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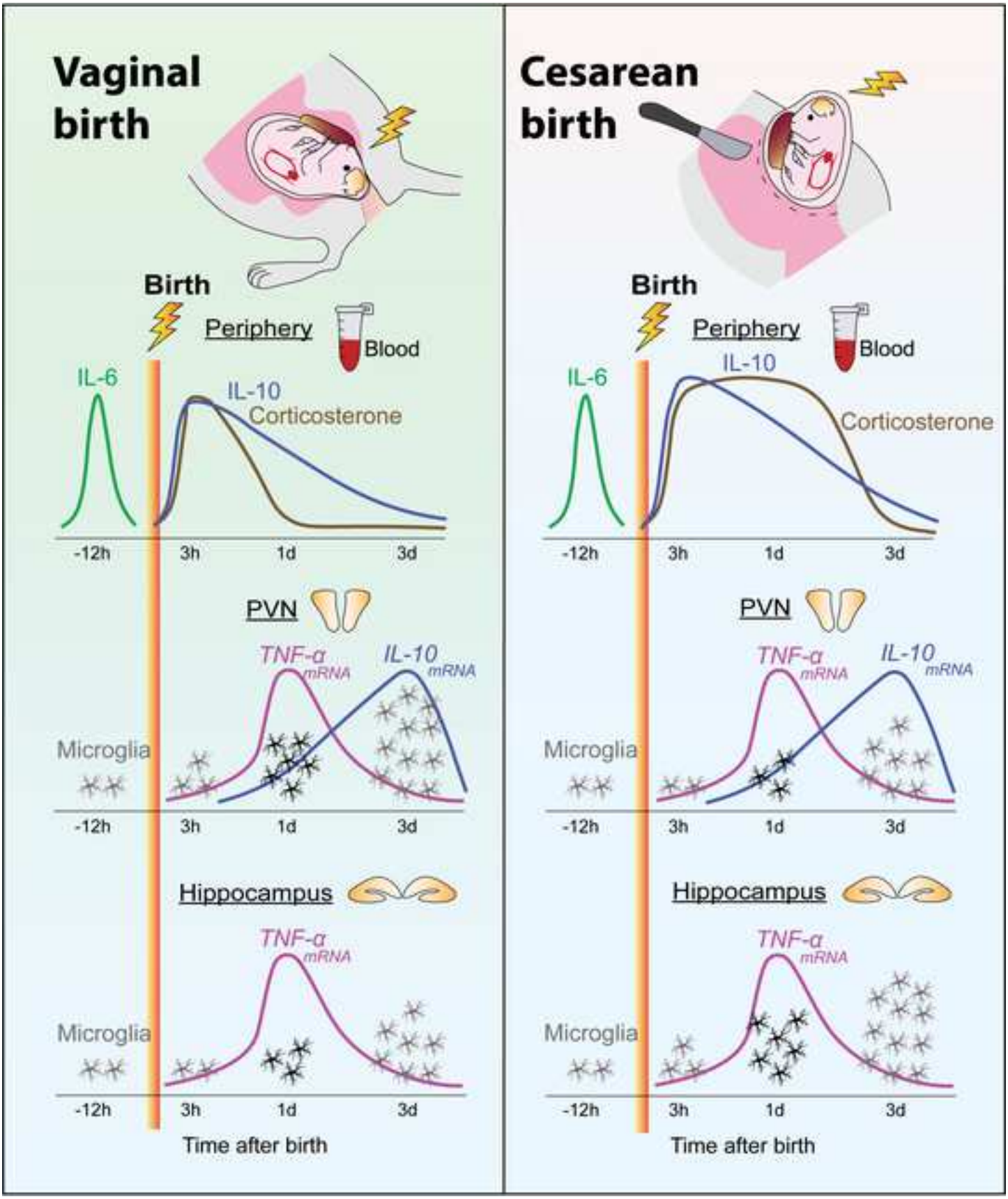

Birth triggers an inflammatory response in the neonatal periphery and brain

Alexandra Castillo-Ruiz[a,*], Carla D. Cisternas[b], Hannah Sturgeon[a], Nancy G. Forger[a]

[a]Neuroscience Institute, Georgia State University, Atlanta, Georgia 30303, USA

[b]Instituto de Investigación Médica Mercedes y Martín Ferreyra INIMEC-CONICET-UNC, Córdoba, Argentina

*Corresponding author:

Alexandra Castillo-Ruiz

PO Box 5030

Atlanta, GA 30302-5030

404-413-5460

Email: acastilloruiz@gsu.edu

Abstract

Birth is preceded by inflammation at the fetal/maternal interface. Additionally, the newborn experiences stimuli that under any other circumstance could elicit an immune response. It is unknown, however, whether birth elicits an inflammatory response in the newborn that extends to the brain. Moreover, it is unknown whether birth mode may alter such a response. To study these questions, we first measured corticosterone and pro- and anti-inflammatory cytokines in plasma of mouse offspring at several timepoints spaced closely before and after a vaginal or Cesarean birth. We found highest levels of IL-6 one day before birth and surges in corticosterone and IL-10 just after birth, regardless of birth mode. We next examined the neuroimmune response by measuring cytokine mRNA expression and microglial number and morphology in the paraventricular nucleus of the hypothalamus and hippocampus around the time of birth. We found a marked increase in TNF-α expression in both brain regions a day after birth, and rapid increases in microglial cell number in the first three days postnatal, with subtle differences by birth mode. To test whether the association between birth and cytokine production or expansion of microglia is *causal*, we manipulated birth timing. Remarkably, advancing birth by a day advanced the increases in all of the markers tested. Thus, birth triggers an immune response in the body and brain of offspring. Our results may provide a mechanism for effects of birth (e.g., acute changes in cell death and neural activation) previously reported in the newborn brain.

Keywords: Iba1, prenatal, neonatal, paraventricular nucleus, CA1 oriens, cytokines, corticosterone, birth mode

1. Introduction

Birth is an extraordinary event for a placental mammal. The fetus experiences hormonal surges, mechanical forces and hypoxia during labor, and an onslaught of microbes and other environmental antigens at delivery. Additionally, the onset of labor depends on a state of "sterile inflammation" in the mother, characterized by infiltration of the uterus and cervix by immune cells and increased proinflammatory cytokine synthesis beginning late in gestation (Golightly et al., 2011; Keelan, 2018; Shynlova et al., 2013a; Thomson et al., 1999; Young et al., 2002). Cytokines are also increased in tissues with more direct access to the fetus, such as the placenta, decidua, and amniotic fluid during late gestation (Keelan, 2018; Osman et al., 2003; Shynlova et al., 2013b; Young et al., 2002), and human neonates exhibit an acute phase reaction, characterized by elevations of plasma interleukin (IL)-6 and C-reactive protein, fever, and other markers typically associated with immune activation (Marchini et al., 2000). These observations suggest that birth is an inflammatory event for the newborn.

Peripheral inflammation is communicated to the brain via several routes (Dantzer, 2018; Hosoi et al., 2002; Silverman et al., 2005), suggesting that the inflammatory events of birth could affect the perinatal brain. Brain regions such as the paraventricular nucleus of the hypothalamus (PVN) and the hippocampus play important roles in coordinating the neural response to an immune challenge and are activated by peripheral inflammation (Elmquist and Saper, 1996; Frenois et al., 2007; Hoogland et al., 2015; Sawchenko et al., 1996; Tarr et al., 2012; Whylings et al., 2021). Even low-grade inflammation activates the PVN (Tarr et al., 2012), and we previously found marked activation of PVN neurons at birth in mice (Hoffiz et al., 2021).

The brain responds to an inflammatory event with increased cytokine production, as well as the proliferation of microglia, which are a major source of brain cytokines (Hanisch, 2002; Hoogland et al., 2015; Perry, 2016; Schwarz et al., 2013; Shankaran et al., 2007); whether birth triggers such a reaction in the brain is unknown.

Activation of the newborn immune system may differ by birth mode in humans. Compared to babies delivered vaginally, for example, those delivered by Cesarean-section have an altered number or function (i.e., response to challenge *in vitro*) of several peripheral immune system cells (Bessler et al., 1998; Hasan et al., 1993; Molloy et al., 2004; Shen et al., 2009; Thilaganathan et al., 1994). Some studies find higher levels of specific cytokines in umbilical cord blood after a vaginal birth compared to an elective Cesarean delivery (Chan et al., 2013; Mir et al., 2018), whereas others report no consistent effect of birth mode on blood cytokines (Denihan et al., 2013; Ly et al., 2006; Nandanan et al., 2019; Werlang et al., 2018). However, it is difficult in human studies to distinguish effects of birth mode, per se, from other aspects of Cesarean delivery that could affect immune functioning, such as birth timing (elective Cesarean sections that are scheduled prior to full term), anesthesia, surgery, and antibiotic treatment of the mother.

Here, we used a mouse model to address these limitations and to probe three questions: 1) are inflammatory markers elevated in the peripheral blood of newborns?; 2) does inflammation experienced at birth extend to the newborn brain?; and 3) do effects of birth on peripheral or central inflammation differ by birth mode? We first measured plasma cytokines at multiple time points during the last three days of gestation and first few postnatal days in mice.

Circulating glucocorticoids, which are released in response to inflammation, and modulate the immune response peripherally and centrally (Ehrchen et al., 2019), were also measured. To determine whether inflammation at birth extends to the brain, we measured cytokine gene expression pre- and postnatally, and quantified microglial number, morphology and staining intensity across perinatal ages in the PVN and hippocampus. Whether peripheral or central inflammation differs by birth mode was tested by comparing offspring born vaginally or by Cesarean section, with Cesarean births yoked to vaginal births, and all animals cross-fostered to carefully control for gestation length, circadian time of parturition, and maternal factors. Finally, we manipulated the *timing* of birth to test two competing hypotheses: that the association of birth with inflammatory markers reflects a developmental program or, alternatively, that birth triggers (i.e., causes) the observed changes.

