Research Article

Prenatal two-hit stress affects maternal and offspring pregnancy outcomes and uterine gene expression in rats: match or mismatch?[†]

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Abstract

Maternal stress and inflammation excesses can lead to adverse pregnancy outcomes and offspring development. We evaluated whether distinct prenatal stressors affect pregnancy, maternal and offspring outcomes, and uterine gene expression differently when combined than either alone. Long-Evans dams were exposed to psychological or/and (two-hit) immune stress (interleukin-1 beta [IL-1 β]), on gestational days 12–18 and 17-delivery, respectively. Gestational length, maternal weight gain, glycaemia and corticosterone levels, offspring weight, and gender effects were recorded. Maternal and offspring uteri were collected at weaning and on postnatal day 160 correspondingly. Uterine expression of genes involved in local progesterone metabolism, neuroendocrine and immune systems were analyzed using quantitative real-time polymerase chain reaction. Maternal two-hit stress increased gestational length variation and the occurrence of adverse pregnancy outcomes while reducing gestational weight gain. Pup weight was negatively affected by prenatal stressors in a gender-specific way. In dams, IL-1 β upregulated gene expression of neuroendocrine (*Crh*, *Crhr1*) and cytokine genes (*Il1b*, *Il1rn*, *Il6*, and *Il10*). Conversely, transcriptional patterns in offspring uteri were more variable with gene-specific up- or downregulation by each stressor separately, while exposure to both extensively reduced the expression of neuroendocrine (*Hsd11b1*), cytokine (*Il1a*, *Il1rn*, *Il6*), and IL-1 receptor genes. In conclusion, maternal stress affects physiological and molecular processes in dams and their offspring; two hits have different effects than single stressors. Outcomes appear generation-, gender-, and stressor-specific. Dampening of offspring uterine gene expression after exposure to multiple stressors could fit within the match/mismatch hypothesis of perinatal programming, with offspring preparing for a stressful life.

Summary Sentence

The combination of two distinct prenatal stressors, psychological and immune stress, induces adverse outcomes and differentially affects expression of genes related to progesterone metabolism, stress and the immune system in the rat uterus.

Key words: stress, pregnancy, parturition, gene expression, fetal development, early development, developmental origins of health and disease, corticotropin-releasing hormone (CRH/CRH receptor), progesterone, transcriptional regulation, steroid hormones/steroid hormone receptors, cytokines, uterus, rodents.

Introduction

Adverse pregnancy outcomes like preterm birth (PTB) and low birth weight not only affect newborn outcomes but have also been recognized as determinants of health later in life within the framework of perinatal programming [1, 2]. PTB, defined as delivery prior to 37 completed weeks of pregnancy, and low birth weight, resulting from intrauterine growth restriction and/or PTB, are the leading causes of perinatal morbidity and under-five mortality [3]. These adverse pregnancy outcomes have been shown to cause neurodevelopmental delays [4], cognitive and behavioral deficits [5, 6], sensory impairments [7] as well as chronic lung disease [8], among others. On the other hand, both PTB and low birth weight can predispose individuals to adult disease, including cardiovascular and pulmonary [9, 10], metabolic and endocrine [11, 12], as well as renal [13] and mental health disorders [14]. Also, women who were born preterm have an increased risk of adverse outcomes in their own pregnancies [15]. Indeed, a personal or family history of PTB remains the best predictor for preterm delivery to date [16].

Maternal stress and inflammation have both been implicated as possible causes of adverse pregnancy outcomes and are regarded as important factors in the parturition cascade in which (interactions between) the hypothalamic-pituitary-adrenal (HPA) axis and the immune system play a key role [17–19]. Moreover, inflammation is essential to maintenance of pregnancy and parturition, with an increased inflammatory response being associated with both PTB and intrauterine growth restriction [20–22]. The transformation of the uterus from being relatively quiescent during gestation to an active laboring organ is an inflammatory process which is mediated, at least in part, by interleukin (IL)-1 β , IL-6, and the anti-inflammatory cytokine IL-10 [23–25]. IL-10 is a crucial mediator of inflammation [26] and also plays a role in the establishment and maintenance of pregnancy [27, 28]. Whether it transpires term or preterm, parturition is thought to occur as a result of a common terminal pathway which can be initiated by a plethora of factors [29, 30]. These can include excessive inflammation and infection as well as maternal stress, potentially through neuroendocrine-immune system interactions [31, 32].

