



## Full-length Article

## Postpartum increases in cerebral edema and inflammation in response to placental ischemia during pregnancy

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## ARTICLE INFO

## Article history:

Received 27 October 2017

Received in revised form 15 March 2018

Accepted 23 March 2018

Available online 26 March 2018

## Keywords:

Cerebral edema

Postpartum

Preeclampsia

Cerebral inflammation

Tight junction proteins

Neuroglia

Astrocyte

Microglia

Blood-brain barrier

## ABSTRACT

Reduced placental blood flow results in placental ischemia, an initiating event in the pathophysiology of preeclampsia, a hypertensive pregnancy disorder. While studies show increased mortality risk from Alzheimer's disease, stroke, and cerebrovascular complications in women with a history of preeclampsia, the underlying mechanisms are unknown. During pregnancy, placental ischemia, induced by reducing uterine perfusion pressure (RUPP), leads to cerebral edema and increased blood-brain barrier (BBB) permeability; however whether these complications persist after delivery is not known. Therefore, we tested the hypothesis that placental ischemia contributes to postpartum cerebral edema and neuroinflammation. On gestational day 14, time-pregnant Sprague Dawley rats underwent Sham ( $n = 10$ ) or RUPP ( $n = 9$ ) surgery and brain tissue collected 2 months post-delivery. Water content increased in posterior cortex but not hippocampus, striatum, or anterior cerebrum following RUPP. Using a rat cytokine multi-plex kit, posterior cortical IL-17, IL-1 $\alpha$ , IL-1 $\beta$ , Leptin, and MIP2 increased while hippocampal IL-4, IL-12(p70) and RANTES increased and IL-18 decreased following RUPP. Western blot analysis showed no changes in astrocyte marker, Glial Fibrillary Acidic Protein (GFAP); however, the microglia marker, ionized calcium binding adaptor molecule (Iba1) tended to increase in hippocampus of RUPP-exposed rats. Immunofluorescence staining revealed reduced number of posterior cortical microglia but increased activated (Type 4) microglia in RUPP. Astrocyte number increased in both regions but area covered by astrocytes increased only in posterior cortex following RUPP. BBB-associated proteins, Claudin-1, Aquaporin-4, and zonular occludens-1 expression were unaltered; however, posterior cortical occludin decreased. These results suggest that 2 months postpartum, neuroinflammation, along with decreased occludin expression, may partly explain posterior cortical edema in rats with history of placental ischemia.

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## 1. Introduction

Preeclampsia, a hypertensive disorder of pregnancy, is often associated with reduced utero-placental perfusion and placental ischemia. Following delivery of the fetus and placenta, blood pressure returns to normal for most patients, and other symptoms resolve, making delivery the only “treatment strategy” for preeclampsia patients. There is strong evidence that a preeclampsia-complicated pregnancy predisposes women to increased cardiovascular and cerebrovascular risk (Brown et al., 2013). Indeed, women with a history of a preeclampsia-

complicated pregnancy have increased risk of mortality from Alzheimer's disease, stroke, and cerebrovascular complications (Theilen et al., 2016). While some potential mechanisms contributing to cerebrovascular complication during preeclampsia and placental ischemia have been identified, the mechanisms underlying the increased risk for postpartum cerebrovascular complications later in life are not well understood.

One potential mechanism could be increased cerebral tissue inflammation. During pregnancy, preeclampsia patients present with increased circulating cytokines such as TNF $\alpha$  (LaMarca et al., 2005; Kalantar et al., 2013; Lau et al., 2013; Zhou et al., 2012), IL-17 (Darmochwal-Kolarz et al., 2012; Martínez-García et al., 2011; Toldi et al., 2011), IL-18 (El-Kabarity and Naguib, 2011; Huang et al., 2005; Seol et al., 2009), interferon gamma (Ozkan et al., 2014; Yang et al., 2014), IL-6 (Gadonski et al.,

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2006), and other cytokines/chemokines, leading to the idea that preeclampsia is an inflammatory disorder (Pinheiro et al., 2013). There is significant evidence that placental ischemia is also associated with increased circulating cytokines (Gadonski et al., 2006; LaMarca et al., 2011; LaMarca et al., 2005), suggesting that the ischemic placenta is the primary source of the circulating cytokines. Our laboratory recently demonstrated that the rat model of placental ischemia displays increased levels of cerebrospinal fluid cytokines during pregnancy (Warrington, 2015); however, whether this increased inflammatory environment persists after delivery and whether cerebral tissue cytokine levels are increased in the postpartum period following exposure to placental ischemia are not known.

Both inflammation and placental ischemia have been shown to contribute to cerebral edema (Warrington et al., 2015; Warrington et al., 2014) during pregnancy, a consequence of impaired cerebrovascular function. Magnetic Resonance Imaging (MRI) and other imaging methods reveal that preeclampsia patients present with abnormalities consistent with edema and blood–brain barrier (BBB) leakage during pregnancy (Matsuda et al., 2005). Moreover, several studies have reported the presence of white matter lesions in previously preeclamptic women (Aukes et al., 2012; Siepmann et al., 2017; Wiegman et al., 2014). Nevertheless, it is not known whether features of edema persist in the postpartum period following placental ischemia or whether cerebral edema occurs along with cerebral tissue inflammation.

We utilize the rat placental ischemia model, a well-characterized rodent model of preeclampsia, sharing numerous characteristics as the preeclampsia patient. Reduced uterine perfusion pressure (RUPP) is induced on gestational day 14, the start of the third trimester in the rat, to mimic the clinical condition of preeclampsia which is diagnosed after the second half of pregnancy. Like preeclampsia patients, RUPP rats develop hypertension, have increased circulating factors such as inflammatory cytokines TNF $\alpha$  (LaMarca et al., 2005) and interleukin-6 (Gadonski et al., 2006) and anti-angiogenic factors soluble endoglin (Gilbert et al., 2009) and soluble Fms-like tyrosine kinase -1 (sFlt) (Gilbert et al., 2007), proteinuria, and cerebrovascular abnormalities towards the end of pregnancy. Thus, the RUPP model is ideal for assessing underlying pathophysiological mechanisms underlying preeclampsia associated with placental ischemia.

While studies have begun to identify potential mechanisms underlying cerebrovascular abnormalities in response to placental ischemia during pregnancy, studies in the postpartum period are lacking. Therefore, we assessed cerebral changes in rats subjected to placental ischemia at two months postpartum for two (2) major reasons. First, a recent study (Paaauw et al., 2017), from which the brains used in the current study were obtained, showed that at 2 months postpartum, rats subjected to placental ischemia have reduced renal and cardiac function, suggesting that other organs such as brain may be affected as well. Secondly, several clinical studies report white matter lesions and other subtle cognitive deficits in women with a history of preeclampsia as early as 4–9 years postpartum (Aukes et al., 2012; Postma et al., 2014, 2016; Siepmann et al., 2017), which according to (Sengupta, 2013) is equivalent to 1.3–3 months in the rat.

In this study, we tested the hypothesis that exposure to gestational placental ischemia contributes to increased cytokines/chemokines and water content in specific brain regions during the postpartum period. To test this hypothesis, we used the rat model of placental ischemia and measured changes in water content in the anterior cerebrum, striatum, hippocampus, and posterior cortex at 2 months postpartum. We also measured posterior cortical and hippocampal levels of cytokine/chemokines and determined whether increases in brain water content was associated with changes in expression of tight junction proteins, neuroglia

markers, or astrocyte and microglia activation at 2 months postpartum in rats exposed to sham or placental ischemic pregnancy.

## 2. Materials and methods

### 2.1. Animals

A subset of Sprague Dawley rats, randomly selected from the larger study used in (Paaauw et al., 2017) were used (10 Shams and 9 RUPP). Only rats with brain samples collected for brain water content, protein analysis, and/or immunofluorescence analysis are included in this study. Time-pregnant rats arrived from Harlan Laboratories (Indianapolis, IN) at gestational day 10 or 11 and housed in the Lab Animal Facilities at the University of Mississippi Medical Center and maintained on a 12 h light, 12 h dark cycle with continuous access to food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee at UMMC before experiments were performed.

### 2.2. Induction of Placental Ischemia (Reduced Uterine Perfusion Pressure, RUPP)

On gestational day (GD)14, pregnant rats were anesthetized using isoflurane (3% induction, 2.5% maintenance). Silver clips were placed on the abdominal aorta (0.203 mm) and on both branches of the uterine arteries between the ovaries and the first pup (0.103 mm). Sham surgery involved the exteriorization of the uterine horn containing the pups with no placement of clips. All rats received Carprofen (5 mg/kg) as a pre-operative analgesic. Following delivery, pups were removed from the dams within 24 h to avoid lactation effects.

