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SHORT COMMUNICATION

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# Gestational stress alters maternal behavior and inflammatory markers in the olfactory bulb of lactating mice

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## Abstract

Inflammatory markers represent important candidates responsible for the altered behavior and physiology observed after stressful experiences. In the maternal brain, the olfactory bulb (OB) is a key constituent of the neural circuit that mediates the reciprocal interaction between mother and infant. This study aimed to investigate the effects of stress during pregnancy on maternal behavior and inflammatory changes in the olfactory bulb of lactating mice. Female Balb/c mice were divided into two groups: control (CT) and restraint stress (RS). Maternal behavior was performed during the first 8 days of life of the offspring. On the 10th day after parturition, corticosterone, gene, and protein expression were assessed. Stress during pregnancy decreased the maternal index at postnatal day 4 and the nuclear factor-κB 1 (NFκB1) gene expression in the OB. Moreover, females from the RS group showed increased interleukin (IL-1β) protein expression. In contrast, stressed females exhibited a decreased tumor necrosis factor (TNF-α) protein expression in the OB. In conclusion, exposure to stress during pregnancy was able to induce specific postnatal effects on maternal behavior and balance of inflammatory mediators in the OB.

**KEYWORDS**
inflammation, maternal stress, olfactory bulb, restraint stress

  

## 1 | INTRODUCTION

Stress during pregnancy is a high prevalent health problem that affects about 9% to 22% of women worldwide (Fairbrother et al., 2016). Evidence report that exposure to chronic stress during pregnancy is an important component to increase the vulnerability to postpartum depression, anxiety, and decreased mother–infant interaction (Haim et al., 2014; Leuner et al., 2014; Racine et al., 2019; Xia et al., 2016). Inflammatory markers represent important candidates responsible for the altered behavior and physiology observed after stressful experiences (Garcia-Bueno et al., 2008). In particular, the hyper-activation of the hypothalamic–pituitary–adrenal (HPA) axis participates by altering the glucocorticoid sensitivity and promoting upregulation of genes related to the pro-inflammatory response, as nuclear factor-κB (NFκB) and cytokines (Ross et al., 2019; Silverman & Sternberg, 2012). However, little is known on the effects of stressors during pregnancy on inflammatory markers, especially in the postpartum period. The upregulation of inflammatory mediators after stress is observed both at peripheral level and in brain-specific cells, which is positively correlated with abnormal maternal behavior (Bekhbat et al., 2019; Coussons-Read et al., 2007).

In the maternal brain, the olfactory bulb (OB) represents a key region of the neural circuitry that mediates the reciprocal interaction between mother and infant (Corona & Levy, 2015). Its functions are strongly regulated by hormonal alterations and studies have shown that maternal stress induces detrimental effects in the OB of lactating rodents, including decreased neural differentiation, proliferation, and morphological alterations (Belnoue et al., 2016; Czarnabay et al., 2019). Furthermore, it has been shown that increased inflammation in the OB promotes atrophy, activation of glial cells and astrocytes, in addition to increased cell death (Doursout et al., 2013; Hasegawa-Ishii et al., 2017, 2020).

Nevertheless, it is not clear whether maternal stress during pregnancy promotes changes on inflammatory markers in the OB of lactating mice. Thus, considering the importance of the OB to the mother-offspring interaction, this study aimed to investigate the effects of stress during pregnancy on maternal behavior and inflammatory markers in the OB of lactating mice.

## 2 | MATERIAL AND METHODS

### 2.1 | Animals and experimental design

Balb/c female mice were randomized into two groups: control (CT) ($n = 5$) and restraint stress (RS) ($n = 5$). The estrous cycle was monitored through vaginal smear visualization. During the fertile period, two females were paired with one male and mating confirmed by the presence of a vaginal plug (G1 - day 1 of gestation). After birth, litters were adjusted to four to six animals (sex ratio at birth: CT = 0.33 and RS = 0.53). Maternal behavior was evaluated during the first 8 days of life of the offspring. On day 10 postpartum, mothers were euthanized, by conscious decapitation, and samples collected. The experimental protocol was approved by the University Ethics Committee for the Use of Experimental Animals (number 8465), and all mice were manipulated according to national guidelines (CONCEA). Experimental design is shown in Figure 1a.