2. Methods

2.1. Animals

Wildtype C57BL/6J mice were obtained from our breeding program at Georgia State University or purchased from the Jackson Laboratory (Sacramento, CA, USA). Mice were maintained on a 12:12 light-dark cycle with *ad libitum* access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee at Georgia State University and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Timed pregnancies and birth-delivery mode

Timed pregnancies were established by pairing males and females within an hour of lights off and removing the males 1-2h after lights on the next morning; this was considered embryonic day (E) 0. Collection timepoints were spaced closely around the expected time of parturition, i.e., 19.3 days for C57BL/6J mice (Murray et al., 2010). Brains and blood plasma from male and female offspring were collected prenatally on E16.5, E18.5, and E19, or postnatally at 3h, postnatal day (P)1, P3, and at weaning on P23. Sex was determined based on anogenital distance and presence of scrotal pigmentation for all ages analyzed, excepting E16.5 for which these characteristics were not obvious. For prenatal collections, dams (*N*= 8 at E16.5; *N*= 6 at E18.5; *N*= 12 at E19) were exposed to 2% CO_2 and rapidly decapitated. An abdominal incision was made to expose the uterine horns, and fetuses were extruded, decapitated, and their trunk blood and brains collected.

Animals collected at postnatal time points were born vaginally or by Cesarean section. We performed hourly, around-the-clock checks of 50 timed-pregnant dams for births beginning on the eve of E19, with checks during the dark period performed under red light illumination. Cesarean deliveries (*N*= 27) were yoked to vaginal births (*N*= 23), so that when a dam had vaginally delivered one pup, a female showing no signs of labor was randomly selected and a Cesarean section performed as described above. In this way, we controlled for both gestation length and time of day of parturition between birth mode groups. Pups were stimulated to breath by gently prodding the torso with a sterile cotton tip applicator. Pups born vaginally were removed from dams within an hour of birth and, together with Cesarean-born mice, were

placed as a litter in a small artificial nest and held on a heating pad kept at ~ 32°C. Three hours after birth, tissues from a subset of Cesarean- and vaginally-born pups were collected. The remaining pups were removed from the heating pad, tattooed for identification and randomly assigned to foster dams (*N* = 26 dams; 6-8 pups per foster dam), with each foster litter comprising a mixture of vaginally- and Cesarean-born pups.

2.3. Maternal access

To address whether maternal absence right after birth, or cross-fostering, might themselves affect the immune response, we examined immune markers in vaginally-born pups that remained with their mother. Timed-pregnancies were generated and offspring of both sexes were collected prenatally at E18.5 (*N*= 3 dams) and after a vaginal delivery (*N*= 13 dams) at 3-4h, P1, and P3.

2.4. Advanced birth

Although most births in C57BL/6J mice occur at 19 days post-conception (Murray et al., 2010), a range of 18-20 days is considered normal (Donahue, 2012). A separate cohort of timed-pregnancies was established to compare immune activation after *on-time* and *advanced* (early) births, with both groups born within the normal range of gestation. Male and female offspring were collected at E16.5 and E18.5 for prenatal time-points (from *N*= 7 and 4 dams, respectively). Advanced births were generated by performing Cesarean deliveries on E18.5 (*N*=

8), and the remaining dams were allowed to deliver vaginally on-time (*N*= 6). Newborns were collected 3h and 24h after birth, following the procedures described above, including assigning pups older than 3h to foster dams (*N*= 10).

2.5. Cytokine multiplex immunoassay and corticosterone radioimmunoassay

Trunk blood was collected immediately after decapitation using heparinized capillary tubes (Thermo Fisher Scientific, Pittsburg, PA, USA). To fulfill assay sample requirements, we pooled samples as necessary: whole litters for E16.5 and 1-3 offspring of the same sex for other timepoints. We also collected trunk blood from 4 dams on E18.5, for comparison of fetal and dam IL-6 levels. Blood samples were transferred to microcentrifuge tubes on ice and spun at 2300xg for 5 min at 4°C. The plasma layer was collected and stored at -80°C. Cytokine levels were measured by the Advanced Analytics Core at the University of North Carolina, using Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) mouse kits to detect IL-6, IL-1β, IL-10, and tumor necrosis factor (TNF)-α. Corticosterone assessment was performed at the Center for Research in Reproduction at the University of Virginia, using a mouse double antibody ^{125}I radioimmunoassay kit (MP Biomedicals LLC, Orangeburg, NY, USA). Samples were run in duplicate, or as singlets for IL-10 in the maternal access experiment, and values below detection were replaced with the lowest detectable value of the standard curve for the plate.

2.6. Reverse transcription polymerase chain reaction (RT-PCR) for brain cytokine expression

Brains were immediately frozen upon collection and stored at -80°C until processing. PVN and hippocampal punches of 0.8mm inner diameter were taken in a cryostat and homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was precipitated, and concentration and purity determined using standard methods. Reverse transcription was performed with a Superscript IV kit (Invitrogen) in a thermal cycler (Applied Biosystems Inc., Foster City, CA, USA) and real time PCR was performed in the LightCycler 96 System (Roche, Mannheim, Germany) using a FastStart Essential DNA Green Master Kit (Roche) according to the manufacturer's instructions. Primers used were for *IL-6, IL-1β, IL-10, TNF-α*, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; all validated primers from Qiagen Inc., Valencia, CA, USA). We confirmed the efficacy of these primers in an adult mouse via an intraperitoneal injection of the bacterial mimetic lipopolysaccharide, which within 5h boosted the expression of the pro-inflammatory cytokines *IL-1β*, *IL-6*, and *TNF-α* in the brain (not shown). Each reaction was run in duplicate, and negative controls were run for each cytokine. Quantitative cycle (Cq) values from duplicate samples were averaged, and cytokine expression relative to expression of the control gene (GAPDH) was calculated for each animal using the Pfaffl method (Pfaffl, 2001). Fold-change values were obtained by dividing each experimental value by the average value for the E16.5 or E18.5 group.

2.7. Immunohistochemistry

Brains were fixed in 5% acrolein for 24h and transferred to 30% sucrose followed by cryoprotectant solution until sectioning. Brains were frozen-sectioned coronally into four series at 40 µm. Sections were collected into cryoprotectant solution and stored at -20°C. Two non-consecutive series were processed for the immunohistochemical detection of ionized calcium binding adaptor molecule 1 (Iba1). Unless otherwise stated, tissue was washed between steps in 1X tris buffered saline (TBS) and all steps were carried out at room temperature. Epitope retrieval was performed with 0.05 M sodium citrate for 1h and unreacted aldehyde blockade with 0.1 M glycine for 30 min. Tissue was then incubated in a blocking solution (20% normal goat serum (NGS), 1% H_2O_2, 0.3% Triton X in TBS), followed by an overnight incubation with the primary antibody: rabbit anti-Iba1 (Wako, Chuo-Ku, Osaka, Japan; 1:3,000-1:10,000 (held constant within experiment); 2% NGS, 0.3% Triton X in TBS). Sections were washed in a dilute blocking solution (1% NGS, 0.02% Triton X in TBS), incubated for 1h in a goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:1,000; 0.32% Triton X in TBS), washed in 1X TBS-0.2% Triton X, and incubated for 1h in an avidin-biotin solution (Vector Laboratories; 1:1,000 in 1X TBS). Tissue was washed in acetate buffer and incubated in 0.02% diaminobenzidine tetrahydrochloride, 2% nickel sulfate, and 0.0025% H_2O_2 made in the same buffer. Sections were mounted onto gelatin-coated slides, dehydrated, and coverslipped.

2.8. Brain regions examined and quantification

For the evaluation of microglial numbers, phenotype and staining intensity, slides were scanned using a Hamamatsu Nanozoomer slide scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and Iba1 quantification was performed with Aperio Image Scope software (Leica Biosystems Inc., Buffalo Grove, IL, USA). Contours were drawn around areas of interest, and the number and, if applicable, microglial cell phenotype (ameboid, stout, transitioning, or ramified, as previously described in Castillo-Ruiz et al., 2018a; Strahan et al., 2017) within each contour was recorded. Iba1+ cells were counted only when the soma was clearly visible within the section. The sum of Iba1+ cells across all sections per animal was divided by total area sampled, and then multiplied by section thickness to obtain the density of Iba1+ cells per mm^3. Although microglia constitutively express Iba1, its expression is increased in response to activation/inflammation (Ito et al., 1998; Ito et al., 2001). Therefore, we also evaluated average microglial staining intensity using the pixel count algorithm in Aperio Image Scope by dividing the staining intensity of Iba1+ positive pixels by the number of Iba1+ pixels. All analyses were performed by investigators blinded to experimental condition.

Microglial counts were performed bilaterally in two alternate sections of the PVN and CA1 oriens layer of the dorsal hippocampus. Microglial staining intensity was evaluated bilaterally throughout the PVN and in alternate sections of the dorsal CA1 oriens. For the PVN, our sampling started where the nucleus has a long-tubular shape (Plate 64 and 65; Paxinos et al., 2007), and for the CA1 oriens, where the dentate gyrus makes its rostral-most appearance (Plate 65 and 67; Paxinos et al., 2007).

2.9. Statistics

We combined the data for males and females in all analyses below because we found no significant effects of sex for any of the variables assessed (note that the effect of sex was not tested for E16.5 as sex was not determined at this age, see Section 2.2). One-way ANOVAs were used to evaluate effects of age on cytokine levels (mRNA and protein) as well as microglial numbers and staining intensity when examining birth modes separately. Two-way ANOVA was used to evaluate birth mode- or birth timing-by-age effects on the dependent variables described above. For the analysis of microglia phenotype, we used a mixed design ANOVA with age as the between-subjects factor and cell phenotype as a within-subjects factor. When applicable, ANOVA was followed by Fisher's least significant difference. Non-parametric tests (Kruskal-Wallis or Dunn's tests) were performed when data did not conform to homogeneity of variance.

3. Results

Figures depict results for each birth mode separately to illustrate the analysis of age-related changes, and combined, to directly compare results by birth mode.

3.1. Birth is associated with dynamic changes in peripheral cytokine and corticosterone levels

We first evaluated the association of birth with anti- (IL-10) and pro-inflammatory (IL-6, TNF-α, IL-1β) cytokines in plasma. Animals were collected prenatally during the last few days of

gestation, and postnatally after a vaginal- or Cesarean delivery. IL-10 tripled within 3h of birth, remained elevated at P1, and returned to prenatal levels by P3, which was reflected in a significant main effect of age for both vaginally- and Cesarean-born pups (H_7= 61.45, p< 0.0001; H_7= 56.99, p< 0.0001, respectively) (Fig. 1A,B). Across all postnatal time-points, however, levels were slightly (~20%) higher following a Cesarean birth ($F_{1,102}$= 4.71, p= 0.03) (Fig. 1C).