### 2.3. Implantation of Carotid Catheters and Blood pressure recording

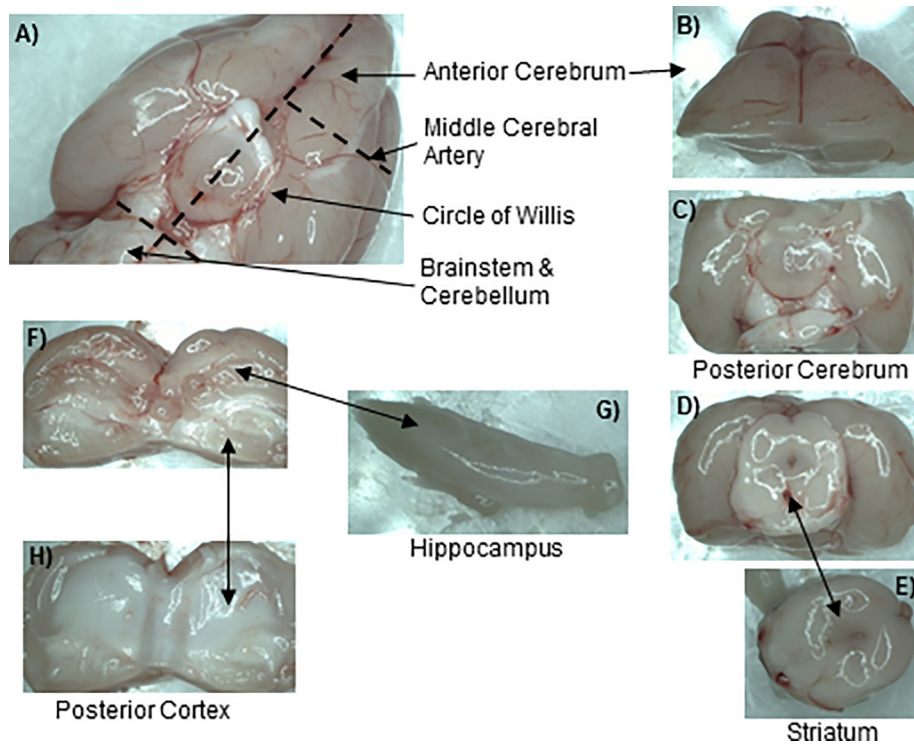
At 8 weeks postpartum, rats were instrumented with carotid catheters. The following day, rats were placed in restrainer cages and blood pressure was determined after 60 min acclimation using Lab Chart software and PowerLab pressure transducers (ADInstruments, Colorado Springs, CO). Blood pressure was recorded for 30 min. Two rats (1 from each group) underwent surgery for the placement of carotid catheters on GD18 followed by blood pressure recording on GD19. These 2 rats did not undergo additional surgery for blood pressure monitoring at 8 weeks postpartum.

### 2.4. Urine collection and measurement of urinary albumin

Twenty four hour urine was collected from rats at 8 weeks postpartum. Albumin concentration was measured using Exocell Nephra kit (Philadelphia, PA) and multiplied by the volume of urine excreted over 24 h.

### 2.5. Dissection of Brain Regions for brain water content and homogenization

Brains were removed, cerebellum and brainstem dissected and discarded. The remaining cerebrum was then hemisected. Brains to be used for water content and homogenization ( $n = 5$  per group) had both hemispheres further dissected to obtain the following regions: anterior cerebrum, hippocampus, striatum, and posterior cortex. Fig. 1A depicts the landmarks used for dissection of brain regions. Briefly, each hemisphere was cut along the most lateral portion of the middle cerebral artery to separate into anterior (Fig. 1B) and posterior cerebrum (Fig. 1C). The posterior cerebrum (Fig. 1D) was then further dissected by removing the striatum (Fig. 1E), and carefully peeling off the hippocampus (Fig. 1G). The



**Fig. 1.** Dissection of Brain Regions. Brains were removed and cerebellum and brainstem were cut and discarded (A). The anterior cerebrum was isolated by cutting along the most lateral portion of the middle cerebral artery (A and B). The posterior cerebrum (C and D) was then dissected to remove the striatum (E). (F) Remaining tissue consisting of the hippocampus (G) and posterior cortex (H).

overlying tissue was the posterior cortex ((Fig. 1H) corpus callosum also included). Other brains ( $n = 5$  Sham and  $n = 4$  RUPP) were dissected for brain water content and the right hemisphere collected for immunofluorescence staining.

## 2.6. Brain water content

The left subregions were weighed (wet weight) and then incubated at 60 °C for 72 h and then reweighed (dry weight). Tissue water content was then calculated as a percentage of the difference between wet and dry weight divided by wet weight.

## 2.7. Measurement of cerebral cytokines

Posterior cortical and hippocampal brain regions from the right hemisphere, corresponding to the regions used for determination of brain water content, were homogenized in RIPA lysis buffer containing Protease Inhibitors. Protein concentration was determined using a BCA kit. Equal volume of protein (25  $\mu$ L) was used in the Rat Cytokine/ Chemokine Magnetic Bead Panel (RECY-MAG65K27PMX, EMD Millipore; Billerica, MA) following manufacturer's directions. Samples were run alongside kit standards and controls in duplicate and standard curve for each analyte was used to calculate tissue levels. All samples were run on the same plate (96-well). Resulting concentrations were normalized to tissue protein concentration and represented as pg/mg protein.

## 2.8. Western blot

Equal concentrations of protein were loaded into pre-cast 26-well gels (4–20%, Bio-Rad) and separated using electrophoresis. Proteins were transferred onto nitrocellulose membrane, blocked using Odyssey blocking buffer, and followed by incubation with respective primary antibodies: 1:1000 Rabbit anti-AQP4 (abcam,

ab46182), 1:2500 Rabbit anti-Claudin1 (abcam, ab15098), 1:20,000 Rabbit anti-GFAP (abcam, ab7260), 1:1000 Goat anti-Iba1 (abcam, ab5076), 1:1000 Rabbit anti-occludin (Thermo Scientific, 71–1500), or 1:500 Rabbit anti-ZO-1 (Thermo Scientific, 61–7300) plus 1:2500 chicken anti- $\beta$ Actin overnight at 4 °C. Following washes ( $4 \times 5$  min), membranes were incubated using respective secondary antibodies (Donkey anti-chicken IRDye 680, Donkey anti-Rabbit IRDye 800 or Donkey anti-goat 800; 1:15,000) for 1 h at room temperature. Images were obtained using the Odyssey Imager and band intensity was determined using Image Studio Lite software (Licor) and normalized to the intensity of  $\beta$ Actin.

## 2.9. Preparation of Tissues for Immunofluorescence staining

Half brains ( $n = 5$  Sham and  $n = 4$  RUPP) were post-fixed in 4% paraformaldehyde overnight followed by cryoprotection in 30% sucrose solution, embedding in Cryo-Gel, and frozen. Molds were stored at  $-80$  °C until processing. From the posterior region, 20  $\mu$ m-thick coronal sections were cut and transferred to slides. Sections used for staining and quantification were approximately 4.36–4.80 mm posterior to Bregma and were 40  $\mu$ m apart (Paxinos and Watson, 2007).

### 2.9.1. Quantification of astrocyte morphology

Following blocking in 10% normal donkey serum and 1% bovine serum albumin (BSA) for 1 h at room temperature, sections were stained using Rabbit anti-GFAP (abcam, Cat #: ab7260) at 1:2000 overnight at 4 °C. Following washes ( $4 \times 10$  min), sections were incubated for 1 h in rhodamine (TRITC)-conjugated Donkey anti-rabbit IgG (Jackson Immuno, Cat # 131591) at 1:400. Z-stacks ( $512 \times 512$  pixels and 0.5  $\mu$ m steps) were captured using confocal microscopy (Nikon) at 60X magnification in the cortical regions (retrosplenial, parietal, and visual cortex) and hippocampus. Using ImageJ, images were thresholded and the number of



astrocytes and % area covered by astrocytes were determined by an investigator blinded to the groups (ABG). A total of 3–6 images per slide and region was used for analysis. Values were averaged per section (2 sections) and animal.

### 2.9.2. Quantification of microglia morphology

Following blocking in 10% normal donkey serum and 1% bovine serum albumin (BSA) for 2 h at room temperature, sections were stained using Rabbit anti-Iba1 (Wako, Cat #: 019-19741) at 1:500 overnight at 4 °C. Following washes ( $4 \times 10$  min), sections were incubated for 2 h at room temperature in 1:600 rhodamine (TRITC)-conjugated Donkey anti-rabbit IgG (Jackson Immuno, Cat # 131591). Z-stacks ( $1\mu\text{m}$  steps) were captured using microscopy (Nikon) at 60X magnification in the cortical regions (retrosplenial, parietal, cortex and visual cortex) and hippocampus. Using ImageJ, number of microglia and activation status were determined by an investigator blinded to the groups as described previously (Johnson et al., 2014; Kettenmann et al., 2011). A total of 3 images per section per slide was used for analysis. Type 1 microglia were identified by their small cell body and long, thin (ramified) processes. Type 2 microglia included those with asymmetrical cell body and long processes, Type 3 had rounded cell bodies and shorter, thicker processes, and Type 4 included those with round, enlarged cell bodies and short to no visible processes. Representative images of each activation state are shown in Fig. 6D.

