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Maternal immune activation by toll-like receptor 7 agonist during mid-gestation increases susceptibility to blood-brain barrier leakage after puberty

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Abstract

Maternal immune activation (MIA), a maternal stressor, increases risk for neuropsychiatric diseases, such as Major Depressive Disorder in offspring. MIA of toll-like receptor 7 (TLR7) initiates an immune response in mother and fetuses in a sex-selective manner. The paraventricular nucleus of the hypothalamus (PVN), a brain region that is sexually dimorphic and regulates hypothalamic-pituitary-adrenal (HPA) stress responses, have been tied to stress-related behaviors (i.e., depression, anxiety, social impairments). The current study characterized the sex-selective impact of mid-gestational TLR7 activation on PVN vasculature of adult offspring based on a prior study of excess prenatal glucocorticoid stress. The PVN of offspring were evaluated to determine if fetal MIA impacted vascular leakage in the brains of adult mice with or without restraint stress. Timed-pregnant female mice were administered the TLR7 agonist Resiquimod (RQ) or saline vehicle on embryonic day (E) 12.5. Basal and restraint stress-induced corticosterone was measured to examine changes in stress response. Mice were perfused transcardially with fluorescein isothiocyanate (FITC) to assess blood vessel integrity. Sections with FITC-labeled blood vessels through the PVN of offspring were immunolabeled for Glial Fibrillary Acidic Protein (GFAP; astrocytic end feet) and IBA-1 (microglia). MIA with RQ led to elevated levels

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CRediT authorship contribution statement

Stuart A. Tobet: Writing – review & editing, Supervision, Software, Resources, Investigation, Funding acquisition, Conceptualization. **Luke A. Schwerdtfeger:** Writing – review & editing, Investigation, Conceptualization. **Jonathan R. Christenson:** Writing – review & editing, Investigation, Data curation. **Julietta A Sheng:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbii.2024.100081.

of plasma corticosterone 60-minutes after restraint in offspring, suggesting prenatal RQ impairs glucocorticoid negative feedback. Blood-brain barrier integrity was assessed. Adult offspring of RQ injected dams showed greater leakage in the PVN (greater in males than females). GFAP+ colocalization with FITC-labeled vessels was lower in the PVN of offspring from RQ treated dams, potentially contributing to the observed increased FITC leakage. Microglia were examined in relation to the vasculature as an indicator of a neuroimmune response. Data show IBA-1+ cells greater in size and number in the PVN with closer proximity to blood vessels after maternal injection of RQ in a male-selective manner. Microglia were unchanged in females from RQ-treated dams but were smaller in size after restraint. This study provides support for sex-selective influences of fetal immune antecedents for altered brain vascular and blood brain barrier development and adult neuroendocrine function that could indicate a PVN locus for increased susceptibility for adult disorders.

Keywords

Maternal immune activation; neurovascular; blood-brain barrier; astrocytes; microglia; depression

1. Introduction

Fetal brain programming is heavily influenced by the physiological environment of the mother(Sheng et al., 2023). Stressors ranging from immune insults to psychological stressors lead to elevated maternal glucocorticoids (GCs) and inflammatory cytokines(Schepanski et al., 2018; Sheng et al., 2020). Stimulation or inhibition of the maternal immune system during middle to late gestation can lead to dysregulation of fetal brain circuitry, behavior, and cerebral vasculature(Frahm and Tobet, 2015; Gilman et al., 2016; Goldstein et al., 2021; Goldstein et al., 2014). Such phenotypes increase risk for associated mood and autonomic disorders, including Major depressive disorder (MDD) and cardiometabolic disease(Bale et al., 2010; Dearing et al., 2022; Goldstein et al., 2014; Madhavpeddi et al., 2022). MDD is sex-selective, with a 2x higher incidence in women(Handa et al., 2022; Sheng et al., 2021), while heart disease shows twice the incidence in men than women(Bots et al., 2017).

The paraventricular nucleus (PVN) is a sexually dimorphic nucleus in the brain and the central regulator of the HPA axis (Borrow et al., 2019; Heck et al., 2020). The PVN is a nexus for the integration of inputs from other brain regions(Cottrell and Seckl, 2009), and plays a key role in the central response to environmental stressors. Dysregulation of PVN development or circuitry negatively alters the stress response and increases susceptibility for stress-related disorders later in life in rodent (Brunton, 2013; Grundwald and Brunton, 2015) and human studies (Bale, 2011; Lautarescu et al., 2020). The current study examined neuroendocrine stress outputs in adults, indicative of potential HPA stress axis changes that were programmed in utero when mice were exposed to maternal immune activation (MIA). In previous experiments, a toll-like receptor (TLR) 7 agonist Resiquimod (RQ) was injected during mid-gestation leading to an increase in peripheral immune responses in mother and fetus(Sheng and Tobet, 2024). Offspring of RQ-injected mothers exhibited developmental delay and stress-related behavioral symptoms, including social impairments,

and anxiety- and anhedonia-like behaviors, in a sex-dependent manner. As these behavioral phenotypes can be regulated by PVN neurons, the current study focused on investigating cellular changes, such as vasculature impairment, in this region(Frahm et al., 2018; Frahm and Tobet, 2015).

The PVN is 2–3 times more densely vascularized than surrounding regions in the brain(Frahm et al., 2012; Palkovits et al., 1984). The blood-brain barrier (BBB) offers protection from potentially harmful peripheral compounds and is comprised of endothelial cells connected by tight junctions, astrocytic end-feet, and pericytes. Overexposure to glucocorticoids (GC) during fetal development lowered blood vessel density in the PVN of juvenile mice(Frahm and Tobet, 2015) and revealed a potential relationship between BBB integrity in the PVN and depression-like behavior in adulthood (Frahm, 2018). Studies in humans have associated BBB disruption with MDD and other neurological diseases(Labonte et al., 2017; Menard et al., 2017), driven by reduced tight junctions between endothelial cells(Dion-Albert et al., 2022b; Greene et al., 2020) and alterations in astrocytes surrounding capillary endothelia(Rajkowska and Miguel-Hidalgo, 2019; Rajkowska and Stockmeier, 2013). Microglia are important regulators of central immune function and may indicate locations of impaired BBB function and elevated vascular leakage in disease(Ronaldson and Davis, 2020; Sequeira and Bolton, 2023). Several studies with rodent offspring of mothers exposed to TLR 3 activation by poly I:C reported changes in microglial morphology and secreted chemokines and cytokines in areas of tissue damage or infiltration of harmful substances (Block et al., 2022; Guma et al., 2021; Loayza et al., 2023; Loewen et al., 2023; Zhao et al., 2022). As a follow up to a previous report on offspring behavioral changes (Sheng and Tobet, 2024), the current study tests the hypothesis that exposure to fetal immune stress predisposed the offspring to be more sensitive to a second stressor in adulthood with an altered acute stress response and impaired BBB integrity in the adult PVN.