In contrast, IL-6 levels peaked one day before birth (E18.5), before dropping to very low levels by P1 (main effect of age for vaginal birth: H_7= 48.91, p< 0.0001; Cesarean birth: H_7= 48.92, p< 0.0001) (Fig. 1D,E), and did not differ by birth mode (Fig. 1F). To test whether the elevation of IL-6 in offspring was related to heightened maternal levels, we compared levels in fetuses and dams at E18.5 and found that IL-6 levels were 7-fold higher in fetuses (13.9 ± 3.3 pg/mL vs. 1.9 ± 0.3 pg/mL for fetuses (N=3) and dams (N=4), respectively; p= 0.001).

Plasma levels of TNF-α and IL-1β did not change during the perinatal period: TNF-α was elevated at all perinatal ages tested in comparison to weaning age (main effects of age for vaginal birth: H_7= 44.07, p< 0.0001; Cesarean birth: H_7= 23.31, p= 0.0007) (Fig. 1G,H) and IL-1β did not show any effect of age (vaginal birth: H_6= 6.42, p= 0.27; Cesarean birth: H_6= 11.61, p= 0.04 but post-hoc comparisons were not significant for any timepoint) (Fig. 1J,K). Although overall levels and patterns of TNF-α and IL-1β were similar in animals born vaginally or by Cesarean section, there was an interaction between birth mode and age for both cytokines ($F_{3,99}$= 4.43, p= 0.006; $F_{3,86}$= 3.53, p= 0.02, respectively), with Cesarean-born mice having lower levels of TNF-α at P3 (Fig. 1I) and higher levels of IL-1β at weaning (Fig.1L). Thus, birth was

associated with a prenatal elevation of IL-6, followed by a postnatal peak of plasma IL-10, and this was true regardless of birth mode.

Glucocorticoids, including corticosterone, modulate the immune system and can upregulate the production of IL-10 (Ehrchen et al., 2019; Marchant et al., 1996; Mozo et al., 2004), which in our study tripled 3h after birth. We therefore evaluated the association of birth with plasma corticosterone levels between E16.5 and P3 in mice born vaginally or by Cesarean section. There was a main effect of age on corticosterone levels ($F_{4,24}$= 37.97, p< 0.0001) (Fig. 2A) in vaginally-born mice, with an overall decline between late prenatal and early postnatal ages. These findings are consistent with the decrease in baseline glucocorticoid production that characterizes the stress hypo-responsive period in perinatal rats and mice (Dupouy et al., 1975; Montano et al., 1991; Schmidt et al., 2003). However, a surge in corticosterone 3h after a vaginal birth was superimposed upon this pattern of declining levels (Fig. 2A) and a nearly identical surge in corticosterone was seen in Cesarean-born mice at 3h (p> 0.99 for vaginal vs. Cesarean at 3h) (Fig. 2B). There was, nonetheless, a significant effect of birth mode ($F_{1,30}$= 7.19, p= 0.012) and birth mode-by-age interaction ($F_{2,30}$= 3.92, p= 0.03) on postnatal corticosterone levels, due to significantly higher corticosterone in Cesarean-born mice at P1 (p= 0.002) (Fig. 2C). Thus, despite the perinatal stress hypo-responsive period, birth causes an acute surge in corticosterone, and a Cesarean birth may prolong this surge.

3.2. Birth is associated with dynamic changes in cytokine expression in the brain

To determine whether the brain mounts an inflammatory response in relation to birth, and whether this response differs by birth mode, we examined cytokine gene expression in punches of the PVN area and hippocampus. These two brain regions are highly responsive to birth, as determined by neural activation and acute changes in cell death in our previous studies (Castillo-Ruiz et al., 2020; Castillo-Ruiz et al., 2018b; Hoffiz et al., 2021; Mosley et al., 2017). In addition, the PVN and hippocampus play key roles in the brain's response to stress and immune challenges, and are highly responsive to stress hormones, including glucocorticoids (Herman et al., 2016; Sapolsky, 1996; Wrona, 2006).

In the PVN, *IL-10* expression varied significantly by age (vaginal birth: H_7= 27.91, p< 0.0001; Cesarean birth: H_7= 35.41, p< 0.0001), with greatest expression on P3 (Fig. 3A,B), and a nearly identical pattern in vaginally- and Cesarean-delivered pups ($F_{1,77}$= 0.07, p= 0.79) (Fig. 3C). *IL-6* expression in the PVN differed by age only in vaginally-born pups (H_7= 15.93, p= 0.01). There appeared to be a modest elevation in *IL-6* at 3h postnatal in the vaginal group, but these levels did not differ significantly from prenatal timepoints (Fig. 3D), and across all ages, *IL-6* expression in the PVN did not differ by birth mode ($F_{1,81}$= 0.60, p= 0.44) (Fig. 3F). The most striking finding was a tripling of *TNF-α* expression on P1 relative to prenatal values (vaginal birth: H_7= 41.08, p< 0.0001; Cesarean birth: H_7= 38.54, p< 0.0001) (Fig. 3G,H), with a nearly identical increase for both birth modes ($F_{1,83}$= 0.93, p= 0.34). Thus, the PVN was characterized by a peak in pro-inflammatory *TNF-α* on P1, followed by elevated anti-inflammatory *IL-10* on P3, irrespective of birth mode.

The hippocampus did not exhibit the striking peak of *IL-10* expression seen in the PVN on P3, but expression at all perinatal ages was elevated in comparison to weaning age, and this was true for both birth modes (Fig. 4A-C). Hippocampal *IL-6* expression was highest at 3h postnatal in vaginally-delivered mice (main effect of age: H_7= 16.58, p= 0.01), although 3h did not differ significantly from other perinatal timepoints (Fig. 4D), and direct comparisons between birth modes revealed a significant interaction between birth mode and age ($F_{3,73}$= 3.07, p= 0.03), with Cesarean-born mice having lower *IL-6* expression at 3h and higher expression at P1 (Fig. 4F). Strikingly, we again found a tripling of *TNF-α* on P1 (vaginal birth: H_7= 45.18, p< 0.0001; Cesarean birth: H_7= 41.17, p< 0.0001), with no difference between birth modes ($F_{1,85}$= 0.06, p= 0.81) (Fig. 4I), as was seen for the PVN. The spike in *TNF-α* on the day after birth may in fact be widespread, as *TNF-α* expression was also elevated at P1 compared to P0 in whole forebrain samples in a follow-up analysis (Supplementary Fig. 1).

Results for *IL-1β* expression in the PVN and hippocampus were not interpretable due to extremely variable signal between biological replicates, and therefore we refrain from presenting and interpreting those data here.

3.3. Changes in cytokine and corticosterone production associated with birth are independent of maternal access

We found above that birth, regardless of mode, is associated with a rapid surge in IL-10 and corticosterone peripherally, and an increase in *TNF-α* in the PVN and hippocampus centrally. However, because our experimental design involved placing newborn pups on a

heating pad for 3h right after birth, followed by cross-fostering, it was possible that our findings were caused by maternal absence immediately postpartum or the cross-fostering manipulation. To test this possibility, we examined the same immune markers in a separate cohort of mice with continuous access to their biological mother. Offspring were collected prenatally at E18.5 or postnatally at 3-4h, P1, and P3 after a vaginal delivery.

As before, IL-10 and corticosterone levels in the periphery peaked within hours of birth (H_4= 21.65, p< 0.0001; $F_{2,23}$= 4.56, p= 0.02, respectively) (Fig. 5A-B). Moreover, we again found a marked elevation in *TNF-α* gene expression on P1 in both the PVN and hippocampus (H_4= 14.99, p= 0.002; $F_{3,21}$= 30.09, p< 0.0001, respectively) (Fig. 5C-D). Thus, these findings replicate the increases in all markers seen above, and demonstrate that the immune responses occur whether or not the pup remains with its mother. Quantitative comparisons between this experiment and those shown above (Sections 3.1 & 3.2) must be made with caution, as the assays were run separately, and many months apart. Nonetheless, the fold-change in IL-10 in the current experiment was larger, and the corticosterone response more subtle, than what was seen above. In contrast, the fold-changes in central *TNF-α* expression were very similar across experiments.

3.4. Rapid postnatal increases in microglial numbers and staining intensity

Microglia are the resident immune cells and main producers of cytokines in the brain, and they also express receptors for cytokines (Harry, 2013) and glucocorticoids (Sierra et al., 2008; Tanaka et al., 1997). Thus, microglia are well-positioned to contribute to and/or respond

to the changes in inflammatory markers reported here. Previous developmental studies have shown an increase in microglial number during the first two postnatal weeks (Dalmau et al., 2003; Sharaf et al., 2013), although a fine-grained analysis centered on the few days before and after birth has not been reported. An effect of birth mode on microglia has also not been explored. Thus, we examined microglial number, morphology, and Iba1 staining intensity in the PVN and the hippocampus at two prenatal timepoints (E18.5 and E19), and at three postnatal timepoints (3h, P1, and P3) after a vaginal or Cesarean birth. Within the hippocampus, we focused on the CA1 oriens layer, because this region in particular shows microglia-dependent changes in cell death in the neonatal brain (Jacobs et al., 2019).

Microglial numbers increased significantly in both the PVN (vaginal birth: $F_{4,50}$= 16.40, p< 0.0001; Cesarean birth: $F_{4,46}$= 7.49, p< 0.0001) and CA1 oriens (vaginal birth: $F_{4,49}$= 14.84, p< 0.0001; Cesarean birth H_5= 29.90, p< 0.0001) between E18.5 and P3 (Fig. 6). For vaginally-born mice, there was a significant increase in microglial number between P0 (3h) and P1, and another significant increase between P1 and P3 in both brain regions (Fig. 6E,L). The increase in the hippocampus was especially striking, with more than a doubling in the number of microglia between P0 and P3 (Fig. 6L). Such rapid increases in microglial cell number immediately after birth may be region specific, as the suprachiasmatic nucleus of the hypothalamus, for example, did not show perinatal changes in microglial numbers (Supplementary Fig. 2). The pattern of changes in microglial cell number was similar for Cesarean-born mice, although direct comparisons between birth modes revealed that Cesarean-born mice had fewer (9%) microglia in the PVN ($F_{1,54}$= 9.90, p= 0.003) and more (16%) in the CA1 oriens ($F_{1,63}$= 5.16, p= 0.03) across all postnatal ages (Fig. 6G,N).