### 2.10. Statistical Analysis

All differences between sham and RUPP groups were determined by unpaired *t*-test (equal variances) or Mann-Whitney *U* tests (for unequal variance). We performed ROUT test to identify outliers ( $Q = 1\%$ ) but no data point was removed from final analysis and results. Differences in microglia activation state and astrocyte number and area were analyzed using Two-Way Analysis of Variance followed by Fisher LSD post-hoc test. A *p*-value of  $<0.05$  was considered significant. All statistical analyses and graphs were performed or generated using GraphPad Prism software (version 7.02).

## 3. Results

**General Characteristics:** At 2 months postpartum, there was no significant difference in mean ( $124 \pm 3$  mmHg vs.  $125 \pm 4$  mmHg,  $p = 0.429$ ), systolic ( $139 \pm 3$  mmHg vs.  $142 \pm 4$  mmHg,  $p = 0.281$ ), or diastolic ( $106 \pm 3$  mmHg vs.  $107 \pm 5$  mmHg,  $p = 0.470$ ) blood pressure in rats exposed to sham or placental ischemic pregnancy respectively. Body weight ( $270 \pm 3$  g vs.  $259 \pm 5$  g,  $p = 0.044$ ) was reduced and urinary albumin excretion ( $1.08 \pm 0.28$  mg/24 h vs.

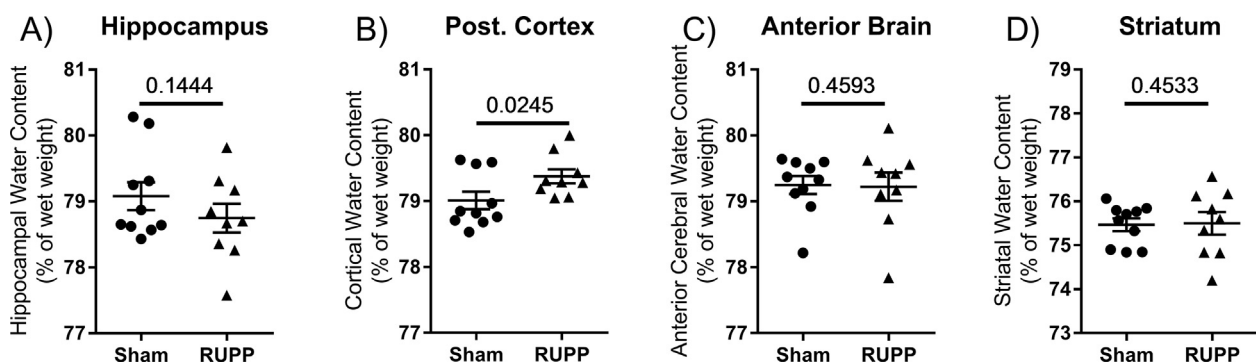
$1.79 \pm 0.41$  mg/24 h,  $p = 0.083$ ) tended to increase in RUPP group ( $n = 9$ –10 per group).

**Exposure to gestational placental ischemia leads to increased posterior cortical water content postpartum.** Water content was measured in anterior cerebrum, hippocampus, posterior cortex, and striatum at 8 weeks postpartum in sham and placental ischemic rats (Fig. 2). There was no difference in water content in the anterior cerebrum ( $79.2 \pm 0.1\%$  in sham vs.  $79.2 \pm 0.2\%$  in RUPP,  $p = 0.463$ ), hippocampus ( $79.1 \pm 0.2\%$  in sham vs.  $78.7 \pm 0.2\%$  in RUPP,  $p = 0.144$ ), or striatum ( $75.5 \pm 0.1\%$  in sham vs.  $75.5 \pm 0.3\%$  in RUPP,  $p = 0.455$ ); however, posterior cortical water content significantly increased in rats exposed to gestational placental ischemia ( $79.4 \pm 0.1\%$  in RUPP vs.  $79.0 \pm 0.1\%$ ;  $p = 0.025$ ) at 2 months postpartum.

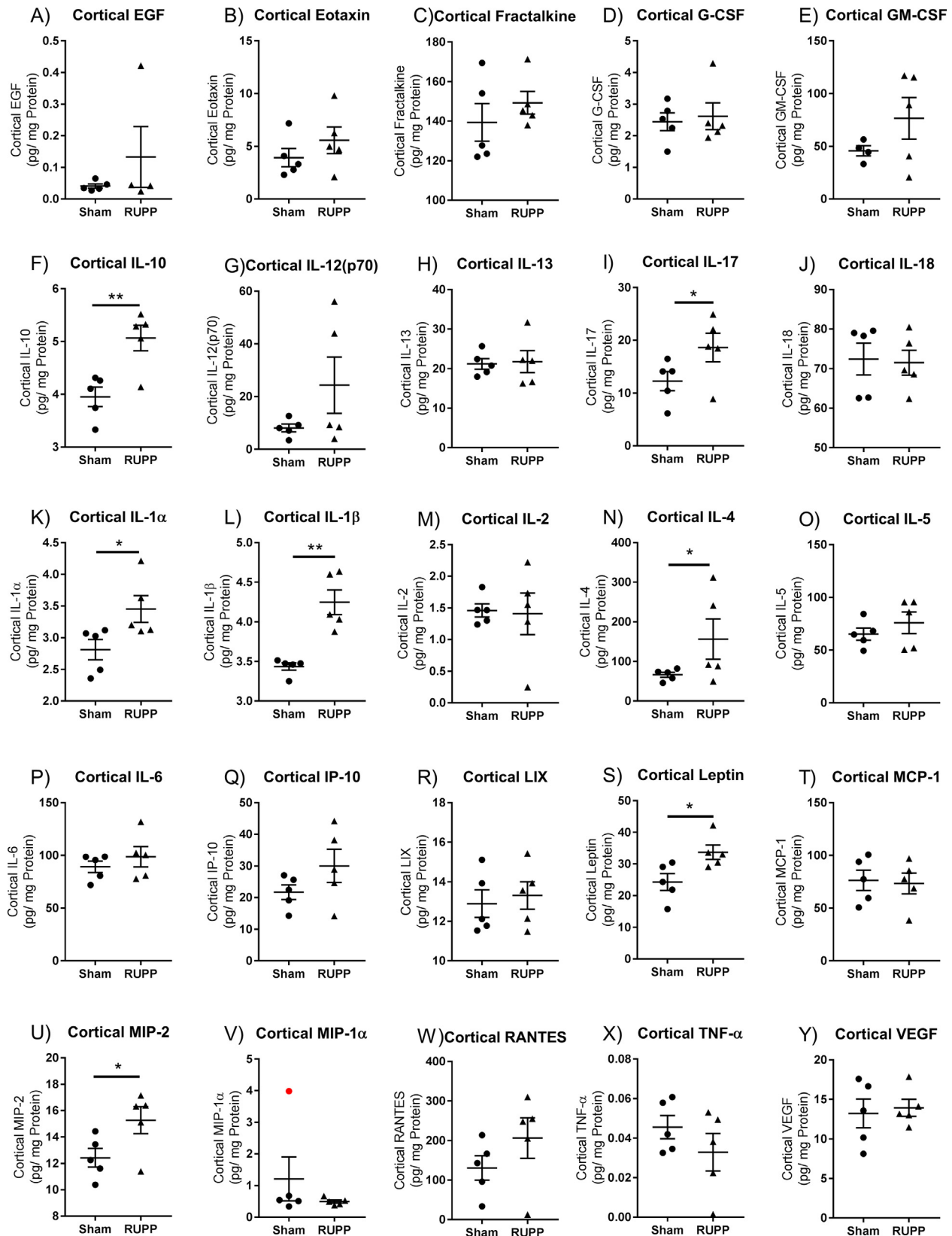
**Exposure to gestational placental ischemia contributes to cerebral inflammation at 2 months postpartum.** A multi-plex kit was used to assess the levels of 27 cytokines/chemokines in the hippocampus and posterior cortex at 2 months postpartum in rats exposed to sham or placental ischemia during pregnancy. Fig. 3 shows the results of 25 out of 27 detectable cytokines/chemokines in the hippocampal tissue samples. There was a significant increase in the anti-inflammatory factor IL-4 ( $p = 0.007$ , Fig. 3N), a decrease in the pro-inflammatory factor IL-18 ( $p = 0.033$ , Fig. 3J), and increase in IL-12(p70) ( $p = 0.007$ , Fig. 3G) and RANTES ( $p = 0.022$ , Fig. 3W) at 2 months postpartum in rats exposed to gestational placental ischemia.