2. Methods

2.1. Mice

Adult female C57BL/6 N female mice (sexually naïve, 6–8 weeks old) were monitored daily by vaginal lavage for 1 week to identify estrous cyclicity. Females on day of proestrus were time-mated with a C57BL/6 N adult stud male (8 weeks old) and removed the following day to their own cage. This day was noted as embryonic day (E) 0. Upon successful pregnancy, females were injected with RQ (HY-13740, MedChemExpress; s.c. 2 mg/kg body weight) dissolved in phosphate buffered saline (0.05 M PBS) or vehicle (VEH; PBS) on E12.5. Pregnant females were allowed to parturition and noted as postnatal day (P) 0 (Fig. 1A). Sex of neonates was determined by anogenital separation on P0 and all litters were culled to 6 pups (3 male, 3 female; randomized selection of pups for each sex) to avoid litter sex bias(Agnish and Keller, 1997). Litters were culled to equal numbers of males and females to reduce variability of maternal behavior toward one sex (usually males) and litter-size to optimize pup growth and development. One pup of each sex from each litter was used in all studies to avoid a litter effect. Mice were housed with *ad libitum* access to food and water and on a 12:12 light: dark cycle (lights on at 06:00 and off at

18:00). All mice were euthanized by isoflurane delivered in a sealed chamber at a fill rate of 30 – 70 % chamber volume per minute until breathing ceased, consistent with Colorado State University's Institutional Animal Care and Use Committee. This was followed by exsanguination by intracardial perfusion at 4 mL/min with fluorescein isothiocyanate (FITC; 1 μg/mL) phosphate buffered saline and 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) to fix tissues according to American Veterinary Medical Association approved methods. FITC is a small, 496 kDa molecule, which can bind to proteins in extracellular matrix and cell surfaces in and outside of blood vessels allows visualization of blood vessels and surrounding tissues when leakage occurs (Frahm 2014, modified from (Miyata and Morita, 2011)). All procedures were approved by Colorado University Lab Animal Resources and Institutional Animal Care and Use Committee Guidelines under protocol #1567.

2.2. Acute restraint and plasma corticosterone assays

Adult (P52) C57 male and female offspring were restrained inside a plastic 50 mL conical for 20-minutes with restricted movement followed by 60-minutes of recovery. The 50 mL conical was a spatially constricted tube with a breathing hole on one end and holes along the lateral sides for increased ventilation. After the 60-minutes of recovery in the home cage, the animal was euthanized by inhalation of isoflurane in a sealed chamber until breathing was ceased. Animal was euthanized by intracardial perfusion as previously described above(Frahm and Tobet, 2015). Restraint stress was performed between 09:00 and 14:00 to avoid diurnal elevations in corticosterone. Cardiac blood was collected and placed into chilled 0.5 M EDTA/aprotinin tubes 2μg/mL; Sigma-Aldrich, St. Louis, MO). Blood was centrifuged in a Beckman J6 centrifuge at 2000 rpm at 4 °C for 10 minutes. Separated plasma was stored at −20° C until assayed. Plasma corticosterone levels were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) per manufacturer's guidelines (Arbor Assays, Ann Arbor, MI; cat no. K014-H1; Limit of detection 7.7 μg/mL mean intra-assay CV = 8.5 %). 5uL of plasma samples (run in duplicates) were combined with dissociation reagent provided with the ELISA kit (Catalog # X058. This allowed dissociation of the corticosterone from corticosteroid binding globulin. The optical density of each sample was determined at a wavelength of 450 nm in Azure biosystems AO microplate reader (Azure Biosystems, Inc, Dublin, CA). The optical density readings for the standards and samples were used to calculate the concentration of corticosterone. A standard curve was generated using the online tool from "MyAssays" through Arbor Assays. The sample concentrations were calculated from the %B/B0 curve and multiplied by the dilution factor to obtain the neat sample values. Values were analyzed with GraphPad Prism (v10, La Jolla, CA) by 3-Way ANOVA to examine the effect of prenatal RQ treatment X sex X restraint. Šídák's multiple comparisons post-hoc analysis was performed, where appropriate.

2.3. Immunolabeling for blood vessels (FITC), astrocytes (GFAP), and microglia (IBA-1)

Mice were perfused intracardially with FITC; (1 μg/mL) in PBS (pH 7.4.), followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4.) (as described above). Brains were dissected and post-fixed in 4 % paraformaldehyde overnight followed by immersion in 0.05 M PBS until processing. Immunohistochemistry methods were followed as previously described(Frahm et al., 2018; Frahm and Tobet, 2015). Briefly, brains from male and female

mice were sectioned coronally at 50 μm with a vibrating microtome (Leica VT1000S). Free-floating sections were collected in 0.05 M PBS, followed by treatment with 0.1 M glycine to neutralize unreacted aldehydes. Sections were then incubated in 0.5 % sodium borohydride prior to being placed in blocking serum [0.5 % Triton X-100 (Tx), 1 % hydrogen peroxide, 5 % normal goat serum (NGS)]. Sections were then incubated in primary antisera for 48 h at 4°C against glial fibrillary acidic protein (GFAP; 1:250, AB_10013382; Z0334, Dako) for astrocytes and ionized calcium binding adaptor molecule 1 (IBA-1; 1:1000, 0.1 mg/mL, Cat# 019–19741, Wako) for microglia. After 48 hours, all sections were washed at room temperature in 0.05 M PBS with 1 % NGS and 0.02 % Tx. The tissue was incubated in secondary antiserum containing Cy3 conjugated anti-rabbit (1:500), 1 % NGS, and 0.32 % Tx for 2 hours and washed in 0.05 M PBS. Sections were mounted onto SuperFrost slides and cover-slipped with Aqua-Poly/Mount prior to imaging.