### 2.2 | Restraint stress

Prenatal stress was performed as described previously (Luft, Levices, da Costa, et al., 2020; Luft, Levices, Pedrazza, et al., 2020). Briefly, pregnant mice were restrained for 30 minutes, on intercalated days, from the 8th day of gestation. Mice from the control group were kept undisturbed without any interventions during pregnancy, except for routine husbandry.

### 2.3 | Maternal behavior

After birth, mothers were individually housed and maternal behavior monitored daily during the light (10 AM, 1 PM, and 4 PM) and dark cycles (7 PM), in the first 8 postpartum days, by two independent trained observers. Each behavioral observation lasted for 72 min, with recordings performed every 3 min, totaling 100 observations per mother per day. The following behaviors were considered maternal care: mother licking the pups (body surface or anogenital region), breastfeeding (arched-back posture, blanked posture, and supine posture), and building the nest. No interaction with pups and eating/drinking were also evaluated. The maternal index was obtained by the frequency of the maternal care behaviors described above divided by the number of all observations (de Souza et al., 2012).

### 2.4 | Corticosterone

The maternal serum levels of corticosterone were determined using a commercial ELISA kit (Diagnostics Biochem Canada Inc, Ontario, Canada), according to the manufacturer's instructions. The final results were expressed as μg/dL. The lowest limit of detection was 0.69 μg/dl.

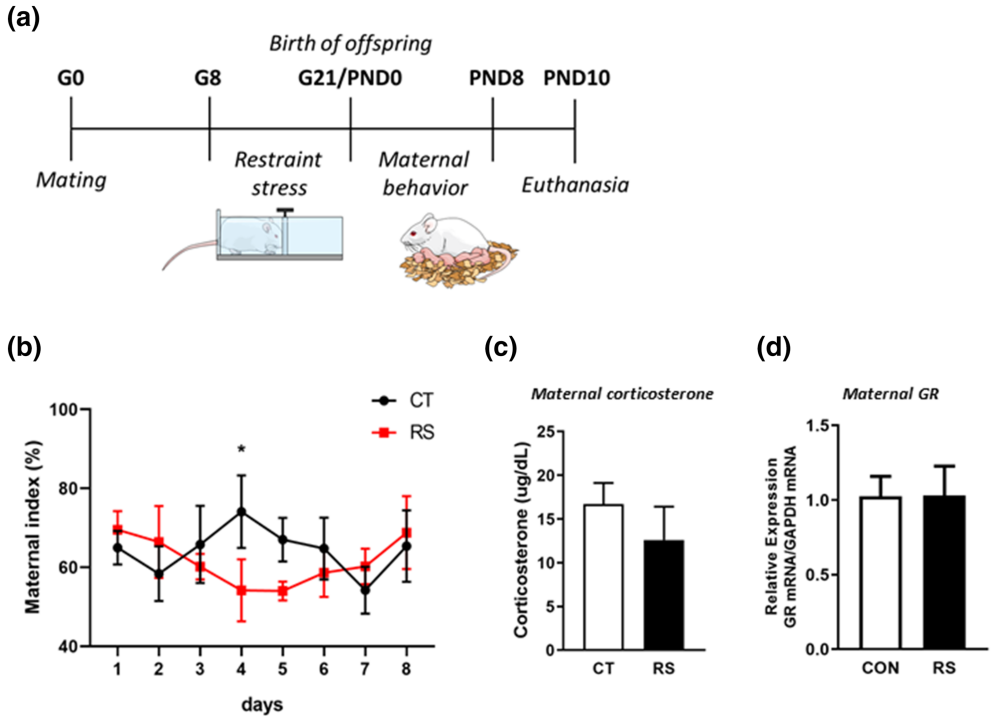


**FIGURE 1** Experimental design of the study (a), maternal behavior (b), corticosterone levels (c), and GR gene expression (d). Significant effects for group ($F(8,56) = 6.66$, $p < 0.0001$; two-way ANOVA) were found for the maternal index analysis. Stress during pregnancy significantly decreased the maternal index on postpartum day 4 ($p = 0.04$, Fisher's test, five animals per group). No significant differences were found regarding maternal plasma corticosterone concentration ($t(10) = 0.91$, $p = 0.38$; Student's $t$ test, six animals per group) and GR expression ($t(6) = 0.014$, $p = 0.98$). G0: gestational day 0; G8: gestational day 8; G21: gestational day 21; PND0: postnatal day 0, PND8: postnatal day 8, PND10: postnatal day 10. Data are shown as mean ± SEM. CT: control; RS: restraint stress from the second week of pregnancy