In the posterior cortex, where water content was increased, the anti-inflammatory factors, IL-10 ( $p = 0.003$ , Fig. 4F) and IL-4 ( $p = 0.048$ , Fig. 4N) were increased. There was a significant increase in IL-17 ( $p = 0.043$ , Fig. 4I), IL-1 $\alpha$  ( $p = 0.021$ , Fig. 4K), IL-1 $\beta$  ( $p = 0.004$ , Fig. 4L), Leptin ( $p = 0.014$ , Fig. 4S), and MIP-2 ( $p = 0.026$ , Fig. 4U) at 2 months postpartum.

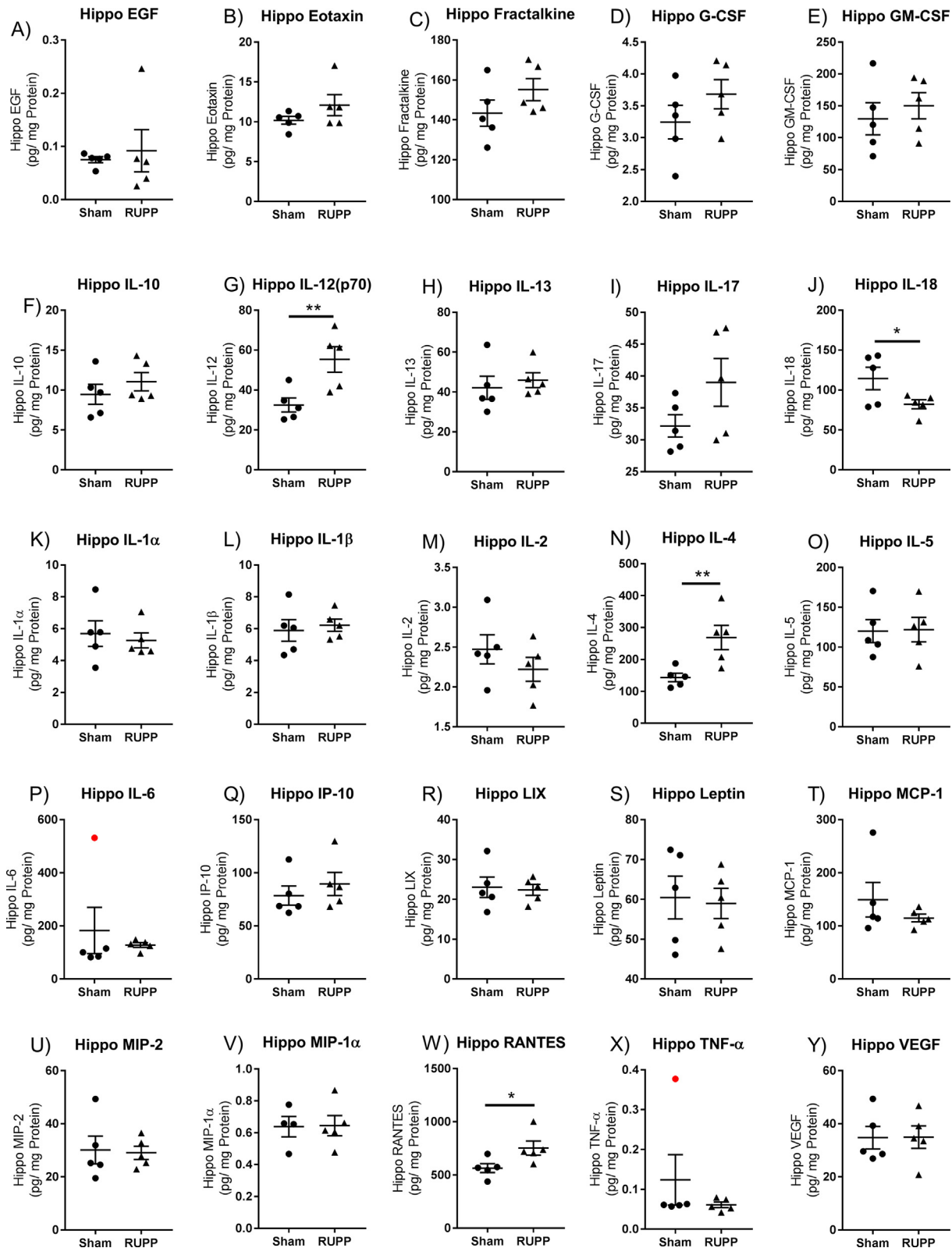
**Exposure to placental ischemia contributes to increased number and percent area covered by astrocytes at 2 months postpartum.** To determine whether increased cytokine/chemokine levels are associated with changes in astrocytes, we measured GFAP expression in the hippocampal and posterior cortical regions using Western blot and immunofluorescence staining. As shown in Fig. 5A, there was no difference in the expression of GFAP in the hippocampus ( $3.26 \pm 0.40$  in sham vs.  $3.52 \pm 0.26$  in RUPP) or posterior cortex ( $3.77 \pm 0.56$  in sham vs.  $3.02 \pm 0.13$  in RUPP) of rats exposed to placental ischemia at 2 months postpartum. Representative images of GFAP staining in the posterior cortex and hippocampus are shown in Fig. 5B. There was a significant increase in the number of astrocytes in the posterior cortex and hippocampus and an increase in the area covered by astrocytes in the posterior cortex of RUPP rats (Fig. 5C). These data suggest that increased astrocyte number and area are associated with increased pro-inflammatory cytokines in the posterior cortex following RUPP.



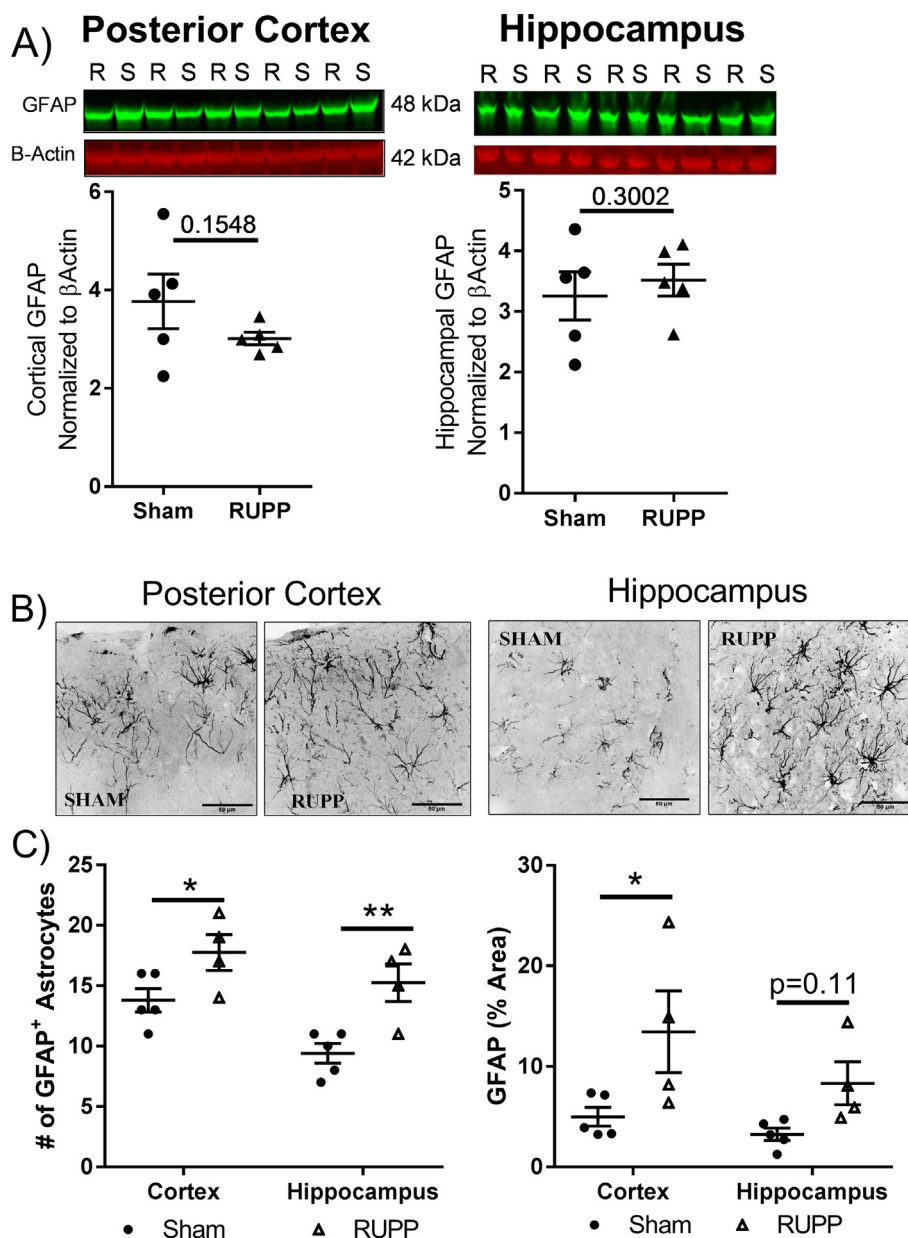
**Fig. 2.** Exposure to placental ischemia during pregnancy leads to posterior cortical edema postpartum. Water content (% of dry weight) in the (A) hippocampus, (B) posterior cortex, (C) anterior cerebrum, and (D) striatum of sham and placental ischemic rats at 2 months postpartum. Values for individual rats ( $n = 9$ –10 per group) are shown along with the Mean  $\pm$  SEM.