2.4. Imaging and analysis

FITC was detected using a 505/530 nm emission filter on a confocal microscope (Carl Zeiss LSM880) with an Axiocam 503 monochromatic camera. GFAP and IBA-1 immunoreactivity (-ir) were imaged with Cy3, detected with a 585/615 emission filter. Images were acquired for the PVN and two control regions [cerebral cortex (CX) and lateral hypothalamus (LH)]. All images were taken in 10 μm z-stacks with two optical sections for every 1 μm using a 40x/0.95 Corr M27 (Plan-Apochromat) oil immersion objective. Images were analyzed by an investigator blinded to treatment groups and sex. For FITC extravascular leakage, z-stack images were compiled in FIJI (ImageJ, v1.54 f) using a maximum intensity z-projection. The mean gray area of a 2.97 ×2.97 μm selection (area = 8.793μm^2) inside (intravascular FITC) and outside (extravascular FITC), directly adjacent to the selection on the inside of the blood vessel, was measured. The ratio of extravascular FITC to intravascular FITC was then determined by dividing the mean gray area of the outside selection by that of the inside adjacent selection to account for differences in quality of the perfusion. This ratio was taken 12 times for each image and averaged for the single output value per image. For GFAP and IBA-1 in proximity to FITC (blood vessels), images of FITC-labeled blood vessels and GFAP-ir or IBA-1-ir were independently z-projected to max intensity and thresholded in FIJI (GFAP threshold to 20 % intensity, IBA-1 threshold to 10 % intensity). The colocalization plug-in was used to determine the percent of colocalization of GFAP or IBA-1-ir with proximity (within 2 pixels = 1.18 μm) of FITC-labeled blood vessels. The number, area size, and total percent immunoreactivity of IBA-1 positive cells was also measured using Analyze Particles on thresholded images in ImageJ. Total percent area immunoreactivity was also measured for GFAP-labeled cells. FITC leakage analysis and immunolabeled protein values were statistically analyzed with GraphPad Prism (v10, La Jolla, CA) by 3-Way ANOVA to examine the effect of prenatal sex x RQ treatment X restraint. Šídák correction factor was used for multiple comparisons post-hoc analysis.

3. Results

3.1. Plasma corticosterone indicative of impaired HPA axis stress reactivity in adult offspring

Plasma CORT levels were assessed following a 60 min recovery period after a 20 min restraint stress (Fig. 1). ANOVA (3-way; Sex x RQ treatment x Restraint) revealed main effects of prenatal RQ treatment [F (1, 46) = 25.16, P = 0.0001], restraint [F (1, 46) = 51.17, P = 0.0001] and sex [F (1, 46) = 83.05, P = 0.0193] (Fig. 1B). An interaction of sex x restraint [F (1, 46) = 5.884, P = 0.0193] was evident in the graph (Fig. 1B) as females had reliably greater CORT responses than males after restraint. An interaction of prenatal RQ treatment x restraint [F (1, 46) = 7.903, P = 0.0072] (Fig. 1B) was evident in the more reliably greater CORT levels in prenatal RQ-offspring after restraint. The 3-way interaction was not statistically significant (P > 0.10). Detailed post hoc tests showed that VEH-treated female offspring exhibited 20 % higher levels of CORT by restraint (VEH-NR vs. VEH-R p = 0.0131) and a 27.1 % increase in CORT in RQ-treated animals with restraint (RQ-NR vs. RQ-R p = 0.0001). RQ-R females additionally showed 11.7 % higher CORT than VEH-R females (p = 0.0064). In males, post hoc Šídák multiple comparisons tests showed a significant difference in levels of plasma CORT in RQ males that underwent restraint compared to VEH males (28.5 % higher in RQ-R vs. VEH-R p = 0.0032). RQ-R males showed 26.5 % higher levels of plasma CORT after restraint compared to RQ-NR (p = 0.0129). In contrast, CORT levels in VEH-R males were not different from levels seen in VEH non-restrained controls within an hour of the restraint.

3.2. BBB permeability in the PVN was impaired in adult offspring of RQ injected mothers

Total vascular area in the PVN as indicated by the area of the fluorescent blood vessels in the region of the mid-PVN was similar across all offspring. The permeability of the BBB in the PVN was greater in adult offspring exposed to MIA (Fig. 2). Vascular leakage was determined by the ratio of extravascular to intravascular FITC (mean gray area intensity). ANOVA (3-way; Sex x RQ treatment x Restraint) showed significantly greater leakage from FITC-labeled blood vessels in the PVN of males and females with main effects of prenatal RQ treatment (Fig. 2A; [F (1, 65) = 144.8, P = 0.0001]) and restraint [F (1, 65) = 16.21, P = 0.0002]. Interestingly, the 3-way ANOVA showed a significant interaction of sex x restraint [F (1, 65) = 16.21, P = 0.0027] was driven by evidence that restraint appeared to selectively drive additional leakage in male offspring independent of prenatal exposure. In males the comparisons between VEH-NR and VEH-R (p = 0.0045) and between RQ-NR and RQ-R (p = 0.0190) were both significant while neither were noticeably different in females. A significant interaction of sex x RQ treatment [F (1, 65) = 4.940, P = 0.027] was driven by evidence that RQ-offspring regardless of restraint stress showed more leakage in RQ-males than RQ-females. RQ-Males had 33.3 % higher FITC leakage regardless of restraint (VEH-NR vs. RQ-NR p = 0.0001, VEH-R vs. RQ-R p = 0.0001, Šídák's post-hoc test). RQ females also displayed 25.6 % greater FITC leakage in the PVN of VEH-NR vs. RQ-NR (p = 0.00011) and VEH-R and RQ-R (p = 0.0001) groups. Leakage from FITC-labeled blood vessels was selective to the PVN. As in a prior study (Frahm et al., 2018) (Frahm) two control regions that were in the same sections (LH, CX) showed little evidence of leakage between groups. In the LH, there were main effects of prenatal RQ

treatment [F (1, 55) = 6.551, P = 0.0133] and restraint [F (1, 55) = 12.78, P = 0.0007]. The main effects were tempered by an interaction of prenatal RQ treatment x restraint [F (1, 55) = 12.04, P = 0.0010] due to greater leakage in Veh-offspring exposed to restraint stress. This can be traced by Šídák's post-hoc analysis primarily to a 15 % higher FITC leakage in the LH of females by restraint (VEH-NR vs VEH-R, p = 0.0164) (Fig. 2B). It was similar in males, but not statistically significant. In the CX, 3-Way ANOVA did not reveal a main or interaction effect.

3.3. Astrocytes less localized with PVN vessels of offspring from RQ-injected mothers

Astrocyte localization measured by GFAP-ir relative to blood vessels was altered in offspring of RQ-injected mothers. The percent of FITC-labeled blood vessel coverage by GFAP-ir was measured in the PVN (Fig. 3A). ANOVA (3-way - Sex x RQ treatment x Restraint) revealed a main effect of prenatal RQ treatment in the PVN [F (1, 50) = 46.96, P = 0.0001] indicating that RQ-offspring had reliably less blood vessel coverage than VEH-offspring. The main effect of sex was not significant, however, the significant interaction of sex by prenatal treatment indicated that there was a sex difference among RQ-offspring [F (1, 50) = 5.109, P = 0.0282]. In females, the percent of FITC-labeled blood vessels by GFAP was 30 % less in RQ no restraint and restraint groups compared to VEH groups in the PVN (RQ-NR vs. VEH-NR p = 0.0002, RQ-R vs. VEH-R p = 0.0006). Males showed 20 % less blood vessel coverage by GFAP in offspring of RQ-injected mothers without restraint with Šídák's post-hoc analysis test (RQ-NR vs. VEH-NR p = 0.0451). There was no apparent influence of adult restraint for either main effect of interaction. Overall, there was no significant effect of total GFAP-ir in the PVN (Fig. 3B). Despite notable differences in the relationship of GFAP immunoreactive astrocytes to blood vessels among the groups, there were no significant effects of sex or treatment on the total area of GFAP-ir area (Fig. 3B). Therefore, astrocyte differences were driven by their distribution rather than their size or number.