In addition, it is now largely accepted that maternal stress can be transmitted in both an intergenerational and transgenerational manner, most likely through epigenetic mechanisms [33–37]. This is thought to be independent of the timing of the stressor, whether it occurs preconceptionally as abuse during the mother's childhood or adult life (e.g., interpersonal violence), during gestation or as postnatal stress [34, 38–40]. Furthermore, maternal stress has been shown to affect the birth process and timing of delivery as well as elevate the risk of adverse pregnancy outcomes [41–44]. We have previously shown in a single-hit stress model in rats that ancestral and multigenerational stress lead to adverse pregnancy outcomes for both mothers and newborns beginning with the offspring F1 generation. Moreover, this stress exposure appears to program and worsen these same effects in future generations as well, while further

augmenting the risk of PTB and delayed growth [35]. This has also been demonstrated in human pregnancy [45].

Here, we present our intergenerational two-hit stress model. We hypothesized that two distinct gestational stressors or hits affect pregnancy and health outcomes differently when combined than either hit alone. Furthermore, we proposed that adverse outcomes would be present starting from the exposed generation as compared to single-hit stress. We also studied the effect of prenatal stressors on uterine gene expression in F0 dams as well as their adult F1 daughters, with a particular interest in possible fetal programming effects of maternal stress.

Materials and methods

Animals

Three hundred and sixty-six Long-Evans hooded rats (*Rattus Norvegicus*) were used. Nulliparous females between the age of 110 and 155 days, bred and raised at the vivarium of the University of Lethbridge, AB, Canada, were mated with stress-naïve males ($n = 10$). The latter were obtained from Charles River Laboratories International Inc. (Kingston, NY, USA). Each female of the parental F0 generation was individually paired with a male for 1 h/day until mating. Sessions were video-monitored and time of final mounting was recorded and regarded as the start of pregnancy on gestational day 0. Pregnancy was confirmed by the observation of consistent maternal weight gain. Pregnant females were housed in pairs until gestational day 19, when they were moved to a single housed cage until delivery where gestational length was monitored by continuous infrared cage site recording (CCTV cameras, Panasonic, Newark, NJ, USA). Pregnancy duration was calculated as the number of hours between the time of final mounting and time of emergence of the first pup, which designated the end of pregnancy. For dams euthanized during pregnancy or delivery because of complications, gestational length was calculated based on the day of sacrifice, with delivery time set at 9:00 AM. Pups stayed with their mothers until weaning on postnatal day (P)21 and were then housed with same sex siblings. The animals were housed under standard conditions on a 12-hour light/day cycle with lights on at 7:30 AM, with room temperature maintained at 20°C and relative humidity of 30%. All experiments were approved by the Animal Welfare Committee of the University of Lethbridge (Protocol #1210) according to guidelines from the Canadian Council on Animal Care.

Experimental design (Figure 1)

Timed-pregnant female rats ($n = 31$) of the F0 filial generation were bred under standardized conditions with food and water ad libitum. Dams and their offspring were split in groups according to treatment. Data referring to mothers are provided in reference to gestational days (GD) and postpartum lactational days (LD), while data regarding does and pups refer to postnatal days (P).

Figure 1. Time line illustrating the experimental design of the two-hit stress model. Dams were exposed to psychological stress (restraint 20 min/day and forced swimming 5 min/day) and/or IL-1 β injections (5 μ g/day i.p.) in the second half of gestation, GD12-18 and GD17-delivery, respectively. Saline was used as sham. Syringes (red online and grey in print) indicate blood draws from the F0 generation; the white syringe signifies daily intraperitoneal injections. Dams were sacrificed at weaning of their offspring, their daughters as virgin adults on P160. F: filial generation; GD: gestational day; IL: interleukin; LD: lactational day; P: postnatal day. This figure is available in color at *Biology of Reproduction* online.

Maternal health was monitored closely pre- and postpartum with serial weighing as well as blood sampling for monitoring of glycaemia and corticosterone levels. Dams were weighed prior to pregnancy as well as three times per week during gestation and additionally on GD11, GD18, and GD21. Offspring gender was determined on P1 ($n = 318$), and pup weights were measured on P1, P7, P14, P21 (weaning), and P30. Male and female progeny were analyzed separately. F1 offspring was also distributed to separate projects to make optimal use of this cohort.