Microglia can rapidly change morphology in response to immune activation, with ameboid and stout microglia representing more activated states than transitioning or ramified types. We therefore also examined the phenotype of the microglia in the PVN and CA1 oriens. As previously reported for the perinatal mouse brain (Castillo-Ruiz et al., 2018a; Strahan et al., 2017), most microglia were stout or transitioning (Supplementary Fig. 3 and 4). We also found significant phenotype-by-age interactions for both brain regions and birth modes (vaginal birth: PVN: $F_{12,150}$= 3.81, p< 0.0001; CA1 oriens: $F_{12,147}$= 3.39, p= 0.0002; Cesarean birth: PVN: $F_{12,138}$= 2.06, p= 0.02; CA1 oriens: $F_{12,144}$= 8.81, p< 0.0001), due to rapid increases in the number of stout and transitioning microglia between E19 and P3 (Supplementary Fig. 3 and 4). Direct comparisons of microglial phenotypes between birth modes revealed no significant differences, with the exception of stout microglia being more numerous in vaginally-born mice in the PVN ($F_{1,54}$= 9.90, p= 0.003) and more numerous in Cesarean-born mice in the CA1 oriens ($F_{1,63}$= 5.16, p= 0.03). Thus, the birth mode effects reported above for microglial number in both regions (Fig. 6G,N) were due to changes in stout microglia.

Although the microglial marker Iba1 is constitutively expressed, it is upregulated following immune activation and density of Iba1 immunoreactivity has been used previously as a proxy for microglial activation (Bosco et al., 2011; Ito et al., 1998; Ito et al., 2001; Qin et al., 2007). We therefore calculated the Iba1 staining intensity of microglia at each age. In the PVN, we found darker Iba1 staining throughout E18.5-P1 in comparison to P3, as reflected in a main effect of age (vaginal: $F_{4,48}$= 6.95, p= 0.0002; Cesarean: $F_{4,47}$= 8.13, p< 0.0001) and a significant elevation between 3h and P1 in vaginally-born pups (Fig. 7). In the CA1 oriens, we again found main effects of age (vaginal: $F_{4,51}$= 5.30, p= 0.001, Cesarean: $F_{4,51}$= 3.54, p= 0.01) with darkest

Iba1 staining at P1 (Fig. 7; also captured in photomicrographs shown in Fig. 6). A direct comparison between vaginally- and Cesarean-delivered pups revealed that the patterns of Iba1 staining intensity were very similar by birth mode (Fig. 7).

3.5. Birth causes the postnatal immune responses in the periphery and brain

The findings above showed that birth is associated with a surge in IL-10 peripherally, and an increase in *TNF-α* and microglia numbers in the PVN and hippocampus centrally. However, the data were correlational and we could not rule out the possibility that postnatal changes in cytokines and microglia are developmentally programmed and only coincidentally related to birth. To test this, we manipulated birth timing. Specifically, we compared IL-10 levels peripherally and *TNF-α* expression centrally, as well as microglia numbers in offspring born "on time" or one day early ("advanced birth"). We reasoned that if birth *causes* the elevations in cytokines and microglia numbers seen previously, then they should be elevated at 3h (IL-10 in the periphery) or 24h (*TNF-α* expression and microglia numbers in the brain) following an early birth (Fig. 8A, top). Alternatively, if these patterns are developmentally programed, then advancing the timing of birth would not advance the immune responses (Fig. 8A, bottom). Because a Cesarean birth did not change the overall pattern of these cytokines (Fig. 1C, 3C, 4C) or microglia (Fig. 6G,N), we generated the advanced birth group via Cesarean section on E18.5. Hormonal manipulations (e.g., prostaglandin and steroid injections) can also be used to generate early births, but were not suitable here as these also have immune effects. We collected mice at E16.5 and E18.5, and postnatally at 3h and 24h after either an advanced

(E18.5) or “on time” (born ~E19.3) birth. For analyses of IL-10, we included data from control animals on E19 from our first experiment (Fig. 1) for visual and statistical comparisons. This was possible due to the consistency in IL-10 results between runs, as evidenced by the values obtained for ages common to both runs ($F_{1,73}$= 0.04, p= 0.85).

IL-10 levels were again quite low prenatally, and very similar to those seen previously (Fig. 1). Using two-way ANOVA (birth timing-by-age) to compare values 3h and 24h after an on-time or advanced birth, we found a significant effect of age in the predicted direction on all tested variables. Specifically, we replicated the sharp increases of IL-10 in plasma at 3h after birth ($F_{1,34}$= 35.72, p< 0.0001) (Fig. 8B), and *TNF-α* expression centrally at 24h (P1) in both the PVN ($F_{1,34}$= 50.42, p< 0.0001) and hippocampus ($F_{1,33}$= 88.48, p< 0.0001) (Fig. 8C,D). The increase in microglial numbers in the PVN and hippocampus between birth and P1 was also replicated in the new cohort of animals (PVN: $F_{1,48}$= 19.52, p< 0.0001; CA1 oriens: $F_{1,48}$= 11.74, p= 0.001) (Fig. 8E,F). Importantly, all measures were advanced by an early birth, indicating that birth is causal for these changes.

4. Discussion

Associations between birth/birth mode and peripheral immune markers have been previously reported in the human clinical literature but, due to limitations inherent in those studies, causation could not be determined. Moreover, no previous study addressed whether birth triggers an immune response in the brain. Using a fined-grained temporal analysis of changes in inflammatory markers, we found that birth is associated with an immune response

in the newborn that is first observed in the periphery and later extends to the brain. In addition, when controlling for gestation length, time of day of delivery, and maternal factors, the overall inflammatory response was similar between animals born vaginally or by Cesarean section, suggesting that labor and a vaginal delivery are not required. By manipulating the timing of parturition, we find that birth indeed *causes* a reproducible inflammatory response in the newborn periphery and brain.

4.1. The peripheral inflammatory response to birth

Peripheral immune markers have previously been reported in newborns. On the day of birth, for example, healthy human newborns experience elevated IL-6 levels that may be as high as those seen in older children with a bacterial infection (Lusyati et al., 2013; Marchini et al., 2000). The inflammatory response we observed in the periphery of mice consisted of an elevation in IL-6 one day prior to birth (E18.5), followed by marked increases of IL-10 and corticosterone at 3h postnatal. Because IL-6 may cross the placenta (Dahlgren et al., 2006), and is elevated in the plasma of mouse dams prior to birth (Orsi et al., 2007; Orsi et al., 2006), it was possible that the mother was the source of the high IL-6 levels observed in near-term fetuses. We found, however, that IL-6 levels were 7-fold higher in fetuses than in dams on E18.5. This underscores the magnitude of the fetal elevation and suggests that the source of IL-6 is the fetus itself, although it is possible that there is also a contribution from the localized inflammation in the maternal/fetal compartments just prior to labor (Keelan, 2018; Shynlova et al., 2013a; Shynlova et al., 2013b).

The anti-inflammatory cytokine IL-10 often increases after an inflammatory event, presumably to limit inflammation. Indeed, IL-10 was sharply elevated at 3h postnatal in the plasma of vaginally-born pups, and this pattern was replicated in pups delivered by Cesarean section. The source of IL-10 is likely to be the newborn itself. IL-10 has a short half-life (Le et al., 1997), making it unlikely that levels measured postnatally in pups originate from the mother. In addition, although milk contains IL-10 (at least in humans; Garofalo, 2010), the high levels of IL-10 in Cesarean-born mice, which had no opportunity to nurse prior to the 3h tissue collection, argues against that possibility.

Corticosterone also exhibited a surge at 3h postnatal, which could be a response to the many stressors that accompany birth. Because glucocorticoids upregulate IL-10 (Ehrchen et al., 2019; Marchant et al., 1996; Mozo et al., 2004) and downregulate pro-inflammatory cytokines, including IL-6 (Keelan et al., 1997; Tobler et al., 1992; Waage et al., 1990; Williams and Coleman, 1995), it is possible that the pattern of cytokines seen at 3h after birth (declining IL-6 and high IL-10) are driven by the corticosterone surge. Moreover, the overall slightly higher levels of corticosterone in Cesarean-born mice may explain the concomitantly higher levels of IL-10 in these mice.

Elevated levels of glucocorticoids following birth have been reported in humans (reviewed in Evers and Wellmann, 2016) and rodents (Cohen, 1976; Dalle et al., 1978; Holt and Oliver, 1968) although, to our knowledge, no previous study has included multiple pre- and postnatal time points to fully characterize the response. Here, we see that the increase in corticosterone is restricted to the immediate post-partum period, with plasma levels dropping

rapidly between 3h and 1 day postnatal in vaginally-delivered offspring. Rodents exhibit a stress hypo-responsive period, characterized by low basal levels of glucocorticoid production and diminished response to stressors during the first two postnatal weeks (Sapolsky and Meaney, 1986; Schmidt, 2019; Schmidt et al., 2003), and our finding of very low corticosterone levels on P1 and P3 is consistent with these observations. One hypothesis posits that the stress hypo-responsive period evolved to protect the developing brain from potentially harmful effects of glucocorticoids (Sapolsky and Meaney, 1986), such that only life-threatening situations override the suppression of the postnatal stress response (Schmidt, 2019). If so, our findings suggest that birth is on par with those extreme situations. Interestingly, the elevation in corticosterone was slightly protracted (through P1) in pups delivered by Cesarean section, suggesting that a Cesarean birth prolongs activation of the stress response in mice.