3.4. Microglial localization and morphology altered in offspring of RQ-injected mothers

Microglia are not a component of the BBB but may serve as indicators of BBB permeability if they respond to leaked molecular signals. Offspring of maternal RQ-injection displayed different microglia localization relative to blood vessels in the PVN, along with alterations in size and number. The percent of IBA-1-positive microglia within 2 pixels (1.18 μm) proximity to FITC-labeled blood vessels were evaluated in the PVN (Fig. 4A). 3-way ANOVA showed main effects of sex [F (1, 53) = 4.141, P = 0.0469], prenatal RQ [F (1, 53) = 27.14, P = 0.0001], and adult restraint [F (1, 53) = 27.14, P = 0.0076]. However, an examination of the significant interaction of sex x prenatal RQ treatment in indicates that the prenatal RQ treatment was selectively impactful in males [F (1, 53) = 6.091, P = 0.0169]. The significant interaction of and sex x restraint [F (1, 53) = 6.129, P = 0.0165] indicates that the acute restraint stress was selectively impactful in females. In RQ-males, microglia were in closer proximity to blood vessels than VEH-males. In females, regardless of RQ fetal exposure, only restraint stress in adults led to microglia to be further from blood vessels. There was 33.3 % more IBA-1 immunoreactive cells in proximity to blood vessels in RQ compared to VEH males (Šídák's post-hoc test, RQ-NR vs. VEH-NR p = 0.0254, RQ-R vs. VEH-R p = 0.0008). In females there was 25.8 % less immunoreactive IBA-1

in proximity to blood vessels in VEH-treated offspring (VEH-R vs. VEH-NR, p = 0.0146). There was no effect of RQ in microglia proximity to FITC-labeled blood vessels in females. There was, however, 20 % more immunoreactive IBA-1 in proximity to FITC-labeled vessels in RQ-R males than RQ-R females (p = 0.0117).

The average size of IBA-1 immunoreactive cells in the PVN (Fig. 4B) differed by fetal RQ treatment. ANOVA indicated a main effect of Restraint [F (1, 57) = 36.44, P = 0.0001] but not sex or prenatal treatment. Adult restraint stress led to smaller IBA1 immunoreactive microglia. In males, IBA-1 positive cells in the PVN were 15.6 % smaller in VEH-treated animals with restraint (VEH-R vs. VEH-NR, p = 0.0483) with Šídák's post-hoc analysis test. In females, IBA-1 positive cells in the PVN were 22.1 % smaller VEH-treated offspring with restraint (VEH-R vs. VEH-NR p – 0.0014) and 12.5 % smaller in RQ-treated offspring with restraint (RQ-R vs. RQ-NR p = 0.0114) according to Šídák's post-hoc analysis test.

The number of IBA-1 immunoreactive cells in mid-PVN sections differed as a function of fetal exposure. There was statistically significant main effect of prenatal exposure [F (1, 54) = 4.423, P = 0.0401]. However, an examination of the significant interaction of sex by prenatal exposure indicates that the prenatal RQ treatment was selectively impactful in males [F (1, 54) = 13.47, P = 0.0006]. The data show that maternal RQ led to greater microglial numbers in RQ-male versus VEH-male, and more IBA-1 labeled cells in the RQ-treated males than RQ-treated females (male RQ-R vs. female RQ-R p = 0.0272, male RQ-NR vs. female RQ-NR p = 0.0276 by Šídák's) (Fig. 4D) regardless of adult restraint. In males, Šídák's post-hoc analysis test showed 25 % more IBA-1 immunoreactive cells in the PVN in RQ-treated compared to VEH-treated offspring (p = 0.0172).

4. Discussion

The current study examined the neuroendocrine stress response and BBB integrity in the PVN of adult offspring from mothers injected with the TLR7/8 agonist RQ. Additionally, an adult stress was tested to evaluate whether exposure to fetal immune stress predisposed offspring to be more sensitive to a second stressor in adulthood. The results suggest HPA axis activity was impaired in offspring of RQ injected mothers. BBB integrity in the PVN was compromised with greater leakage and changes in astrocytes and microglia morphology and vascular proximity in offspring of RQ injected mothers. There was little to no change in BBB integrity in control regions (motor CX or LH) indicating this effect was at least partially PVN-selective. Given that the PVN is more densely vascularized than surrounding regions and plays a critical role regulating homeostasis and stress responses in clinical studies, dysregulation of its BBB could alter neuronal signaling and act as a potential mechanism for increased risk of adult neuropsychiatric disorder.

4.1. Offspring of RQ injected mothers displayed delays in acute-stress induced HPA axis negative feedback in adulthood

The PVN of the hypothalamus acts as the central regulator of the HPA axis stress response. PVN receives signals in relation to stressful stimuli from the environment and subsequently signals to the periphery to stimulate the release of GCs. The data in the current study show females were more sensitive to adult stressors measured by CORT secretion, in agreement