Stress procedures

After verification of mounting followed by confirmation of pregnancy on GD11, dams consecutively received psychological stress (indicated as Stress or S) and/or IL-1 β as immune stressor. Psychological stress consisted of restraint and forced swimming from GD12 to GD18, which have been validated as effective stress procedures [46]. Stress was administered alternately between 8:30 and 9:30 AM and 3:00 and 4:00 PM to avoid habituation. For body restraint, animals were placed in a customized Plexiglas container with perforated ends for ventilation for 20 min [46]. The inner diameter of the container was adjusted to the size of the animal to allow the dam to remain standing while avoiding compression of the body and to prevent turning. Forced swimming occurred for 5 min in a round water tank with a diameter of 45 cm and height of 77 cm, filled up to 50 cm with water at room temperature [47]. For the second stress hit, half of the animals received daily intraperitoneal injections with IL-1 β at a dose of 5 μ g/day from GD17 to delivery (approximately GD21) for a total volume of 0.5 mL, with saline used for sham injections. Injections were administered alternately on each side of the body. This design gave rise to four treatment groups of dams and their offspring: No Stress/Saline (N/Sa), No Stress/IL-1 β (N/IL-1 β), Stress/Saline (S/Sa), and Stress/IL-1 β (S/IL-1 β), $n = 7$ -8-7-9 dams/group, respectively (Figure 1).

Tissue collection

Blood

Blood samples (0.6 mL) were collected from the lateral tail vein of F0 animals between 9:00 and 10:00 AM under 4% isoflurane anesthesia [46]. Sampling occurred on P110 (baseline), on GD11 (before stress), GD18 (last day of psychological stress, second day of IL-1 β) and on the day after delivery (LD1).

Tissues

Dams delivered a single litter before being euthanized on LD21 after weaning of their pups, the earliest possible collection time after delivery. Female F1 offspring were sacrificed as virgin adults on P160. Animals received a cardiac injection of an overdose of Euthasol (Western Drug Distribution Centre Ltd, Edmonton, AB, Canada) or intraperitoneal injection in case of complications during pregnancy. The right uterine horn of each female was dissected rapidly and flash-frozen for mRNA analysis (F0 $n = 6$ -7 samples available/group, F1 $n = 4$ -6/group, except F1 N/Sa for non-cytokine genes $n = 3$). N-numbers per analysis are noted in the figure legends as $n = 1$ -2-3-4 corresponding with the columns from left to right, i.e., N/Sa-N/IL-1 β -S/Sa-S/IL-1 β , respectively.

Biochemical analyses

Blood glucose was tested on a sample acquired between 09:00 and 10:00 AM without prior fasting with an Ascensia Breeze Blood Glucose Meter (Bayer, Toronto, ON, Canada). Plasma corticosterone levels were determined with commercial kits (Corticosterone ELISA kit ab108821, Abcam, Toronto, ON, Canada).

Gene expression analysis

RNA extraction

Total RNA was extracted from the right uterine horn from F0 dams (weaning) and F1 offspring (adult, virgin) using Qiagen RNeasy Mini Kit on QIAcube using QIAzol Lysis Reagent according to the manufacturer's instructions (Qiagen, Toronto, ON, Canada).

Quantitative real-time polymerase chain reaction

To analyze gene expression patterns, we performed quantitative real-time polymerase chain reaction (qRT-PCR) analysis of genes known to be involved in the stress, inflammation, and parturition cascades. These include signal transducer and activator of transcription 5B (*Stat5b*) and zinc-finger E-box-binding homeobox 1 and 2 (*Zeb1* and *Zeb2*) involved in progesterone metabolism [35, 48, 49] as well as corticotropin-releasing hormone *Crb* and the CRH receptors *Crhr1* and *Crhr2* [17]. We also evaluated the expression of the gluco- and mineralocorticoid receptors, nuclear receptor subfamily 3 group c member 1 (*Nr3c1*) and 2 (*Nr3c2*), respectively, and both isoforms of 11 β -hydroxysteroid dehydrogenase, *Hsd11b1* and *Hsd11b2* [50]. Furthermore, we analyzed the mRNA expression of the components of the IL-1 receptor complex, consisting of IL-1 receptors (*Il1r1* and *Il1r2*) and receptor accessory proteins (*Il1racp* and *Il1racpb*) [51], as well as the mRNA abundance of pro-inflammatory cytokines interleukin-1 alpha (*Il1a*) and beta (*Il1b*) [21], interleukin-6 (*Il6*) [52], the endogenous IL-1 receptor antagonist (*Il1rn* also known as *Il1ra*) [53], and the anti-inflammatory interleukin-10 (*Il10*) [28, 54]. An overview of the physiological roles of these genes and their respective proteins is available in Supplemental Table S1.