## 2.5 | Gene expression

The total OB cellular RNA from dams was extracted using the Trizol method (ThermoFisher-Scientific), and 1 μg of RNA was reverse-transcribed using the GoScript™ Reverse Transcription System Protocol (Promega). Gene expression was performed in real-time quantitative PCR (Step One Plus, ThermoFisher-Scientific). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene. The qRT-PCR was performed using the SYBR® Green fluorescence marker (ThermoFisher-Scientific). The following primers were used: glucocorticoid receptor (GR) (forward: 5′ GGAATAGGTGCCAAGGGTCT 3′; reverse 5′ GAGCACACCAGGCAGAGTTT 3′), nuclear factor-κB 1 (NFκB1) (QT00154091), nuclear factor-κB 2 (NFκB2) (QT00129864), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (QT01658692) (Qiagen, Hilden, Germany).

## 2.6 | Protein expression

The OB lysates from dams were prepared with CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) buffer plus protease inhibitors. Protein samples (40 μg) were analyzed by electrophoresis and immunoblotting (Haute et al., 2020). Membranes were incubated with interleukin (IL-1β), tumor necrosis factor (TNF-α), and interleukin-6 (IL-6) at 1:500. Blots were incubated with secondary antibodies at 1:2000. GAPDH was used for normalization of quantitative densitometry values and total expression was evaluated with Image J software (National Institutes of Health, USA). The antibodies used were the following: anti-IL-6 mouse (1:500 dilution, sc-32,296, Santa Cruz Biotechnology, USA), anti-TNF-α mouse (1:500 dilution, sc-52,746, Santa Cruz Biotechnology, USA), and anti-IL-1β mouse (1:500 dilution, sc-12,742, Santa Cruz Biotechnology, USA).

## 2.7 | Statistical analysis

The normality of data was tested using the Shapiro–Wilk test. Outliers were detected with the Grubbs' test and excluded from analyses. Data were expressed using mean and standard error of the mean (SEM). Comparisons between two groups were evaluated by the Student's $t$ test. Two-way ANOVA followed by Fisher's LSD post-test was used for maternal behavior. In all cases,

statistical significance was set at 5%. Data were analyzed using the Prism GraphPad software (version 8.0.1, GraphPad Software Inc., USA).

## 3 | RESULTS

Stress during pregnancy disrupted normal maternal behavior, as demonstrated by the significant decrease in maternal index at day 4 when the RS group was compared to the CT group (Figure 1b). No significant differences were found for corticosterone secretion and GR gene expression in the maternal OB (Figure 1c,d, respectively). Figure 2a shows a significant decrease in the expression of NFκB1 in the maternal OB of the RS group when compared to the CT group. However, no differences were found between groups for the NFκB2 gene expression (Figure 2b).

When the effects of maternal stress on protein levels of inflammatory markers in the OB from dams were investigated, we observed a significant increase in the IL-1β protein expression in the OB of RS females when compared to the CT group (Figure 3a). On the other hand, Figure 3b shows a significant TNF-α protein expression decrease in the RS group compared to CT. No significant differences were found for IL-6 (Figure 3c).

## 4 | DISCUSSION

Although the association between adverse events during pregnancy and negative outcomes on maternal behavior has been widely reported (Belnoue et al., 2016; Gatta et al., 2018; Hakanen et al., 2019), the period, intensity, and frequency of the stressor are determining factors. The maternal HPA axis responsiveness is attenuated during late pregnancy, whereas stress during early pregnancy promotes an increased response and could have more detrimental effects (Brunton, 2013). Our data showed that restraint stress, from the second week of gestation, decreases maternal behavior only at postpartum day 4. Between the third and fourth day of life, important processes occur for the offspring's neurodevelopment, such as increased cerebral synaptogenesis and the formation of sensory barriers (Chen et al., 2017). Alterations during this period may be relevant to the long-term development of different diseases in the offspring. Studies have already shown compromised maternal behavior and increased corticosterone secretion in response to stress in dams submitted to experimental to gestational stress (Bosch et al., 2007; Patin et al., 2002). Moreover, it is well established that glucocorticoid levels during the postpartum period in mice are low and the HPA axis is relatively hyporesponsive (Douglas et al., 2003). Conversely, our study did not find significant differences in the HPA axis activity, as observed by the basal corticosterone secretion and GR gene expression. Although a second-hit stress was not performed, hormonal long-term effects are typically not observed at basal levels (Douglas et al., 2003) (Zoubovsky et al., 2020). Thus, we hypothesize that the behavioral changes observed may be due to mechanisms not directly dependent on the activity of the HPA axis.