with previous studies (Handa et al., 1994, 1985; Simerly et al., 1985). Data further indicate that in adult offspring of RQ-injected mothers, there was hyperactive HPA axis function with impaired negative feedback following acute stress. A dysregulation of the neuroendocrine stress response in adulthood may lead to abnormal levels of stress hormones and associated pathologies. These findings align with previous studies that have shown fetal overexposure to exogenous GCs (e.g., Dexamethasone)(Barbazanges et al., 1996; Frahm et al., 2018; Hiroi et al., 2016; O'Regan et al., 2004; Sheng et al., 2023), maternal high fat diet(Niu et al., 2019; Sasaki et al., 2014; Sheng et al., 2023; Sullivan et al., 2014; Sullivan et al., 2012; Sullivan et al., 2011) and maternal caloric restriction(Akitake et al., 2015; Levay et al., 2008; Sheng et al., 2023) led to impaired HPA axis function and stress-related behavioral phenotypes (Sheng and Tobet, in press). When offspring were examined in adulthood after excess fetal GC treatment, the area of GFAP immunoreactive astrocytes was decreased in females compared to controls, the area of desmin immunoreactive pericytes was greater in males, and elevated depression-like behavior as indicated by a tail-suspension test in males and females(Frahm et al., 2018). The developmental origins of health and disease hypothesis posits perturbations in the maternal environment influence brain development. These fetal derived factors can drive adult risk for neuropsychiatric disorders, including MDD and cardiometabolic disease(Gilman et al., 2016; Goldstein et al., 2011; Grundwald and Brunton, 2015; Harris and Seckl, 2011; Kestering-Ferreira et al., 2021; Lin et al., 2023; Niu et al., 2019; Seckl and Holmes, 2007). Many reports suggest disruption in the PVN, the central regulator of the HPA axis, as a common pathway associated with these disorders(Goldstein et al., 2019; Herman et al., 2012; Herman and Tasker, 2016; Myers et al., 2012). In the current study, fetal exposure to maternal RQ injection led to elevated levels of CORT 60-minutes after restraint, indicating improper negative feedback to the HPA stress axis. Dysregulation of the HPA axis could further be linked to impaired BBB integrity and alter downstream neuroendocrine function and stress-related behaviors(Sheng and Tobet, 2024).

The PVN is an important anatomical region of stress regulation, however, other areas are involved in the HPA axis regulation of negative feedback, including the anterior pituitary and various extrahypothalamic regions (e.g., HIPP). PVN neurons may not be the only contributors to an altered stress response and other pathways to glucocorticoid release may involve more complicated circuits that may or may not include PVN neurons. Such pathways may include activation of the sympathetic nervous system where catecholamines are released by the adrenal medulla and stimulate the adrenal cortex to produce cortisol (Schaeuble and Myers, 2022), circadian rhythms regulated by the SCN (Miller et al., 2022), and low blood glucose levels that trigger the adrenal glands to release glucocorticoids(Dearing et al., 2022). Therefore, plasma levels of corticosterone alone do not provide direct evidence that the PVN is involved in the changes in stress response. Future investigations might benefit from measurements of CRH and/or ACTH to elucidate a more complete picture of the role of maternal RQ injection on HPA axis acute stress circuitry in offspring that differs by sex, in particular given that females in the current study showed higher levels of CORT response to RQ and restraint than males.

4.2. Offspring of RQ injected mothers displayed greater leakage from capillaries in the PVN

The BBB protects the brain by preventing harmful compounds from infiltrating into the CNS(Dion-Albert et al., 2022a). In mice, the BBB begins developing embryonically at ~;E11.5 and continues to mature through postnatal life (Frahm and Tobet, 2015; Haddad-Tovolli et al., 2017; Saili et al., 2017). Maternal stress can perturb BBB formation and lead to downstream influx of toxins and peripheral immune cellular and secretory components into the brain (e.g., inflammatory T cells) (Dudvarski Stankovic et al., 2016; Zhao et al., 2022). Reduced BBB integrity driven by maternal immune stress (i.e., inflammatory response during pregnancy with elevated cytokines, viral or bacterial infection during pregnancy) has been linked with increased susceptibility to neuropsychiatric pathologies, including MDD and autism spectrum disorders, among others(Arnone, 2022; Kealy et al., 2020; Ronaldson and Davis, 2020; Wu et al., 2022). While many maternal immune studies on BBB permeability have focused in cortical, hippocampal, or cerebellar regions and their downstream influence over adult neuropsychiatric disorders, the unusually dense vasculature of the PVN led to the hypothesis that the BBB in the PVN of the hypothalamus might be a site of anatomic and functional importance in the etiology of these disorders (Goldstein et al., 2014). In fact, in previous work by our collaborators in a clinical imaging study of maternal prenatal immune dysregulation on stress response circuitry abnormalities in adult offspring, the anterior hypothalamus (in which PVN is located) and connectivity with the hippocampus, were significantly impacted and by sex, effects that were retained into adulthood(Goldstein et al., 2021). In the present study, BBB permeability as assessed by FITC leakage was higher in the PVN of adult male and female offspring from RQ injected mothers. FITC leakage was selective to the PVN, with little change in leakage in control regions taken from the same sections. This effect was exacerbated with acute restraint stress in males. Such results demonstrate a level of sex-selectivity for susceptibility to BBB leakage in the PVN following a stressor in adulthood if previously exposed to excess maternal immune stimulation during prenatal development.

Astrocytes are an important component of a functional BBB. In the current study, adult offspring of RQ injected mothers showed lower GFAP-ir and coverage of GFAP-labeled astrocyte end feet near blood vessels in the PVN. Reduced coverage of astrocytes surrounding blood vessels of the PVN correlates with increased permeability, as suggested by higher leakiness found of FITC in the adjacent extravascular areas (Supplementary Figure 1). Astrocytic endfeet are shown to mediate water flow through a bi-directional regulatory channel, aquaporin 4 (Rajkowska and Miguel-Hidalgo, 2019; Wang et al., 2018). The current data show that paracellular FITC leaks into extravascular space in the PVN in adult offspring of RQ injected mothers. This effect could be a consequence of reduced GFAP-labeled astrocyte endfeet near blood vessels in the adult offspring.

Mechanisms that influence astrocyte coverage of the blood vessels could be programmed during early development, including genetic mutations or epigenetic modifications involved in end-feet function. Overall, these findings are consistent with a hypothesis that changes in astrocytes might provide a mechanism that leads to a leaky BBB in PVN capillaries in adulthood, a potential causal pathway for understanding associations with neuropsychiatric

disorders. BBB components beyond astrocytes have been examined within the context of prenatal GC exposure (Frahm et al., 2018), and MIA studies have shown disruption in endothelial tight junction proteins and mRNA expression (Claudin-5, Claudin-3, occludin, etc.) (Collignon et al., 2024; Dion-Albert et al., 2022b; Menard et al., 2017). Future studies are needed to determine if maternal injection of RQ has a similar effect on BBB pericytes, endothelia, and tight junction proteins in the PVN of maternally stressed offspring.