Although levels of TNF-α and IL-1β did not show acute changes during the perinatal period, it is noteworthy that TNF-α levels were elevated throughout all perinatal timepoints in comparison to weaning age. Birth mode had age-dependent effects on TNF-α and IL-1β levels, with Cesarean-born mice showing lower TNF-α at P3 and higher IL-1β at P23. In contrast, IL-10 levels were slightly elevated in Cesarean-born offspring throughout all ages tested. Of note, Cesarean-born adult humans also show elevated IL-1β and IL-10 at baseline (i.e., in the absence of any challenge; Dinan et al., 2022), although the functional significance of these differences remains to be determined. The perinatal elevations we see in IL-6, IL-10, and TNF-α may relate to developmental functions that go beyond their immune roles, as these cytokines, for example, participate in embryogenesis and organogenesis (Jaskoll et al., 1994; Wooldridge and Ealy, 2019; You et al., 2021).

4.2. The brain immune response to birth

The PVN and hippocampus are both activated after a peripheral inflammatory challenge (Elmquist and Saper, 1996; Frenois et al., 2007; Sawchenko et al., 1996; Tarr et al., 2012) and showed a robust increase in *TNF-α* expression starting one day after birth. This was followed by increased *IL-10* expression in the PVN on P3. Moreover, nearly identical patterns of *TNF-α* and *IL-10* expression were seen in vaginally- and Cesarean-born offspring. An elevation of *TNF-α* mRNA is a common brain response to peripheral inflammation (Hoogland et al., 2015), and the 3-fold increase in *TNF-α* expression that we observed is comparable to increases in *TNF-α* mRNA or protein previously reported in the brains of rodents after peripheral injection with lipopolysaccharide (Bossu et al., 2012; Qin et al., 2007; Shin et al., 2019; Staikos et al., 2008). This suggests that the neuroinflammatory response to birth is commensurate with the response to a bacterial challenge in this respect.

Overall, birth mode did not affect brain cytokine expression, with the exception of *IL-6* expression in the hippocampus, which was reduced in Cesarean-born mice at 3h and elevated at P1, relative to vaginally-born controls.

Peripheral inflammation is communicated to the brain via several routes, including the vagus nerve, cytokine transport across the blood brain barrier, and cytokine action at circumventricular organs (Dantzer, 2018). The colonization of the body by the microbiota may contribute to the systemic immune response at birth (Lotz et al., 2006; Marchini et al., 2000), and the vagus nerve is well-positioned to broadcast such information to the brain (Gars et al.,

2021). We previously showed that neonatal mice born into germ-free conditions have blunted expression of pro-inflammatory cytokines in the brain, including an 11-fold reduction of *TNF-α* expression compared to conventionally colonized mice (Castillo-Ruiz et al., 2018a). Thus, the microbiota may be an important contributor to the brain's immune response to birth. Other stimuli associated with birth, such as hypoxia, may also affect *TNF-α* gene expression in the brain (Kaur et al., 2013; Silverstein et al., 1997; Szaflarski et al., 1995; Wang et al., 2008).

Inflammatory cytokine production can be triggered in the brain within 2-3h after systemic lipopolysaccharide treatment (Ghosh et al., 2014; Sharma et al., 2018), yet there was a two-day lag (E18.5 to P1) between elevations of peripheral (IL-6) and brain (*TNF-α*) inflammatory cytokine production observed here. This may be due to neuroprotective signals such as vasopressin (Hoffiz et al., 2021; Spoljaric et al., 2017) and oxytocin (Tyzio et al., 2006), which show massive elevations at birth and can inhibit cytokine production following immune challenges (Jan et al., 2017; Kingsbury and Bilbo, 2019; Park et al., 2015).

Cytokines are often associated with their inflammatory roles, but they also function in normal brain development. For example, IL-6 participates in gliogenesis, and TNF-α in neuronal differentiation, cell death and synaptogenesis (Bernardino et al., 2008; Bilbo and Schwarz, 2012; Deverman and Patterson, 2009; Kaneko et al., 2008; Nakanishi et al., 2007; Sedel et al., 2004; Zhang et al., 2006). In addition, neural development is altered in mice with whole-body deletions of the genes for *IL-6*, *IL-10* or *TNF-α* (Barker et al., 2001; Golan et al., 2004; Pratt et al., 2013). Thus, the perinatal elevations in cytokines that we observed may play important roles in the neonatal brain. In fact, if inflammation normally accompanies a mammalian birth, it would

be surprising if immune signals had not been co-opted by evolution to shape neurodevelopment.

Although other cell types contribute (Loram et al., 2012; Semple et al., 2010), microglia are the main producers of cytokines in the brain (Schwarz et al., 2013; Williamson et al., 2011) and may be the source of the brain cytokine expression reported here. The increase in microglial numbers (especially of the stout and transitioning types) observed starting a day after birth in both the PVN and CA1 oriens may support the postnatal increases in cytokine production. Birth mode slightly affected these increases in a region-dependent manner across the ages tested, with the PVN and CA1 oriens of Cesarean-born mice experiencing either a smaller or a larger increase in microglia number, respectively, in comparison to vaginally-born animals. Whether these differences amount to changes in microglial function remains to be addressed.

Microglial staining intensity was highest on the day after birth in the PVN and CA1 oriens, regardless of birth mode. Because Iba1, the marker used to identify microglia, is upregulated following immune challenges (Ito et al., 1998; Ito et al., 2001), our results may indicate increased microglial activation at the time of birth, at least in the brain regions examined. Interestingly, the increase in Iba1 staining coincided with peak TNF-α levels in both regions. Thus, microglia may be the source of TNF- α in newborns, as has been shown at later ages (Hanisch, 2002; Raffaele et al., 2020).

4.3. Birth as a trigger of peripheral and central immune responses

The sharp increases in cytokines (IL-10 in plasma and *TNF-α* in brain) and microglial number within the first day post-partum argued for a causal role for birth in triggering these changes. Alternatively, increases in immune markers may have been caused by a developmental program. To distinguish between these hypotheses, we compared postnatal immune markers in offspring born "on time" or in which birth was advanced by a day. We again found increases in IL-10 peripherally, and *TNF-α* and microglial numbers centrally in the control animals, replicating our earlier observations. Importantly, all of these measures were advanced by an early birth, demonstrating that birth indeed *causes* the immune responses in the periphery and the brain. Together with our previous finding that an early birth advances neuronal cell death patterns (Castillo-Ruiz et al., 2020), these findings position birth as an orchestrator of brain development.

4.4. Limitations of the study

We focused on several key cytokines that comprise the canonical response to inflammation, but there are many more cytokines, chemokines, and other inflammatory markers that were not examined, and that may be regulated by birth. Similarly, while we saw only minor differences between offspring born vaginally versus by Cesarean section, there may be effects of birth mode on inflammatory markers that we did not assess. In support, cytokines can induce cell death and/or neuroprotection (Correale and Villa, 2004; Fontaine et al., 2008; McAdams and Juul, 2012), and we previously found that a vaginal but not a Cesarean birth is

followed by an acute decrease in cell death across the brain (Castillo-Ruiz et al., 2018b). We measured mRNA expression of cytokines in the brain in order to avoid contamination from any peripheral cytokines that may have gained access to the brain. However, we cannot assume that the patterns of protein production in the brain would be identical to what we report here for mRNA. Although, in support, a bacterial challenge doubles *TNF-α* mRNA and protein expression in the hippocampus within a few hours (Walker et al., 2020). In the future it would be of interest to have parallel measures of cytokine mRNAs and proteins in brain.

5. Conclusions

We found that birth triggers a robust and reproducible inflammatory response in the newborn periphery and brain, regardless of delivery mode or maternal presence/absence during the first few hours after birth. Although maternal immune activation has been linked to negative neurodevelopmental consequences for the fetus (Bergdolt and Dunaevsky, 2019; Bilbo et al., 2018; Estes and McAllister, 2016), the immune system also plays important roles in normal brain development. The data presented here identify the time-course and specific cytokines to target in future functional studies that examine the effect of preventing or perturbing the inflammatory response to birth. Previously, we reported that birth causes acute changes in brain activation and neuronal cell death (Castillo-Ruiz et al., 2020; Castillo-Ruiz et al., 2018b; Hoffiz et al., 2021); the immune response to birth may be a mechanism underlying these, and other, effects of birth on the neonatal brain.

Declaration of competing interest

The authors have nothing to disclose.

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Figure captions

Figure 1. Birth is associated with dynamic changes in peripheral cytokines, regardless of delivery mode. While IL-10 plasma levels tripled 3h after birth (A-C), IL-6 levels were elevated a day before birth (D-F). In contrast, levels of TNF-α (G-I) and IL-1β (J-L) did not change during the perinatal period. These cytokine patterns were observed whether pups were born vaginally or by Cesarean-section (left and middle columns), although IL-10 was slightly elevated in Cesarean-born mice across all ages tested (C). In addition, Cesarean-born mice had lower TNF-α at P3 (I) and higher IL-1β at P23 (L). IL-1β values at E16.5 were not interpretable and therefore are not included. Letters denote effects of age within birth modes separately, such that group means with different letters are significantly different from each other. The right column provides direct comparisons between birth modes, with asterisks above brackets representing a main effect of birth mode (**p*= 0.03) and above symbols a significant post-hoc comparison (following a significant interaction) for single timepoints (***p*= 0.02; ****p*= 0.0005). Dashed red lines indicate timing of birth. Data are mean ± SEM. Note that in some cases error bars are smaller than symbols. *N*= 9-17 per group, except for E16.5 (*N*= 5; pooled litters), E18.5 (*N*= 21) and vaginally-delivered at P23 (*N*= 23).

Figure 2. A vaginal birth was associated with an acute surge in corticosterone (A), and a Cesarean birth prolonged this surge (B). Letters denote effects of age within birth modes separately, such that group means with different letters are significantly different from each other. Asterisks denote direct comparisons between birth modes (C), with the asterisk above

the bracket representing a main effect of birth mode (*p= 0.01) and above a symbol, a significant post-hoc comparison (following a significant interaction) for a single timepoint (***p= 0.0007). Dashed red lines indicate timing of birth. Data are mean ± SEM. Note that in some cases error bars are smaller than symbols. N= 5-6 per group.

Figure 3. Birth is followed by increased *TNF-α* and *IL-10* expression in the PVN, regardless of delivery mode. *IL-10* expression peaked on P3 (A-C) and *IL-6* showed a modest elevation at 3h postnatal (D-F). In contrast, a sharp increase in *TNF-α* expression occurred on P1 (G-I). These patterns were observed in both vaginally- and Cesarean-born mice (left and middle columns). Letters denote effects of age within birth modes separately, such that group means with different letters are significantly different from each other. Data are expressed relative to cytokine levels at E16.5. Dashed red lines indicate timing of birth. Data are mean ± SEM. Note that in some cases error bars are smaller than symbols. N= 8-12 per group.