**Fig. 3.** Hippocampal changes in 25 of 27 detectable cytokines/chemokines following placental ischemia are shown. Scatter plots along with Mean  $\pm$  SEM are shown for: (A) EGF, (B) Eotaxin, (C) Fractalkine, (D) G-CSF, (E) GM-CSF, (F) IL-10, (G) IL-12(p70), (H) IL-13, (I) IL-17, (J) IL-18, (K) IL-1 $\alpha$ , (L) IL-1 $\beta$ , (M) IL-2, (N) IL-4, (O) IL-5, (P) IL-6, (Q) IP-10, (R) LIX, (S) Leptin, (T) MCP-1, (U) MIP-2, (V) MIP-1 $\alpha$ , (W) RANTES, (X) TNF- $\alpha$ , and (Y) VEGF. Values for GRO/KC were below detectable range and Interferon gamma levels were not obtained due to standard curve abnormalities. Statistical outliers are depicted in red and were not removed from analysis or final results. \* $p < 0.05$ , \*\* $p < 0.01$ . EGF – epidermal growth factor, G-CSF – granulocyte colony stimulating factor, GM-CSF – granulocyte macrophage colony stimulating factor, GRO/KC/CINC – Growth-regulated oncogene/ keratinocyte chemoattractant/ cytokine-induced neutrophil chemoattractant, IP-10 – Interferon gamma-induced protein 10, LIX – Lipopolysaccharide-induced CXC chemokine, MCP-1 – monocyte chemoattractant protein 1, MIP – macrophage inflammatory protein, RANTES – regulated on activation, normal T cell expressed and secreted, TNF – tumor necrosis factor, VEGF – vascular endothelial growth factor.



**Fig. 4.** Placental ischemia exposure leads to increased posterior cortical cytokines postpartum. Posterior cortical levels of 25 of 27 detectable cytokines/chemokines are shown. Scatter plots along with Mean  $\pm$  SEM are shown for: (A) EGF, (B) Eotaxin, (C) Fractalkine, (D) G-CSF, (E) GM-CSF, (F) IL-10, (G) IL-12(p70), (H) IL-13, (I) IL-17, (J) IL-18, (K) IL-1 $\alpha$ , (L) IL-1 $\beta$ , (M) IL-2, (N) IL-4, (O) IL-5, (P) IL-6, (Q) IP-10, (R) LIX, (S) Leptin, (T) MCP-1, (U) MIP-2, (V) MIP-1 $\alpha$ , (W) RANTES, (X) TNF- $\alpha$ , and (Y) VEGF. Values for GRO/KC were below detectable range and Interferon gamma levels were not obtained due to standard curve abnormalities. Statistical outliers are depicted in red and were not removed from analysis or final results. \*p < 0.05, \*\*p < 0.01. EGF – epidermal growth factor, G-CSF – granulocyte colony stimulating factor, GM-CSF – granulocyte macrophage colony stimulating factor, GRO/KC/CINC – Growth-regulated oncogene/ keratinocyte chemoattractant/ cytokine-induced neutrophil chemoattractant, IP-10 – Interferon gamma-induced protein 10, LIX – Lipopolysaccharide-induced CXC chemokine, MCP-1 – monocyte chemoattractant protein 1, MIP – macrophage inflammatory protein, RANTES – regulated on activation, normal T cell expressed and secreted, TNF – tumor necrosis factor, VEGF – vascular endothelial growth factor.



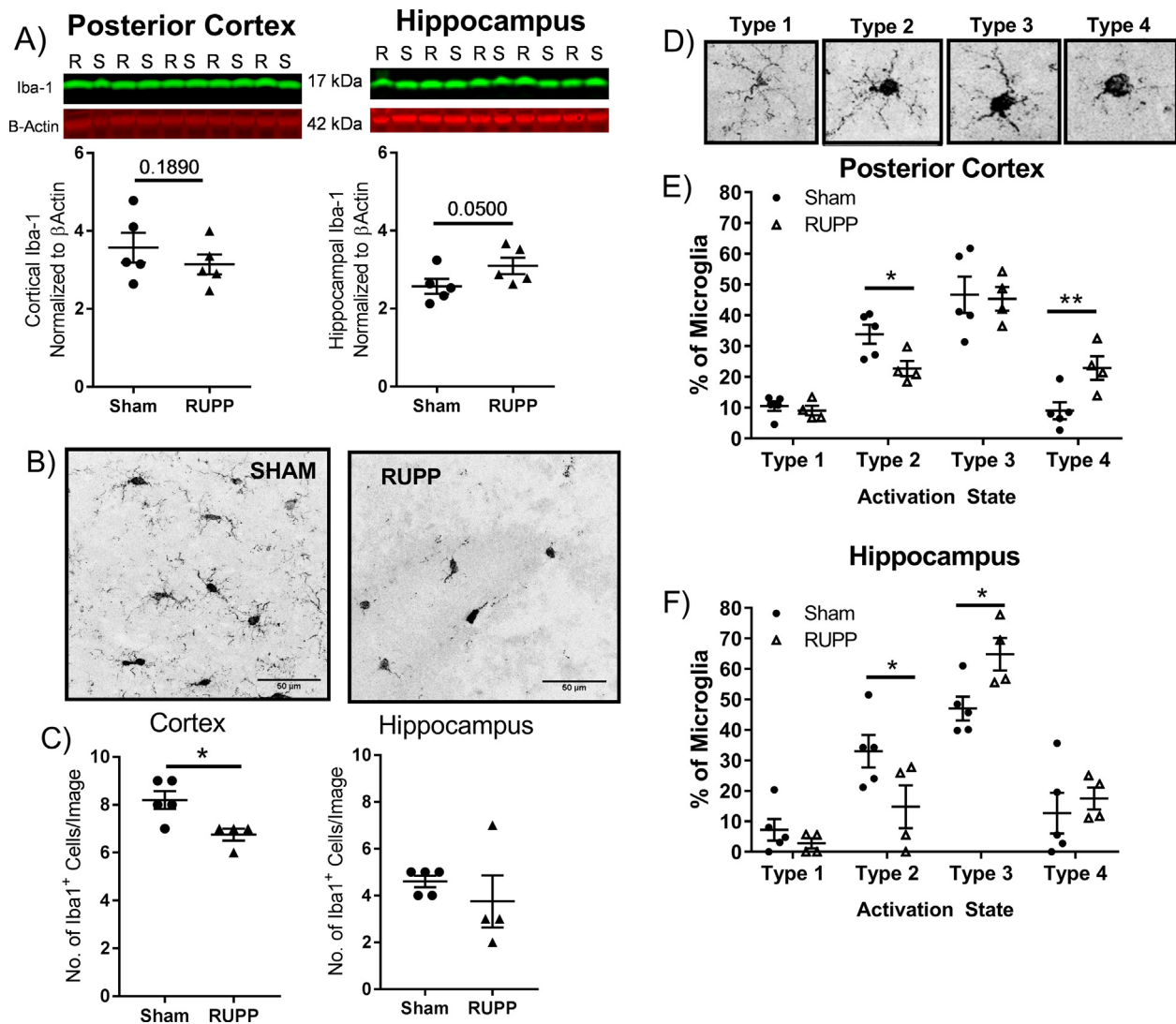
**Fig. 5.** Changes in astrocytes postpartum. No difference in (A) posterior cortical or hippocampal GFAP expression in rats exposed to placental ischemia. Western blot images are shown for each target and their respective  $\beta$ Actin bands. Values for individual rats are shown along with the Mean  $\pm$  SEM. (B) Representative images of GFAP staining in the posterior cortex and hippocampus at 2 months postpartum. Scale bars represent 50  $\mu$ m. (C) Placental ischemia induced increased number of astrocytes in posterior cortex and hippocampus but only increased the percent area covered by astrocytes in posterior cortex. Data points represent mean of 2 brain sections (3 images per section).

Exposure to gestational placental ischemia leads to reduced microglia number but increased activated microglia postpartum. To determine whether increased cytokine/chemokine levels are associated with changes in microglia, we assessed the expression of the microglia marker, Iba1, in the hippocampal and posterior cortical tissue homogenates using Western blot. As shown in Fig. 6, there was no difference in Iba1 expression in the posterior cortex ( $3.57 \pm 0.38$  in sham vs.  $3.14 \pm 0.26$  in RUPP) of rats exposed to placental ischemia at 2 months postpartum; however, there was a trend for increased Iba1 expression in the hippocampus ( $2.57 \pm 0.19$  in sham vs.  $3.10 \pm 0.21$  in RUPP,  $p = 0.05$  Fig. 6A). Representative images of Iba1<sup>+</sup> microglia in Sham and RUPP rats are shown in Fig. 6B. The number of microglia was significantly reduced in the posterior cortex of RUPP-exposed rats but unchanged in hippocampus (Fig. 6C). Fig. 6D shows examples of microglia in each of the activation states assessed. Quantification of the activation state

of the microglia shows that in both the posterior cortex (6E) and hippocampus (6F), type 2 microglia was significantly reduced in the RUPP-exposed rats. Type 4 microglia was increased in the posterior cortex, while Type 3 microglia increased in the hippocampus of RUPP rats at 2 months postpartum. Thus, placental ischemia is associated with increased microglia activation at 2 months postpartum.