4.3. Prenatal RQ treatment led to sex-selective changes in microglia in the PVN

Microglia are resident immune cells in the CNS that drive inflammatory effects after MIA from fetal life to adulthood (Bilbo et al., 2018; Block et al., 2022; Loayza et al., 2023; Ozaki et al., 2020; Prins et al., 2018; Schaafsma et al., 2017). In the present study changes in anatomic localization and morphology of PVN microglia were examined in adult offspring of RQ injected mothers. Microglia were closer to blood vessels in the PVN of RQ-males than VEH males. Females did not show an effect of maternal RQ-injection on microglial coverage of PVN blood vessels but did have lower microglial coverage on PVN blood vessels following restraint stress. These data suggest sex-selective responses of microglial located in the PVN. Other groups have shown that pregnant mice treated with the TLR7 agonist, imiquimod, resulted in greater microglial gene expression, including chemokines (*Ccl2, Ccl6, Cxcl10*) and pro-inflammatory cytokines (*Tnf-α, IL-6*)(Missig et al., 2020). Unfortunately, this study did not assess sex as a variable, a crucial factor when investigating microglia in the context of sex-selective neuropsychiatric disorders(Lenz and McCarthy, 2015; Lenz et al., 2013; McCarthy et al., 2017).

Maternal immune stress leads to morphological changes in adult microglia. The decrease in size induced by restraint in VEH males and all females in the current study could suggest the PVN microglia are reacting to stress, potentially indicating disrupted BBB function or becoming protective to some degree. Additional studies are needed to fully evaluate the role of these microglia. Microglial size did not change with restraint in RQ-males, indicating less functional microglia caused by exposure to maternal injection of RQ. Activated microglia often migrate to “injured” sites, phagocytose harmful compounds or cells in a region, and secrete inflammatory cytokines to recruit additional innate immune cells to help repair damaged tissue and neurons(Sequeira and Bolton, 2023). Data in the current study demonstrate impaired integrity of the BBB within the bounds of the PVN, suggesting a role for microglia to help “repair” leaky vascular tissue by releasing vascular growth factors and phagocytose harmful compounds from the periphery. An examination of microglial secretory products in the current RQ-paradigm might better illuminate the functional role of microglia in BBB integrity following maternal stress.

5. Conclusions

In summary, the current study demonstrated maternal injection with RQ led to neuroendocrine dysfunction that was selective for PVN. Improper PVN function was associated with sex selective differences in vasculature integrity, and alterations in astrocyte and microglial cell interactions with PVN capillaries. Interestingly, FITC leak was exacerbated in males overall compared to females, but these data were not fully paralleled

by other cellular changes (astrocytes, microglia) in the BBB. Future studies may help tease out interactions between these and other cell types in the BBB (e.g., tight junctions, claudins, matrix metalloproteinases) to better understand sex differences in BBB integrity and function. Given the 3-fold greater vascularization of the PVN relative to most other brain regions, this location of injury may be particularly critical. We previously showed that offspring of mothers injected with RQ demonstrated more stress-related behavioral phenotypes linked to HPA axis dysregulation. The data, taken as a whole, are consistent with the hypothesis that maternal immune stress leads to changes in BBB integrity in the PVN that influence neuroendocrine signaling, and potentially influencing susceptibility to neuropsychiatric pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ACTH	Adrenocorticotropic hormone
BBB	Blood-brain barrier
CRH	Corticotropin releasing hormone
CORT	Corticosterone
CX	Cerebral cortex
E	Embryonic day
FITC	Fluorescein isothiocyanate
GC	Glucocorticoid
GFAP	Glial fibrillary acidic protein
HIPP	Hippocampus
HPA	Hypothalamic-pituitary-adrenal
IBA-1	Ionized binding calcium adaptor molecule –1
IL	Interleukin
-ir	Immunoreactivity

MDD	Major depressive disorder
MIA	Maternal immune activation
NR	No restraint
P	Postnatal day
PVN	Paraventricular nucleus
R	Restraint
RQ	Resiquimod
TLR	Toll-like receptor
VEH	Vehicle

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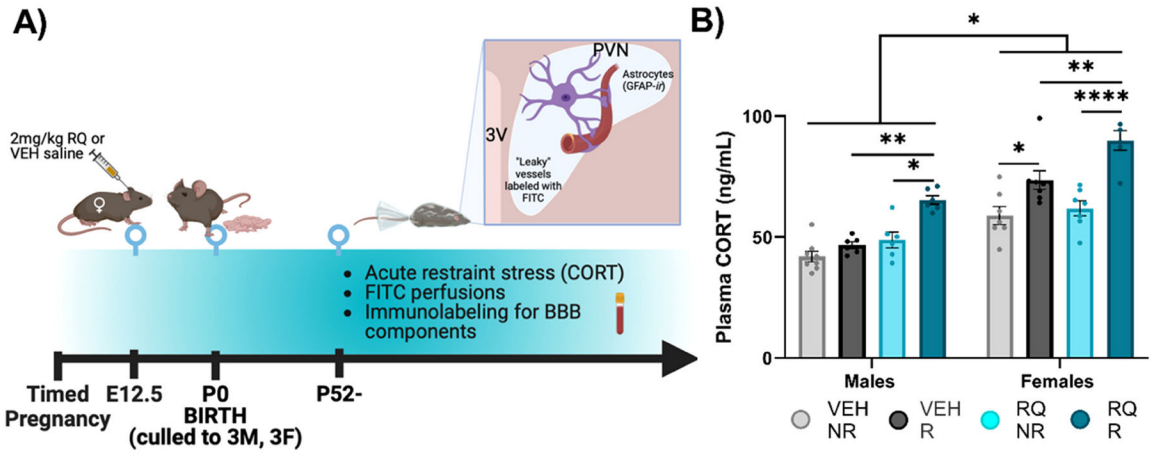

Fig. 1. Experimental timeline and plasma corticosterone (CORT) measurements indicative of impaired HPA axis stress reactivity in adult offspring.

(A) Experimental timeline. (B) In males, plasma CORT was elevated in RQ-R adult offspring compared to the other groups. Plasma CORT was greater in restraint/recovery groups in adult female offspring (VEH-R/RQ-R vs. NR) and overall greater with prenatal RQ treatment (RQ-NR/RQ-R vs. VEH). E = embryonic day, P = postnatal day, HPA = Hypothalamic-Pituitary-Adrenal, VEH = vehicle, RQ = Resiquimod, NR = no restraint, R = restraint H-recovery, CORT = corticosterone, PVN = paraventricular nucleus of the hypothalamus, 3 V = 3rd ventricle, FITC = fluorescein isothiocyanate, IBA-1 = ionizing binding calcium adaptor molecule-1. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented as +/− SEM. n = 6–9 mice / group.