Figure 4. Birth is followed by increased *TNF-α* expression in the hippocampus, regardless of delivery mode. *IL-10* expression was uniformly elevated relative to expression on P23 during all perinatal ages tested (A-C), and *IL-6* showed a modest elevation at 3h postnatal in vaginally-born mice (D-F). In contrast, a sharp increase in *TNF-α* expression occurred on P1 (G-I). Nearly identical patterns were observed in vaginally- and Cesarean born mice, although for *IL-6* Cesarean-born mice had lower expression at 3h and higher at P1 (F). Letters denote effects of age within birth modes separately (left and middle columns), such that group means with

different letters are significantly different from each other. Asterisks denote direct comparisons between birth modes (right column), with asterisks above symbols representing a significant post-hoc comparison (following a significant interaction) for single timepoints (*$p \leq 0.04$). Data are expressed relative to cytokine levels at E16.5. Dashed red lines indicate timing of birth. Data are mean ± SEM. Note that in some cases error bars are smaller than symbols. *N*= 8-12 per group.

Figure 5. Changes in cytokine and corticosterone production associated with birth are independent of maternal access. Vaginally-born offspring that remained with their biological mothers showed changes in peripheral and central hormone/cytokine levels similar to those of offspring without maternal access during the first 3h after birth (Fig.1-4). Relative to levels at E18.5, plasma levels of IL-10 more than tripled (A) and corticosterone increased almost by half (B) 3-4h after birth. No samples were available for corticosterone assay at P3. In addition, a 3-fold increase in *TNF-α* gene expression occurred on P1 in the PVN (C) and hippocampus (D). Group means with different letters are significantly different from each other. Dashed red lines indicate timing of birth. Data are mean ± SEM. Note that in some cases error bars are smaller than symbols. *N*= 7-10 per group, except for IL-10 plasma levels at P3 (A; *N*= 5) and *TNF-α* expression at P3 (C,D; *N*= 3).

Figure 6. Birth is associated with rapid increases in microglial numbers in the PVN (A-G) and CA1 oriens layer of the hippocampus (H-N). Photomicrographs of Iba1+ stained tissue in

representative vaginally-born mice show increases in microglial numbers between E18.5 and P3 in both the PVN (A-D) and CA1 oriens (H-K). Regions of interest are indicated by dashed red lines. 3V, third ventricle; LV, lateral ventricle. Scale bar= 100 µm (A-D, H-K), 50 µm (insets). Quantification of Iba1+ cells revealed a significant increase in microglia between P0 (3h) and P1 and another increase between P1 and P3 in both brain regions (PVN: E-G, CA1 oriens: L-N). Letters denote effects of age within birth modes separately, such that group means with different letters are significantly different from each other. Asterisks denote direct comparisons between birth modes, with asterisks above bracket representing a main effect of birth mode (*p= 0.03, **p= 0.003). Dashed red lines indicate timing of birth. Data are mean ± SEM. Note that in some cases error bars are smaller than symbols. N= 7-12 per group.

Figure 7. Birth is associated with increased microglial staining intensity in the PVN (A-C) and CA1 oriens (D-F), regardless of delivery mode. Staining intensity in both brain regions was highest on P1, and decreased by P3. This pattern is also captured in the insets of Fig.6A-C,G-I. Letters denote effects of age within birth modes separately (left and middle columns), such that group means with different letters are significantly different from each other. Note that the conventional intensity scale has been inverted, such that it is expressed from 0 (brightest) to 255 (darkest) (see gradient provided). Dashed red lines indicate timing of birth. Data are mean ± SEM. N= 9-12 per group.

Figure 8. Birth causes the postnatal inflammatory responses observed in the periphery and the brain. (A) We tested two alternative hypotheses: (H1, top) birth triggers immune responses and (H2, bottom) immune responses are developmentally programmed and only coincidentally associated with birth. For an immune variable that normally peaks a day after birth (post-conception day 20), H1 predicts that mice born a day early (advanced birth group) should have that peak advance by a day. In contrast, H2 predicts that both advanced and on-time birth groups should have that variable peak on the same developmental age (post-conception day 20). Our results support H1 as all tested variables peaked a day earlier in the advanced group; specifically, levels of IL-10 in the periphery (B), *TNF-α* expression in the PVN and Hippocampus (C,D), and microglial number in the PVN and CA1 oriens (E,F). Asterisks next to brackets represent main effects of age (B: E18.5/E19 vs. 3h; C-F: 3h vs. 24h) (**p= 0.001, ****p< 0.0001), and confirm the expected increases. For B, the E19 data used for the on-time group were from the larger run shown in Fig. 1 (see text); this is represented by the dotted-gray line. For C-D, data are expressed relative to *TNF-α* levels at E16.5. Arrows indicate timing of birth. Data are mean ± SEM. Note that in some cases error bars are smaller than symbols. N= 3-15 per group.

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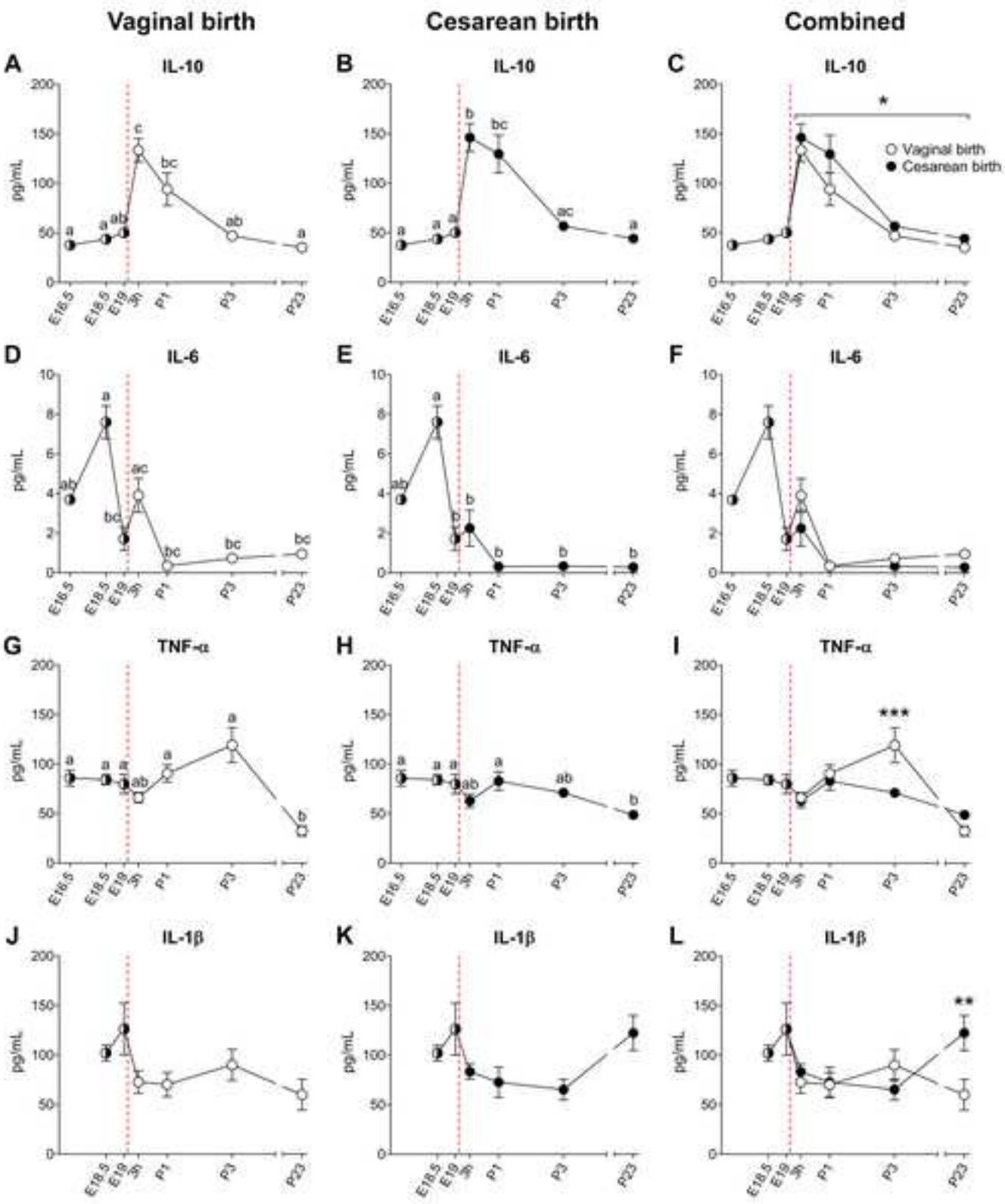
First author: A. Castillo-Ruiz
Abbreviated title: Birth triggers inflammation



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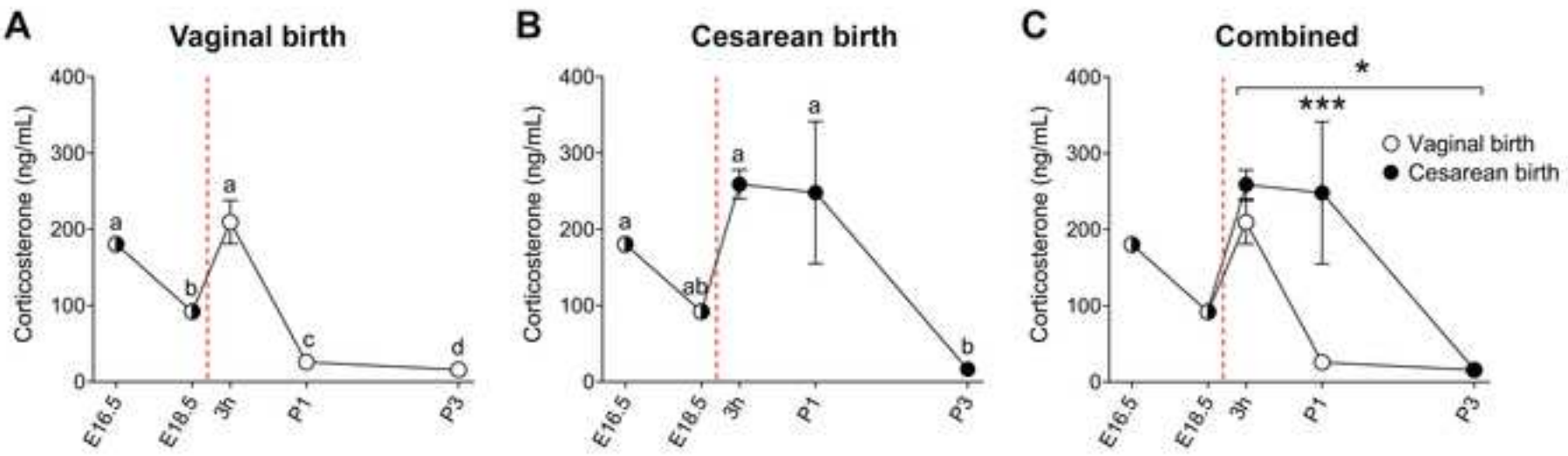