Several studies have shown increased expression of proinflammatory mediators during neuropsychiatric disorders (DiSabato et al., 2020; Mao et al., 2018; Niraula et al., 2019). NF-κB activation elicits the transcription of inflammatory cytokines (Oeckinghaus et al., 2011), although NF-κB1 and NF-κB2 subunits suppress κB-dependent transcription (Oeckinghaus & Ghosh, 2009). Our data demonstrated a decrease in the OB NF-κB1 gene expression in lactating mice submitted to prenatal stress. The reduction of NF-κB1 would contribute to

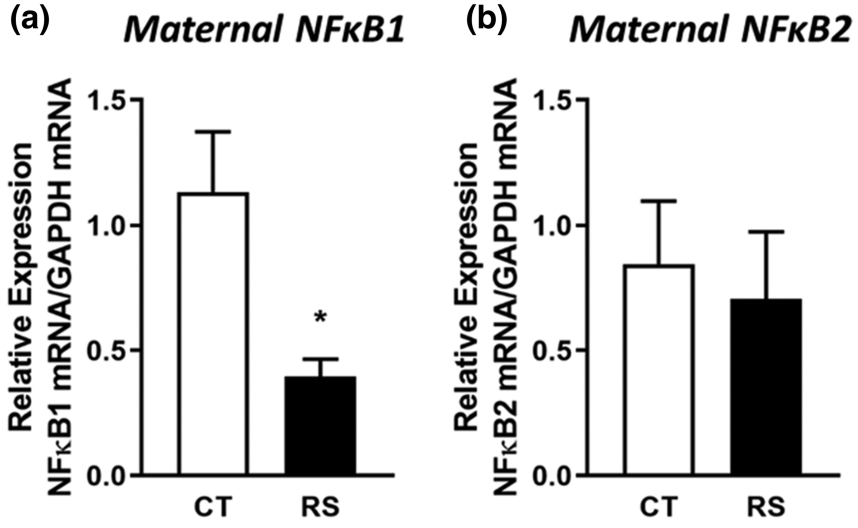


**FIGURE 2** Gene expression in the maternal olfactory bulb. (a) Gestational stress significantly decreased tissue NFκB1 mRNA expression ($t(8) = 2.92$, $p = 0.01$; Student's $t$ test, five animals per group) in the olfactory bulb of dams. (b) No significant differences were found regarding the NFκB2 expression ($t(6) = 0.37$, $p = 0.71$; Student's $t$ test, four animals per group). Data are shown as mean ± SEM. NFκB1: Nuclear factor-κB 1; NFκB2: Nuclear factor-κB 2; CT: control; RS: restraint stress from the second week of pregnancy