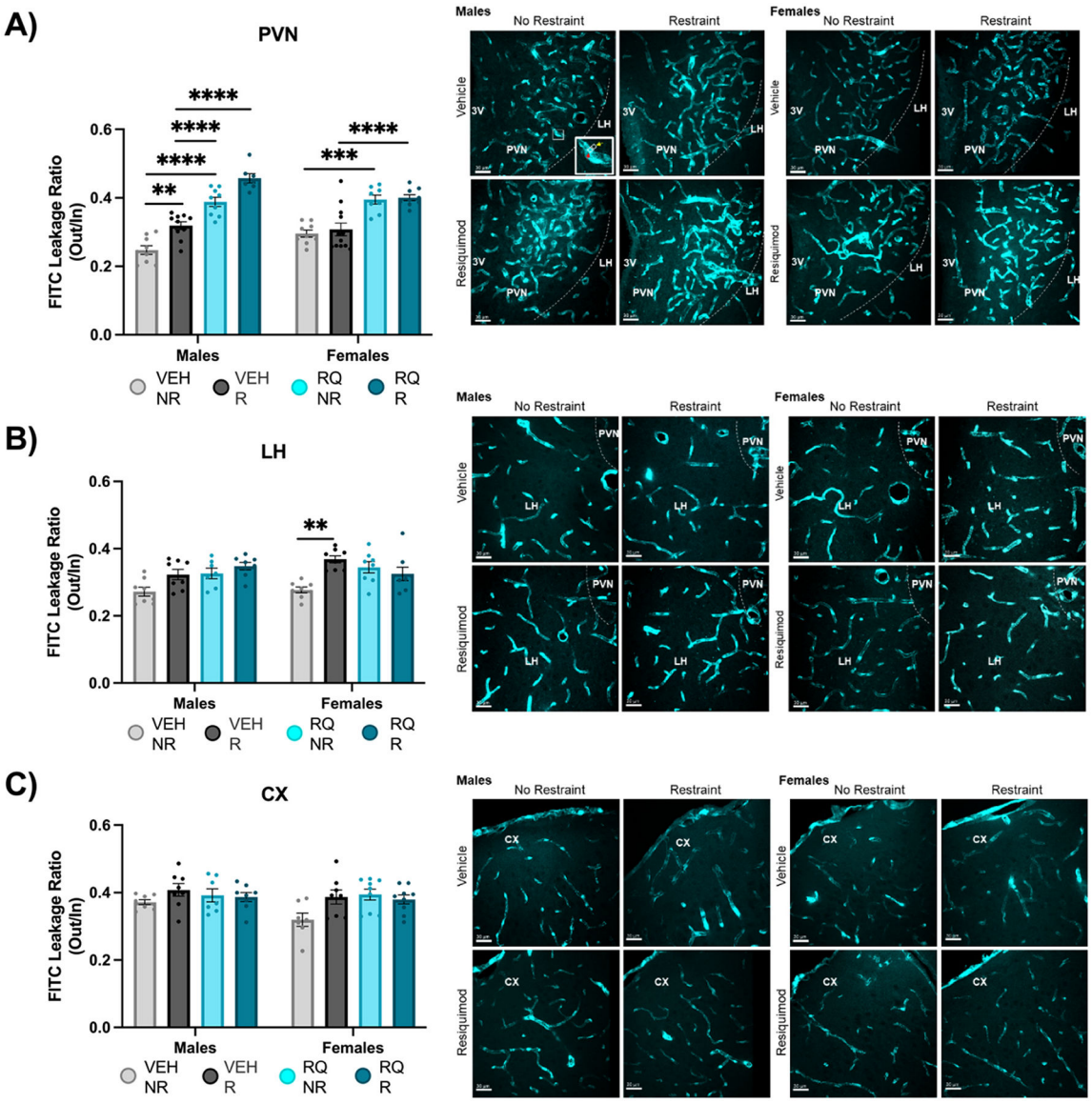

Fig. 2. BBB permeability in the PVN is impaired in adult offspring of RQ injected mothers.

Permeability was determined by FITC leakage in the (A) PVN, (B) LH, and (C) CX. Representative images to the right of each graph. The enlarged image in the bottom right corner of Male VEH-NR in (A) shows how the FITC ratio out (yellow arrow): in (red arrow) was measured. Mean gray area of the extravascular FITC (8.793μm^2 selection) was divided by the mean gray area of the intravascular FITC (8.793μm^2 selection) to obtain FITC leakage ratio. VEH = vehicle, RQ = Resiquimod, NR = no restraint, R = restraint, PVN = Paraventricular Nucleus of the Hypothalamus, LH = Lateral Hypothalamus, CX = Cortex. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented as +/− SEM. n = 8–10 animals / group.