Gestational placental ischemia exposure leads to reduced occludin expression but no change in other tight junction proteins. To determine whether increased brain water content was associated with changes in expression of tight junction proteins at the blood-brain barrier (BBB), we measured changes in expression of major components of the tight junction and adherens junction using Western blot. We found no changes in Claudin-1 (Fig. 7A and E), aquaporin 4 (Fig. 7B and F), or zonular occludens-1 (Fig. 7C and G) in the posterior cortex or hippocampus of rats following placen-





**Fig. 6.** Changes in microglia postpartum. No difference in (A) posterior cortical Iba1 expression but a trend for increased hippocampal Iba1 in rats exposed to placental ischemia. Western blot images are shown for each target and their respective  $\beta$ Actin bands. Values for individual rats are shown along with the Mean  $\pm$  SEM. (B) Representative images of Iba1 staining in the posterior cortex of Sham and RUPP-exposed rats. Scale bar represents 50  $\mu$ m. (C) Decreased number of microglia in the posterior cortex but no change in hippocampal microglia numbers in rats exposed to gestational preeclampsia at 2 months postpartum. (D) Representative images of microglia at each activation stage used during image analysis. (E) Quantification of posterior cortical microglia activation (F) Quantification of hippocampal microglia activation. Data points represent mean of 2 brain sections (3–6 images per section).

tal ischemia but a significant reduction in occludin in the posterior cortex (Fig. 7H) at 2 months postpartum. There were no changes in the major water channel, AQP4, following placental ischemia in the posterior cortex or hippocampus at 2 months postpartum.

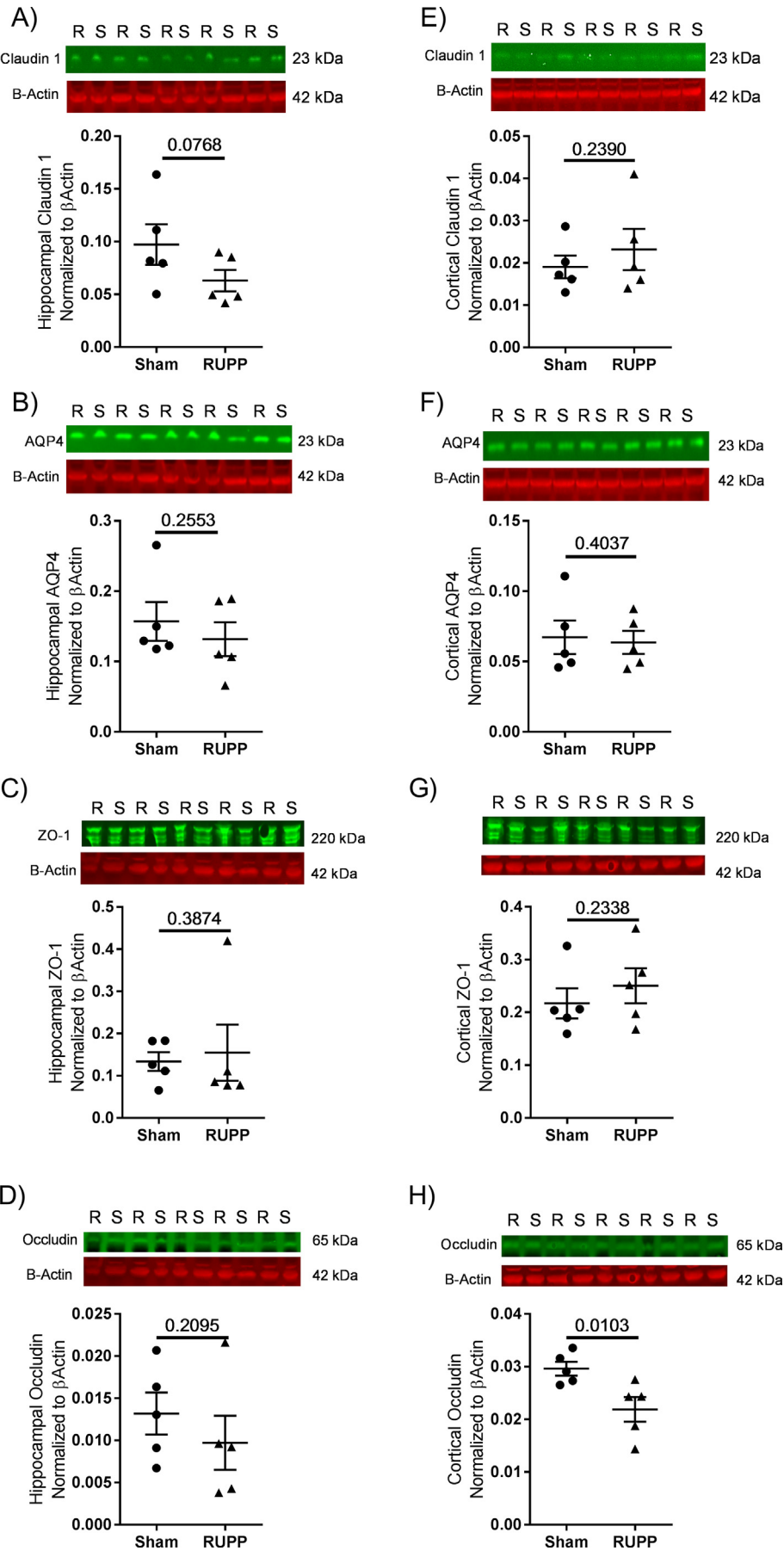
#### 4. Discussion

Preeclampsia is now recognized as a major risk factor for cardiovascular and cerebrovascular events in later life (Brown et al., 2013); the underlying pathophysiological mechanisms for which are not known. Placental ischemia, a recognized contributor to the pathophysiology of preeclampsia, is associated with cerebrovascular complications during pregnancy; however, whether these cerebrovascular complications persist after delivery is not known. In this study, we show that placental ischemia contributes to increased water content and cytokines/chemokines in the posterior cerebral cortex of rats at two months postpartum. This increase in posterior cortical water content was associated with a decrease in occludin protein expression but no significant changes

in other components of the tight and adherens junction at the BBB. While we found no changes in astrocyte or microglia markers via Western blot, we found increased number of GFAP<sup>+</sup> astrocytes in both the posterior cortex and hippocampus of rats exposed to placental ischemia, a reduction in the number of Iba1<sup>+</sup> microglia in the posterior cortex, and an increase in activated microglia in both regions. Importantly, consistent with reports in preeclampsia patients, rats exposed to placental ischemia were no longer hypertensive compared to sham control rats, and albuminuria was normalized at 2 months postpartum.

Our finding of no difference in blood pressure between the sham and RUPP rats at 2 months postpartum is consistent with other studies in humans and animals. As reviewed in (Bramham et al., 2013), elevated blood pressure generally returns to normal within days after delivery, with an estimated 20% of women with a hypertensive pregnancy having persistent hypertension and requiring medication two years postpartum. Importantly, studies using the RUPP model report no difference in blood pressure at 2 months (Pauw et al., 2017) or at 1 or 3 months (Brennan et al., 2016) postpartum after having significantly higher blood





pressures towards the end of gestation. While we have not performed blood pressure measurement at multiple time-points in this study, we are aware of the possibility that hypertension may develop with advancing age in the rats with a history of placental ischemia. This is an area of active investigation.

Previous studies have shown increased water content in the anterior cerebrum of placental ischemic rats during pregnancy, and this increase in water content is associated with increased permeability to Evans blue dye (Warrington et al., 2015, 2014). The differences in water content are very small. In rodents, we generally see a 0.5% difference in water content for the entire cerebrum or in the region of the anterior cerebrum during pregnancy following the RUPP procedure (Warrington et al., 2015, 2014). The current findings of 0.4% increase is consistent, although at 2 months postpartum, there is no difference in anterior cerebral water content but a significant increase in the posterior cortex of rats exposed to placental ischemia. Increased water content, an indicator of tissue edema, is characterized as either cytotoxic (increased water transport into cells through water channels, leading to cell swelling, with BBB intact) or vasogenic (edema involves physical disruption of the BBB resulting in increased water in the extracellular space) (Betz et al., 1989). Although we have not directly measured BBB permeability in this study, our finding of reduced expression of occludin, an important component of the BBB, suggests that vasogenic edema may be involved. It should be noted that because the rats exposed to placental ischemia no longer have increased blood pressure compared to the sham exposed rats, that increased pressure may not be playing an active role in the edema formation unless cerebral vessels, compromised during pregnancy, remain unrepaired. Indeed, a recent study demonstrated impaired vascular function in the mesenteric vascular bed during pregnancy and up to 3 months postpartum (Brennan et al., 2016), supporting our hypothesis that cerebrovascular function may still be impaired during the postpartum period. This possibility will be investigated in future studies.

