activation of MMP-9 in the dorsal hippocampus drives nicotine-induced alterations in spatial working memory (50), and both MMP-2 and MMP-9 are required for cocaine relapse–associated synaptic plasticity (51). Overall, these results suggest that chronic nicotine exposure and nicotine withdrawal increase oxidative stress and modulate the extracellular matrix in the PFC of female mice. These molecular changes may drive the leaky BBB observed in the PFC of female mice, suggesting that therapies that reduce oxidative stress and neuroinflammation may prevent nicotine withdrawal–induced BBB damage and behavioral deficits.

Recent studies have demonstrated the crucial role of microglia in the maintenance of BBB integrity (52) and anxiety disorders (53,54). We also observed that depletion of microglia attenuated nicotine withdrawal–induced anxiety-like behavior in female mice in addition to preserving BBB integrity, suppressing inflammatory signaling, and preventing the loss of TJPs in the PFC during early nicotine withdrawal. Because PLX5622 impacts all myeloid cells, we evaluated Ccr2, a marker of invading peripheral monocytes. Increased *Ccr2* mRNA expression in the PFC of females exposed to nicotine and 48-hour nicotine withdrawal is likely due to the infiltration of the peripheral monocytes in the female PFC through a compromised BBB (55), especially given the fact that mRNA expression of *Ccr2* is nearly undetectable at baseline in saline controls. Furthermore, PLX5622 also reduces peripheral monocyte populations along with microglia (56), and *Ccr2* expression following treatment with PLX5622 in our study was near undetectable regardless of treatment. Therefore, we speculate that infiltrating peripheral immune cells may also contribute to the nicotine withdrawal effects observed in females. Two possible roles include 1) peripheral monocytes infiltrating the PFC and becoming brain-resident macrophages, and/or 2) they are attracted to and become attached

to the perivascular space, thereby resulting in microglial activation. However, future studies examining this question are needed. We and others have observed region-specific differences in microglial dynamics across different brain regions (37,57–60). While we have shown that microglial responses in the nucleus accumbens are important in driving affective nicotine withdrawal endophenotypes in male mice, we did not assess changes in the BBB or examine effects in female mice. This prevents us from assessing at this time whether there may be a dissociation by sex and region regarding microglial function, BBB permeability, and behavioral nicotine withdrawal end points. Further supporting this possibility, though, is the observation of the heterogeneity of the neurovascular units themselves within the brain by sex and region (61,62). However, because this is an emerging field in the substance use disorder realm and more broadly in psychiatry, more experimentation is needed to determine the region-specific causal relationships between BBB function, microglial dynamics, and affective withdrawal endophenotypes.

While we did not examine male subjects beyond the initial experiment, previous work that utilized a model of mecamylamine-induced nicotine withdrawal in male mice observed changes in microglial activation in the PFC concurrent with nicotine withdrawal–induced cognitive deficits (63). However, it is important to note that mecamylamine alone has cognitive-depressive effects in rodents (64), monkeys (65), and even humans (66), which limits the interpretation of these effects. Therefore, future studies that examine microglial dynamics in the PFC of male subjects undergoing spontaneous nicotine withdrawal are needed for a complete evaluation of these processes.

Opportunities for Precision Medicine in Tobacco Use Disorder: Targeting Restoration of BBB Integrity for Smoking Cessation in Women

There are currently 3 FDA–approved smoking cessation therapies: nicotine replacement therapy, varenicline, and bupropion. While FDA-approved, none of these therapies has a better than 20% success rate at 1-year postquit (67). Furthermore, meta-analysis has shown that nicotine replacement therapies are significantly less successful in women than in men (68), suggesting that non-nicotinic approaches for smoking cessation in women may be more viable targets. Our work suggests that BBB disruption and neurovascular dysfunction in women may underlie the reduced success of nicotine replacement therapies in women. These observations may be the mechanistic drivers of more severe nicotine withdrawal symptoms and increased relapse susceptibility in women. Therefore, further clinical research is urgently warranted to explore the utility of BBB- and microglia-related targeted therapies for the management of nicotine withdrawal symptoms in females as smoking cessation aides during early abstinence.

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JRT and CIR obtained funding for the study. JRT and MK designed the experiments. MK performed all the experiments. JK performed real-time reverse transcriptase–polymerase chain reaction analysis. MH assisted with mouse behavioral data analysis. CIR and SPA designed and performed tissue clearing and light sheet microscopy experiments. MK wrote the first draft of the manuscript, and all authors edited subsequent versions and approved the final version of the manuscript.

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ARTICLE INFORMATION

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