Figure 3

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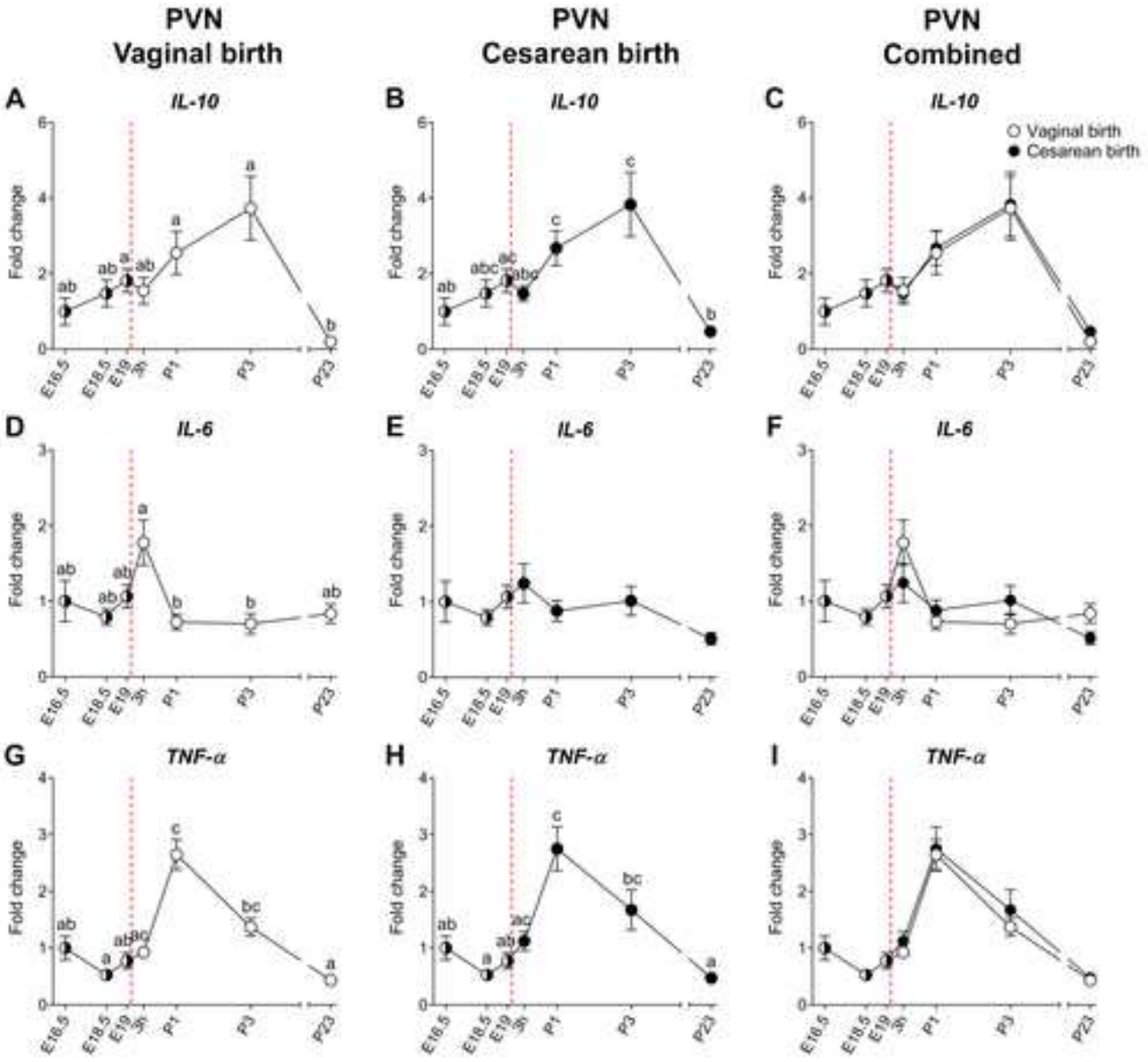

Figure 4

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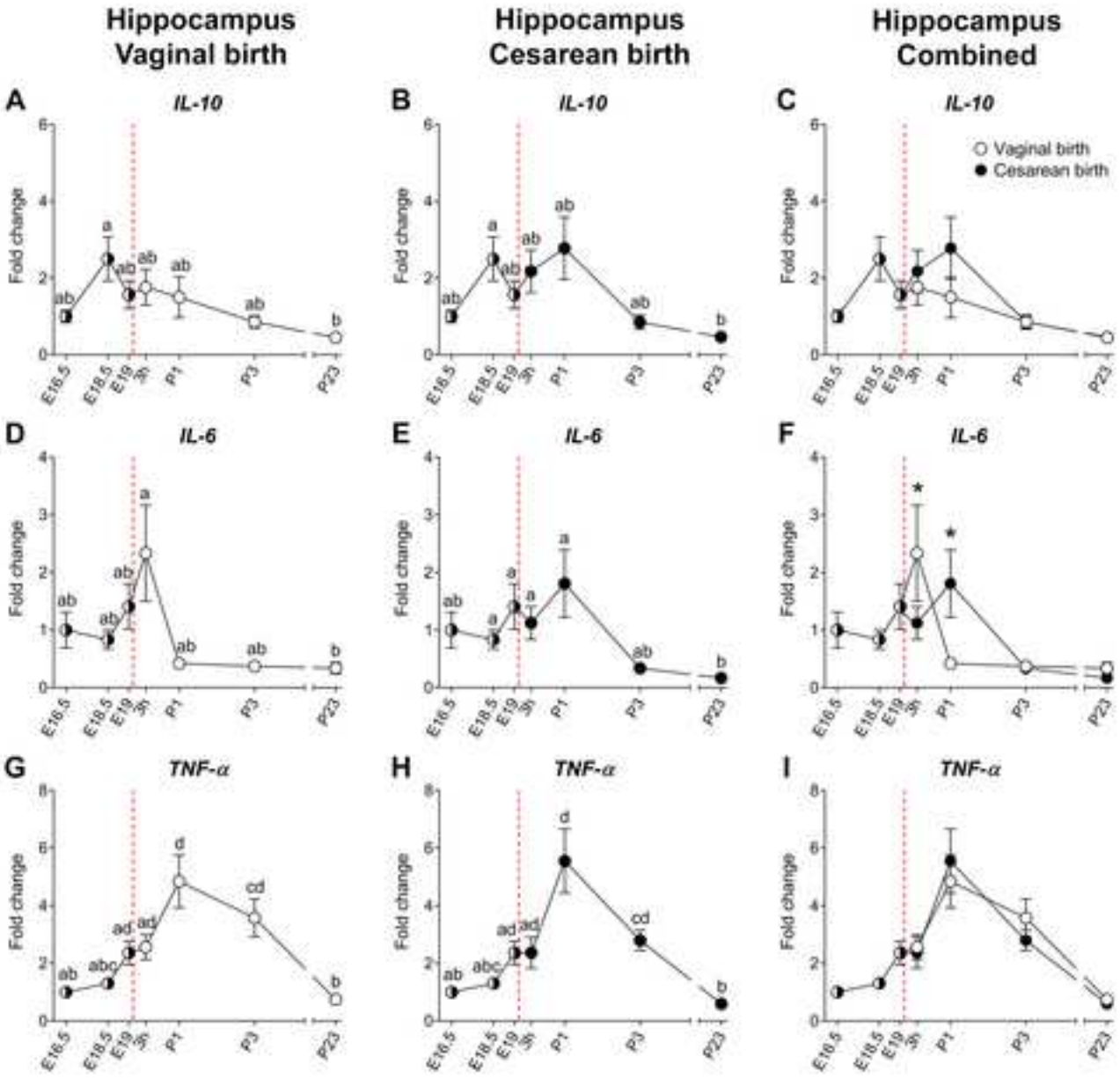