The BBB is formed by the association of several cell types such as endothelial cells, pericytes or smooth muscle cells, and astrocytic end feet to the cerebral vessels. The first interface between the blood and the brain tissue is formed from tight junctions between adjacent endothelial cells lining the lumen of the vessels. The tight junction consists of several proteins that maintain the integrity of the BBB such as claudins, occludins, and zonula occludens (ZO) (Reviewed in (Liu et al., 2012)). Disruption of the BBB and reductions in expression of tight junction proteins lead to increased vessel permeability and edema. We previously measured the relative expression of tight junction proteins in normal pregnant and placental ischemic rats during pregnancy and found that increased tight junction protein expression in placental ischemic rats may protect the posterior cerebrum from edema (Warrington et al., 2014). This is consistent with findings from other studies showing that during late pregnancy, there is no difference in gene expression of claudin-1, claudin-5, occludin, and ZO-1 in cerebral arteries (Cipolla et al., 2011). Aquaporins (AQPs) are channel-forming proteins through which water, glycerol, and other solutes enter cells (Verkman, 2011). Three major AQPs are expressed in the brain: AQP1, AQP4, and AQP9, with AQP4 being the most abundantly expressed (Nicchia et al., 2004). AQP4 is mainly expressed in astrocytic end feet surrounding blood vessels,

and ependymal cells that line the cerebral ventricles and is associated with edema resolution (Fukuda and Badaut, 2012; Nicchia et al., 2004). In a previous study, we found that AQP4 protein is increased in the posterior cerebrum, which is protected from edema formation, but unchanged in the anterior cerebrum of placental ischemic rats during pregnancy. Compared to non-pregnant rats, late pregnant rats have increased AQP4 expression in the posterior brain but similar levels in the anterior brain (Wiegman et al., 2008), consistent with our findings in this study. In the current study, there are no changes in expression of AQP4 in the hippocampus or cortex of rats previously exposed to placental ischemia during the postpartum period. Taken together, our results suggest that cortical edema is associated with reduced expression of occludin at the BBB, potentially secondary to inflammation.

Cerebral inflammation and increased cytokines can influence brain signaling and cerebrovascular function and contribute to cerebral edema formation. Both preeclampsia patients and the rat model of placental ischemia are characterized by increased levels of circulating and placental inflammatory cytokines during pregnancy. Importantly, we recently showed that cerebrospinal fluid levels of the cytokines/chemokines eotaxin, IL-2, IL-17, and IL-18 are increased in the placental ischemic rat (Warrington, 2015; Zhang and Warrington, 2016). In this study, we show that 2 months after delivery, rats exposed to placental ischemia have increased levels of the anti-inflammatory cytokines IL-4 and IL-10, and a significant increase in the pro-inflammatory cytokines, IL-17, IL-1 $\alpha$ , IL-1 $\beta$ , Leptin, and MIP-2 in the posterior cortex where water content was significantly increased. Additionally, in the hippocampus where brain water content was unchanged, IL-18 decreased and IL-4 increased. These data demonstrate that cerebral edema is associated with a pro-inflammatory environment and the absence of a pro-inflammatory tissue environment in the hippocampus is associated with no increase in tissue water content.

During pregnancy, preeclampsia patients have increased levels of IL-17 (Darmochwal-Kolarz et al., 2012; Martínez-García et al., 2011; Toldi et al., 2011) and IL-18 (El-Kabarity and Naguib, 2011; Huang et al., 2005; Seol et al., 2009) in the circulation. Animal studies have demonstrated a direct role for IL-17 in mediating hypertension during pregnancy as infusion of IL-17 into pregnant rats increased mean arterial pressure and oxidative stress (Dhillon et al., 2012) while reducing IL-17 in placental ischemic rats attenuates blood pressure and oxidative stress (Cornelius et al., 2013). Increased circulating IL-17 levels have also been reported in seizure disorders such as epilepsy in the period between seizures or following seizures (Mao et al., 2013) and in CSF between seizure episodes (Mao et al., 2013). Additionally, IL-17 levels were correlated with glutamate levels and BBB disruption in a group of multiple sclerosis patients (Kostic et al., 2014). Furthermore, IL-17 has been shown to induce BBB disruption through the production of reactive oxygen species and down-regulation of occludin (Huppert et al., 2010) which we found to be decreased in the posterior cortex of rats exposed to placental ischemic pregnancy. Thus, the current findings support the hypothesis that increased IL-17 levels in the posterior cortex may contribute to downregulation of occludin, reducing BBB integrity, and contributing to edema formation. Further studies are required to directly test this hypothesis.

An interesting finding in this study is that posterior cortical levels of the anti-inflammatory cytokines IL-4 and IL-10 were

**Fig. 7.** Changes in expression of tight junction proteins associated with the BBB. No difference in hippocampal expression of (A) Claudin 1, (B) AQP4, (C) ZO-1, or (D) Occludin. Similar postpartum posterior cortical levels of (E) Claudin 1, (F) AQP4, (G) ZO-1 but decreased expression of (H) Occludin in posterior cortex of rats exposed to gestational placental ischemia. Western blot images are shown for each target normalized to  $\beta$ Actin. Values for individual rats are shown along with the Mean  $\pm$  SEM. Statistical outliers are depicted in red and were not removed from analysis or final results.

increased even in the presence of increased water content and a pro-inflammatory environment. IL-4 was also increased in the hippocampus of rats exposed to placental ischemia at 2 months postpartum. Clinical studies report reductions in both IL-4 and IL-10 in women with preeclampsia (Daneva et al., 2016) or increased maternal circulating IL-10 in preeclampsia patients compared to controls and a significant increase in preeclampsia patients complicated with intrauterine growth restricted fetuses compared to uncomplicated preeclampsia patients (Celik et al., 2012). In pre-clinical rodent models, there is evidence that during pregnancy, infusion of IL-10 in placental ischemic rats reduces blood pressure by lowering the pro-inflammatory cytokines, IL-6 and TNF $\alpha$  (Harmon et al., 2015). Additionally, treatment with IL-4 and IL-10 prevented hypertension and placental necrosis in a mouse model of preeclampsia by changing the activated maternal immune cell subsets (Chatterjee et al., 2015). Taken together, while clinical studies show both increased and reduced levels of IL-4 and IL-10 in preeclampsia patients, preclinical studies show a beneficial effect of increasing these anti-inflammatory cytokines in different rodent models of preeclampsia. Thus, our finding of increased posterior cortical IL-4 and IL-10 in prior placental ischemic rats support the hypothesis that at 2 months postpartum, compensatory increases in the anti-inflammatory cytokines occur in the posterior cortex. This hypothesis will have to be tested.

A key finding in this study is that gestational exposure to placental ischemia leads to increased posterior cortical levels of the pro-inflammatory cytokines, IL-1 $\alpha$  and IL-1 $\beta$  postpartum. There is evidence that placentas from preeclampsia patients secrete more IL-1 $\beta$  compared to normotensive placentas (Amash et al., 2012). Additionally, intracerebral IL-1 $\beta$  infusion in young rats has been shown to induce BBB breakdown (Anthony et al., 1998). Taken together, increased tissue levels of IL-1 $\beta$  may play a deleterious role at the BBB, contributing to cortical edema formation. Whether IL-1 $\beta$  plays a direct role in mediating posterior cortical edema formation will be assessed in future studies.

We found a significant increase in posterior cortical leptin levels in rats exposed to gestational placental ischemia, the source and effect of which has not been investigated. Studies have shown increased levels of serum leptin in preeclampsia patients compared to controls (Acromite et al., 2004; Taylor et al., 2015) although leptin levels were not independent of body mass index. Other studies have shown no difference (Asnafi et al., 2011; Celik et al., 2004) in leptin levels in preeclampsia patients. While placental ischemia has been associated with reduced circulating leptin levels (Gilbert et al., 2012), changes in cerebral levels of leptin during pregnancy have not been assessed. Nevertheless, infusion of leptin into pregnant rats increases blood pressure and placental expression of TNF $\alpha$  (Palei et al., 2015). Moreover, infusion of exogenous leptin in male mice led to increased brain water content and neurological deficits following intracerebral hemorrhage compared to control mice or leptin deficient mice given exogenous leptin (Kim et al., 2013). Thus, increased cortical leptin could mediate posterior cortical edema formation.

MIP-2 (CXCL2), a strong chemoattractant for neutrophils (Diab et al., 1999), was increased in the posterior cortex of rats with a history of placental ischemia in this study. While there are no studies reporting MIP-2 levels in preeclampsia patients, there have been reports of neurotoxic effects on primary neuron cultures (De Paola et al., 2007). Thus, increased levels of MIP-2 could lead to neurotoxicity, contributing to the vicious inflammatory cycle. This possibility will need to be tested.