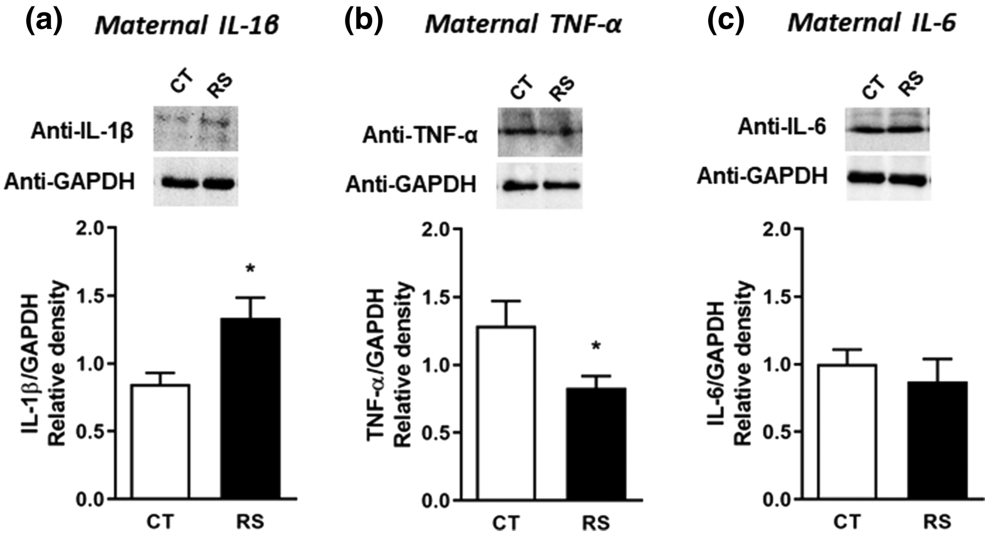


**FIGURE 3** Protein levels of inflammatory markers in the maternal olfactory bulb. Stress during pregnancy significantly increased IL-1β ($t(7) = 2.65$, $p = 0.03$; Student's $t$ test, CT = 4 animals per group, RS = 5 animals per group) protein expression in the olfactory bulb (a). There was a significant decrease ($t(7) = 2.47$, $p = 0.04$; Student's $t$ test, CT = 4 animals per group, RS = 5 animals per group) in the protein expression of TNF-α in the olfactory bulb of dams (B). No significant differences ($t(8) = 0.66$, $p = 0.52$; Student's $t$ test, five animals per group) were found regarding IL-6 protein expression (c). Data are shown as mean ± SEM. IL-1β: nterleukin-1β; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; CT: control; RS: restraint stress from the second week of pregnancy

decrease the inhibition upon NF-κB and thus lead to increased inflammatory markers. Evidence has shown a direct relationship between stress, NF-κB and decreased neuronal proliferation, leading to altered behavior (Koo et al., 2010).

The present results also demonstrated that stress during pregnancy decreased TNF-α in the OB and increased IL-1β protein expression in the postpartum period. The increase in inflammatory markers associated with behavioral changes has been previously described (Salvador et al., 2021). Particularly, IL-1β is a well-recognized cytokine marker for the central nervous system (CNS)-related diseases (Mendiola & Cardona, 2018). For example, increased IL-1β has already been found in different brain regions of rodents with mood disturbances, such as depression and anxiety (Goshen et al., 2008; Rossi et al., 2012). During the perinatal period, there is increased blood levels of IL-1β in depressed women (Corwin et al., 2008; Leff Gelman et al., 2019; O'Mahony et al., 2006). Stressors during pregnancy have also been associated with increased levels of IL-6 and TNF-α (Coussons-Read et al., 2005). In contrast, our data revealed that stress during pregnancy decreases the expression of TNF-α in the OB of lactating mice. It is well established that TNF-α has a key role in several neurological processes, including excitatory transmission, homeostatic synaptic activity, and glutamate release (Olmos & Llado, 2014). Therefore, clinical and experimental studies have demonstrated that decreased signaling of TNF-α in the CNS could contribute to the development of pathological conditions such as neurodegenerative diseases, neuronal impairment, and altered microglia activation (De Lella Ezcurra et al., 2010; McCoy & Tansey, 2008; Paouri et al., 2017). Thus, we believe it is possible to speculate that an imbalance in the expression of this proinflammatory cytokine could impact the normal function of the OB, although further studies would be needed to confirm that.

This study has also limitations, including the limited sample size, which could have prevented us from achieving a higher power for the analyses performed. Furthermore, it is not possible to exclude, from the results presented, that stress during pregnancy may be influencing post-transcriptional processes in the expression of further inflammatory mediators, such as NF-κB. However, we believe present findings may serve as a starting point for future studies to further examine the relationship between prenatal stress and its maternal effects on the postnatal period.

In conclusion, our results have shown that exposure to stress during pregnancy was able to induce specific post-natal effects on maternal behavior and balance of inflammatory mediators in the OB. These findings may not be related to alterations in the HPA axis, as no differences were found for both corticosterone secretion and GR expression. Although these are preliminary results that should be further explored, it may contribute to a better understanding of prenatal stress and maternal postpartum changes.

## AUTHOR CONTRIBUTIONS

CL conceived the work, acquired data, drafted the paper, performed data analysis, and approved the final version. LEWS, MSC, LP, and GLA acquired data, revised the article, and approved the final version. RGO and JRO conceived the work, revised the paper, and approved the final version. MVFD conceived the work, acquired funding, performed data analysis, revised the article, and approved the final version.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

## ETHICS APPROVAL

All the experiments were performed in agreement with the international ethical standards and following the local animal protection guidelines. The experimental protocol was approved by the Ethics Research Committee (protocol number 8465) of the Pontifical Catholic University of Rio Grande do Sul (PUCRS).

## DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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