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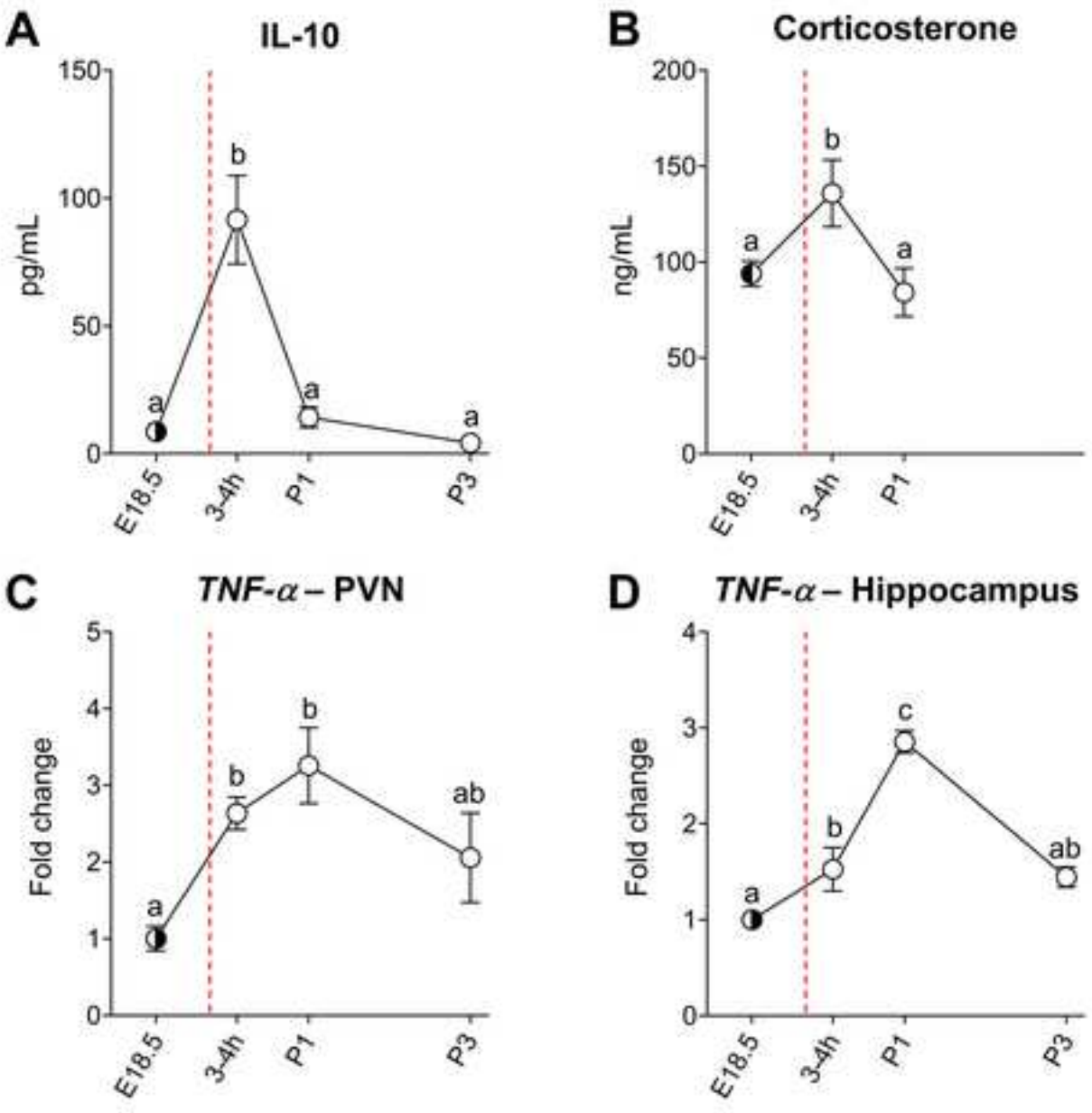

Figure 6 Click here to access/download;Figure;Figure 6.tif

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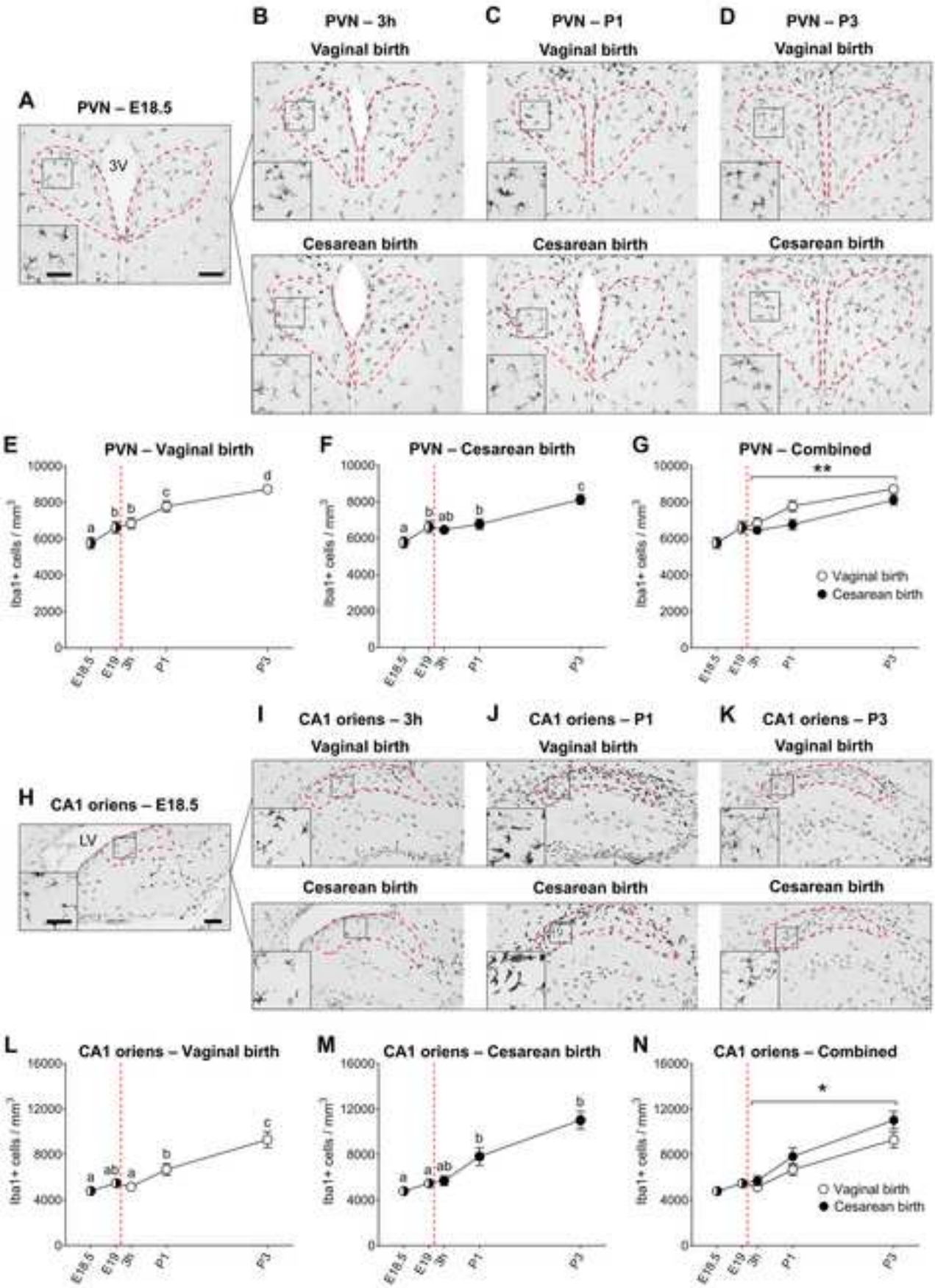

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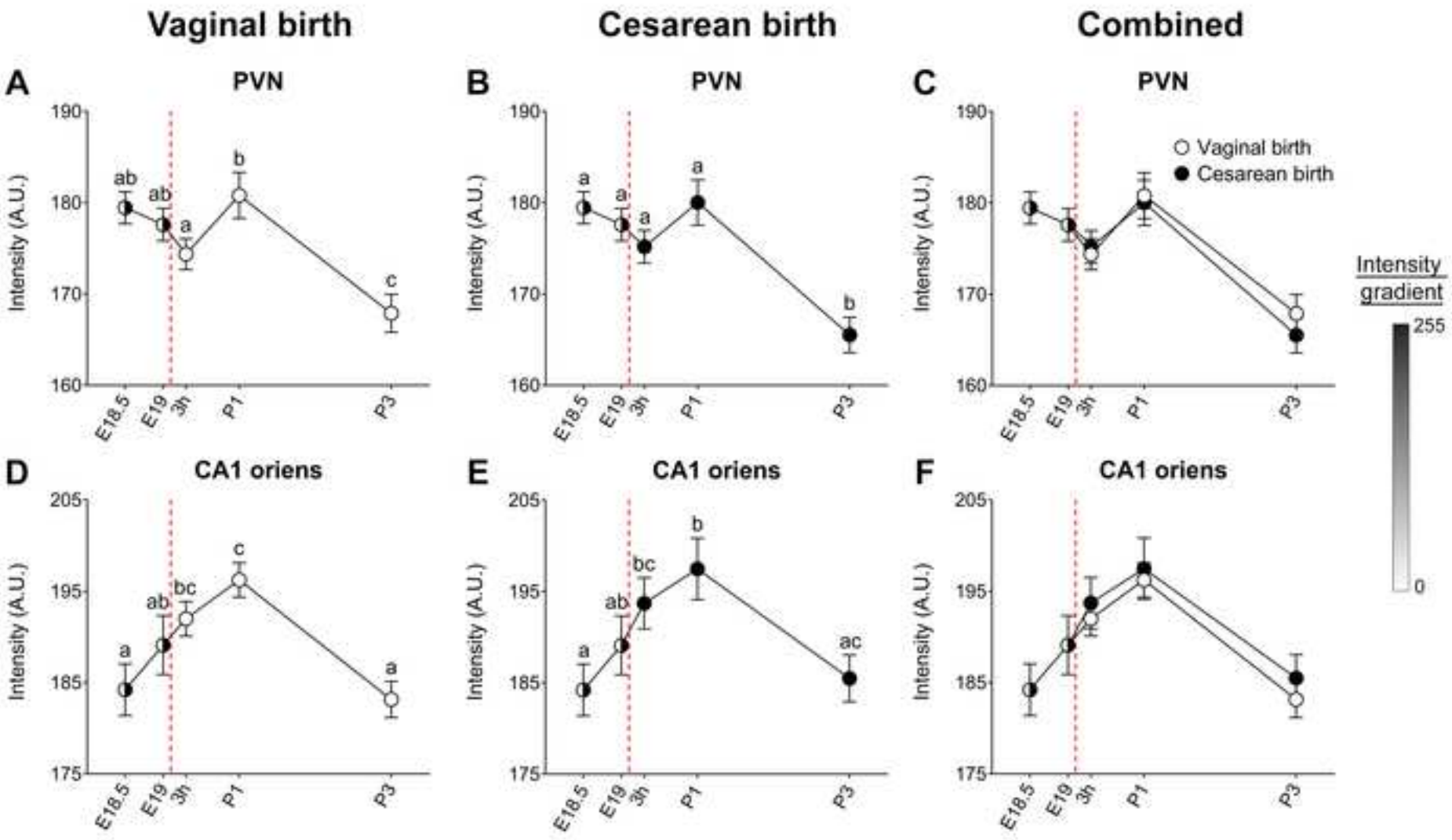

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