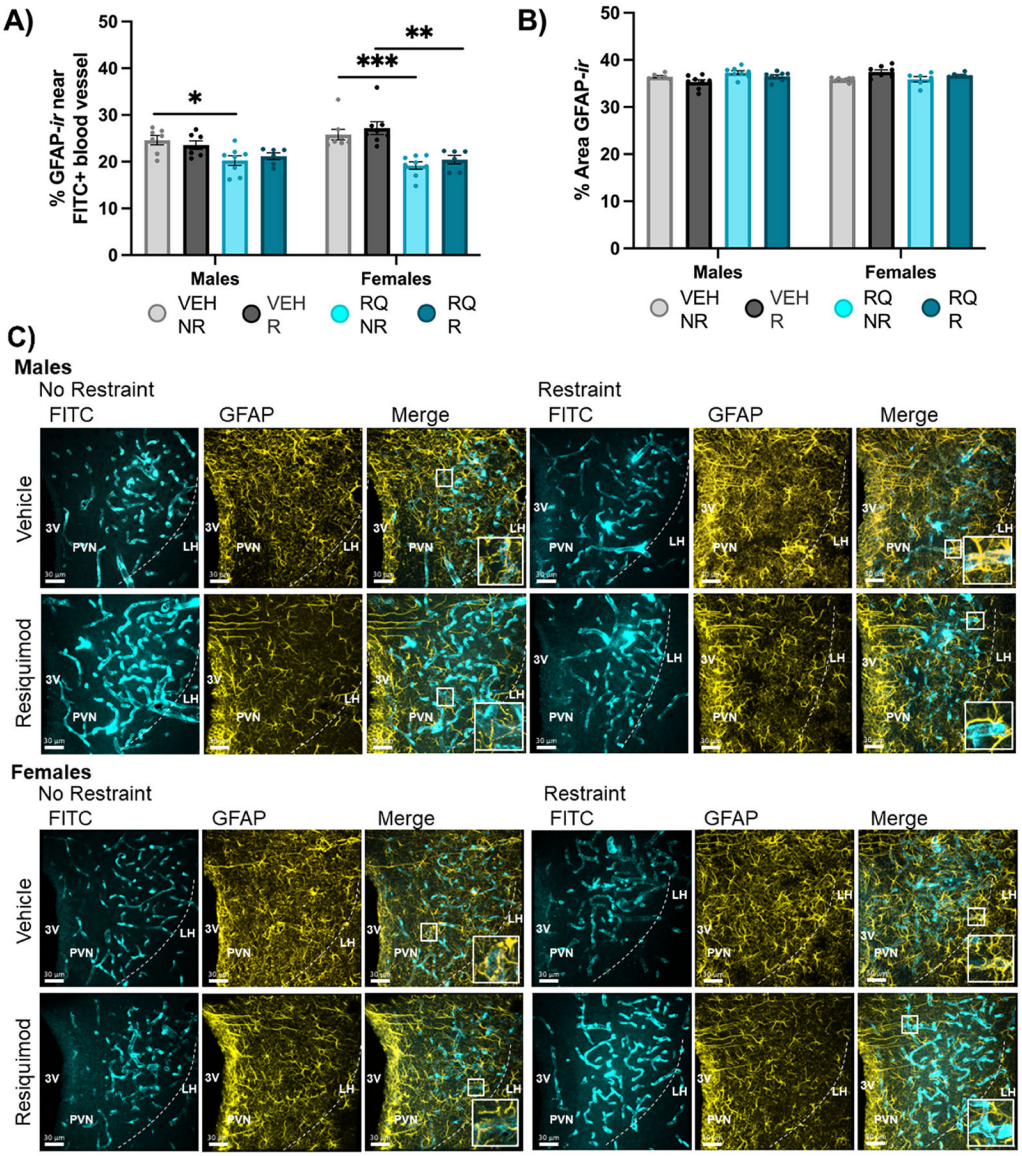

Fig. 3. Astrocytes were less co-localized with PVN vessels of offspring from RQ-injected mothers.

(A) GFAP-labeled astrocyte coverage of PVN blood vessels was lower in male and female adult mice exposed to prenatal RQ. (B) The total immunoreactivity denoted by " % Area GFAP-*ir*" was higher in VEH-R females compared to no restraint counterparts. (C) Representative images. VEH = vehicle, RQ = Resiquimod, NR = no restraint, R = restraint, PVN = Paraventricular Nucleus of the Hypothalamus, 3 V = 3rd ventricle, FITC = fluorescein isothiocyanate, GFAP = glial fibrillary acidic protein, F = Female, M = Male. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented as +/− SEM. n = 6–8 animals / group.

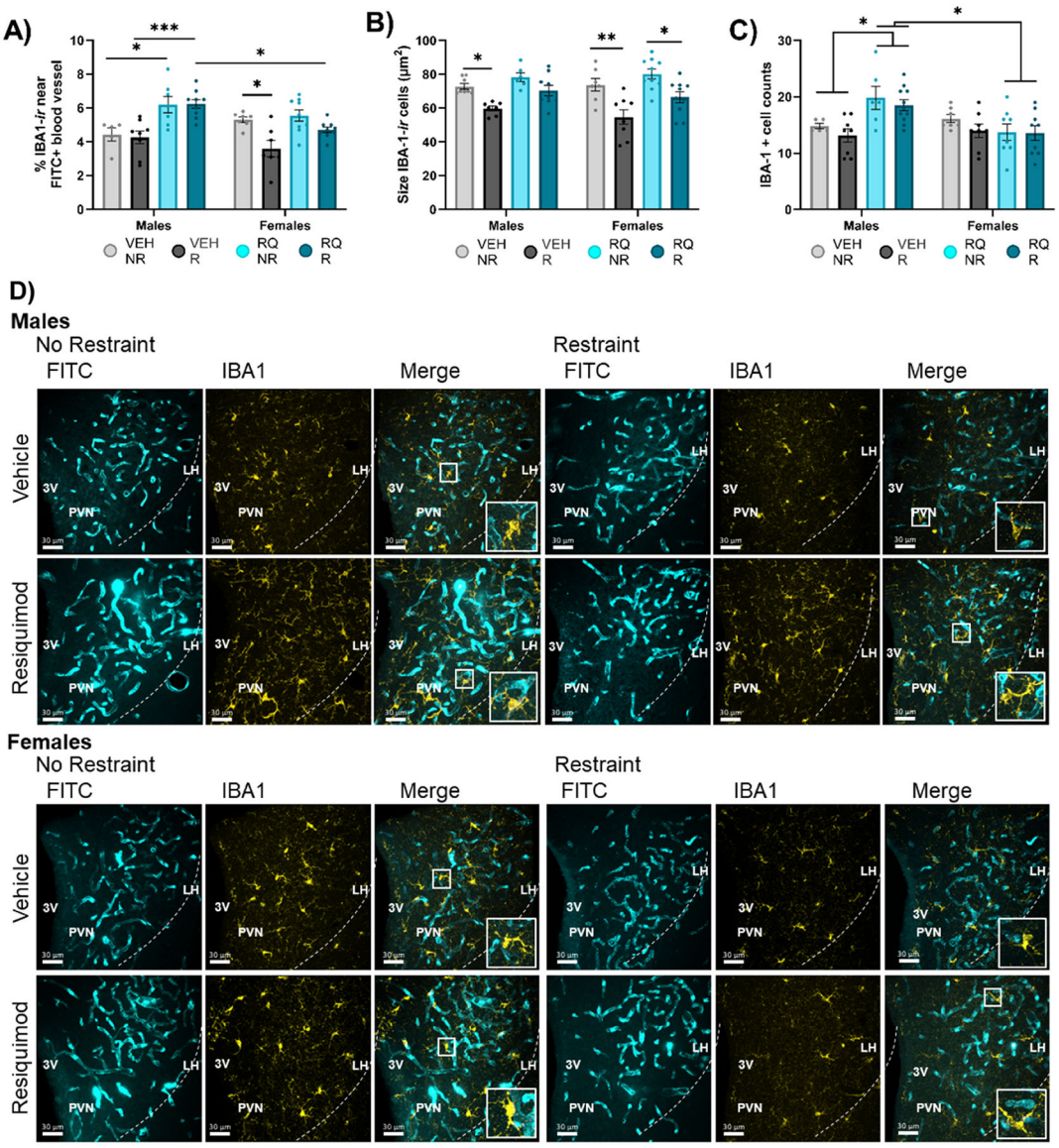

Fig. 4. Microglia localization, size, and number differed among offspring of prenatally injected mothers.

(A) Proximity of microglia to blood vessels (within 1.18 μm), (B) average size of IBA-1-labeled microglia cells, and (C) the number of IBA-1-labeled cells in the PVN. (D) Representative images. VEH = vehicle, RQ = Resiquimod, NR = no restraint, R = restraint, PVN = Paraventricular Nucleus of the Hypothalamus, 3 V = 3rd ventricle, FITC = fluorescein isothiocyanate, IBA1 = ionizing binding calcium adapter molecule-1. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$. Error bars represented as +/− SEM. n = 8–10 animals / group.