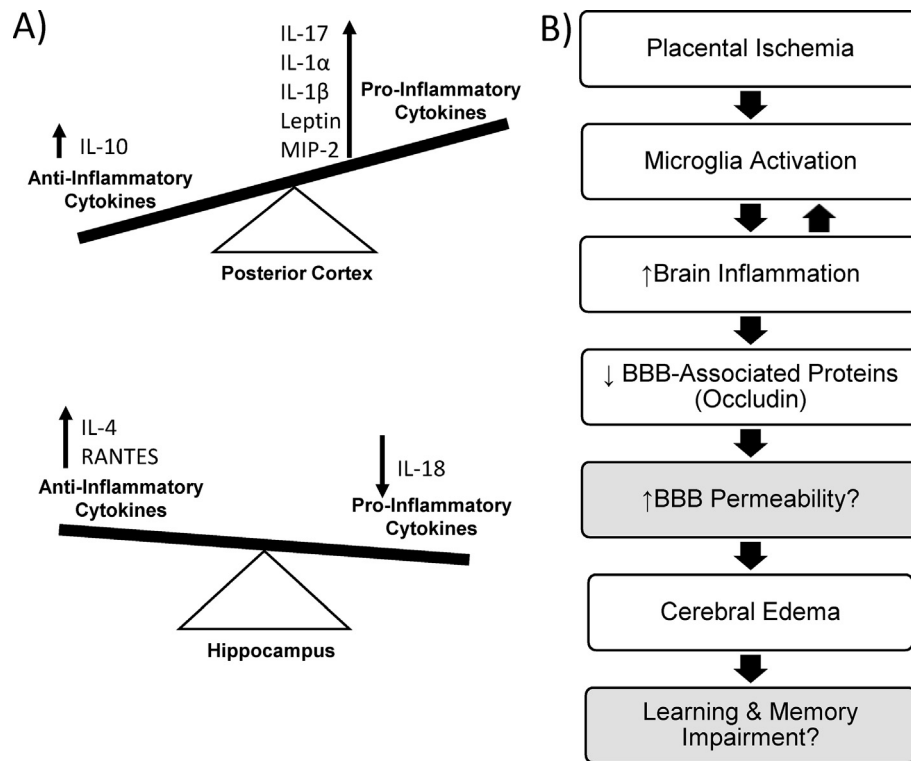
We found significant increases in hippocampal RANTES (CCL5) levels, where water content was unchanged. There is evidence that both plasma and placental levels of RANTES are increased in preeclampsia patients (Hentschke et al., 2012). In addition to being increased in preeclampsia patients, RANTES is also increased in

microvessels from Alzheimer's disease patients; although in vitro studies showed a protective role of RANTES using neuronal cultures (Tripathy et al., 2010). It is therefore possible that increased RANTES in the hippocampus is protective, resulting in lower inflammation and no edema. We also found a significant increase in hippocampal IL-12(p70), a cytokine involved in the differentiation of T-cells. We are unsure of the role of increased IL-12(p70) in this environment but speculate that the increased anti-inflammatory factors and reduced IL-18 levels in the hippocampus outweigh the contribution of IL-12(p70) resulting in a net neuroprotection.

Although we have not measured changes in cytokine/chemokine expression in the cerebral tissue during pregnancy, the cerebrospinal fluid levels are a great reflection of the tissue environment. Products from the extracellular space collect in the cerebrospinal fluid (Iliff and Nedergaard, 2013; Mendelsohn and Larrick, 2013; Plog et al., 2015) primarily by the actions of neuroglia. Rats subjected to placental ischemia and a high cholesterol diet have increased levels of activated microglia (Johnson et al., 2014) suggesting that increased activation of microglia may contribute to increased tissue inflammation. Thus, the findings in pregnant RUPP rats treated with high cholesterol diet are in agreement with our current findings of increased activated microglia in the posterior cortex of postpartum RUPP rats. The current study also found increased astrocytes in posterior cortex and hippocampus of postpartum RUPP rats. Together, these data support the hypothesis that activated microglia and increased astrocytes could be the source of some of the pro-inflammatory cytokines in the posterior cortical region. Of course, the presence of increased neuroglia is not all bad, as they participate in restoring homeostasis to injured tissue and the increase in number of astrocytes and microglia could indicate an active attempt to restore the tissue microenvironment to normal. Studies assessing later postpartum time-points are therefore essential to determining whether the neuroinflammation reported at 2 months in this study is persistent, exaggerated over time, or reversed.

The finding of significant changes only in the posterior cortex was surprising since we hypothesized that any cerebral abnormalities will be global in nature. A search of the literature revealed that our findings may be relevant to the condition of posterior cortical atrophy (PCA). PCA is a neurodegenerative disorder affecting primarily the posterior cortex (parietal, occipital, and occipitotemporal cortex (reviewed in (Crutch et al., 2012). Alzheimer's disease is identified as the most common underlying cause and there has been reports that PCA affects predominantly women (Lehmann et al., 2011; Migliaccio et al., 2009; Snowden et al., 2007). Interestingly, a recent study reported smaller gray matter volumes in the posterior cerebrum, especially localized to the occipital lobe in women with a history of preeclampsia and late life hypertension compared to women with a history of preeclampsia and late-life normotension (Raman et al., 2017). Taken together, it is plausible that a history of preeclampsia may be a contributor to PCA and that the placental ischemia model could be used as a unique tool to assess underlying pathophysiology, with inflammation being a likely factor.

There are several limitations to the current study. Firstly, we used a small sample size and although we found several significant findings, a larger sample size may have yielded additional differences. Secondly, we used Carprofen, a non-steroidal anti-inflammatory drug (NSAID) as an analgesic agent before performing the RUPP and sham surgeries. One study (Thau-Zuchman et al., 2012) found reduced water content, cytokines, and microglia in the lesioned cortex in mice following traumatic brain injury after 7 days of Carprofen treatment. While Carprofen use could have dampened the inflammatory response, both groups received the drug, thereby assuring that increases in cytokines are indeed



**Fig. 8.** Summary and Working Hypothesis. (A) Exposure to placental ischemia during pregnancy induces increased pro-inflammatory environment in the posterior cortex and a balanced environment in the hippocampus of rats at 2 months postpartum. (B) Our working hypothesis is that placental ischemia leads to astrocyte and microglia activation beginning in the posterior cortex, leading to increased production and secretion of pro-inflammatory cytokines into the tissue, down-regulation of BBB-associated proteins and subsequent BBB disruption. Increased BBB permeability induces tissue edema, leading to long-term impairments in learning and memory (cognitive function). Gray boxes indicate hypotheses that have not been tested yet.

evident following placental ischemia exposure. Thirdly, although we assessed change in water content and cytokine expression in different brain regions, there is still heterogeneity within the regions. Immunohistological techniques and use of further sub-region analysis (such as CA1, CA3, dentate gyrus within the hippocampus) will provide additional important information about the measured changes.

In summary, we demonstrate that exposure to placental ischemia during pregnancy induces increased pro-inflammatory cytokines in the posterior cortex and not the hippocampus of rats at 2 months postpartum (Fig. 8A). Increased neuroinflammation was associated with increased water content specifically in the posterior cortex and coincided with decreased expression of occludin protein. Whether this tissue inflammation persists or is exacerbated with time following delivery is to be determined. Additionally, whether posterior cortical edema and neuroinflammation result in behavioral changes associated with impaired learning and memory or increased susceptibility to stroke damage will be the focus of future studies. Our simplified working hypothesis is shown in Fig. 8B. Briefly, placental ischemia leads to astrocyte and microglia activation beginning in the posterior cortex, leading to increased production and secretion of pro-inflammatory cytokines into the tissue and down-regulation of BBB-associated proteins and subsequent BBB disruption. Increased BBB permeability induces tissue edema and via other mechanisms lead to long-term impairments in learning and memory (cognitive) function. The placental ischemia model may serve as a model of postpartum cerebrovascular complications and neuroinflammation; and can be used as a tool to assess the underlying mechanisms contributing to the increased risk of cerebrovascular insults in patients with a history of preeclamptic pregnancies and a subset of patients with posterior cortical atrophy.

## Funding

This study was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P20GM104357 and the National Heart, Lung, And Blood Institute of the National Institutes of Health under Award Number P01HL051971, K99HL129192 (JPW), and a diversity supplement grant R25HL121042 awarded to Michael J. Ryan (AMC). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

## Disclosures

The authors have no disclosures

## Author contributions

AMC, QS, NDP, ABG, and JPW performed the experiments, NPD, JPG, and JPW designed the study. AMC, QS, NDP, JPG, ABG, and JPW reviewed and approved the manuscript.

## Acknowledgements

The author would like to thank Marietta Arany and Kathy Cockrell for their technical assistance.

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