Complementary DNA (cDNA) was generated from 500 ng total RNA using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada) following supplier's instructions. This cDNA acted as template for qRT-PCR using gene-specific primers as described or designed specifically for this study using Primer-BLAST [55] and Beacon Designer (PREMIER Biosoft International, Palo Alto, CA, USA) (see Supplemental Table S2). Samples were run in duplicate with reaction mixes containing 1 μ L cDNA (50 ng/ μ L), 10 μ L iQ SYBR Green Supermix (Bio-Rad Laboratories), 0.5 μ L forward, and 0.5 μ L reverse primer (10 μ M), as well as sterile water for a total reaction volume of 20 μ L.

Quantitative RT-PCR was performed on iCycler iQ thermal cyclers (Bio-Rad Laboratories) with the following reaction protocol: denaturation at 95°C for 10 min followed by annealing and elongation for 15 s at 95°C and 1 min at the primer-specific annealing temperature (Supplemental Table S2). For data analysis, cDNA samples were serially diluted to obtain standard curves, with the slope of each curve used to determine the reaction efficiency ($E = 10^{-1/\text{slope}}$). The average threshold cycles for each sample per gene were corrected for this efficiency and expressed relative to a single N/Sa sample. Data were normalized against housekeeping gene Cyclophilin A (Peptidylprolyl Isomerase A or *Ppia*) according to the formula $\text{Expression ratio} = \frac{(E_{\text{Target}})^{\Delta\text{CT}(\text{Control}-\text{Sample})}}{(E_{\text{Ref}})^{\Delta\text{CT}(\text{Control}-\text{Sample})}}$ with E_{Target} being the reaction efficiency of the gene of interest and E_{Ref} the RT-PCR efficiency of Cyclophilin A [51, 56, 57]. Expression levels of *Ppia* (C_T values) for F0 and F1 samples are shown in Supplemental Figure S1.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics, Version 24.0 (IBM Corp, Armonk, NY, USA). When necessary, transformations of data were conducted to achieve normal distribution and constant variance prior to statistical testing, if achievable. Two-way analysis of variance (ANOVA) was used with Stress and IL-1 β as factors to evaluate interactions between both stressors. Additionally, three-way ANOVA with Sex as the third factor was used for analyzing offspring weight, followed by two-way testing of males and females separately. Significant results were further explored with post hoc testing with correction for multiple comparisons. For correlation analyses, Z-transformation of plasma corticosterone concentrations was applied to calculate correlation coefficients and linear regression

with weighted least squares was performed. Observational data are presented as mean $\pm$ standard error of the mean (SEM) with graphs depicting untransformed data. Data shown for offspring (Figure 3) are marked F for females and M for males in the upper left corner of the graph and depicted in red and blue (grey in print), respectively. F0 data are depicted in purple (grey in print). For gene expression analyses, values are depicted in scatter dot plots showing transformed data where applicable, as indicated on the Y-axis. Logarithmic transformations with base 10 ($\log_{10}$) are shown with the median, whereas other graphs are shown with mean $\pm$ SEM. Maternal data are marked F0, while offspring results are marked F1. Saline injections are shown as circles ($\circ$), whereas IL-1 β treatments are depicted as triangles ($\blacktriangle$). A P -value of ≤ 0.05 was deemed significant. P -values between 0.05 and 0.100 were considered as trends or tendencies towards significance. Asterisks indicate significant differences: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Specific P -values of the asterisks are mentioned in the figure captions.

Results

Prenatal multi-hit stress impacted pregnancy evolution, outcome as well as maternal gestational weight gain

Since we previously showed in a single-hit stress model that exposure to ancestral stress shortened gestational length as of F1 generation pregnancies but not in F0 pregnancies [35], we hypothesized that two different stressors applied during the same gestation would have a similar effect on the F0 generation. With regard to gestational length, gestational exposure to either Stress (525.53 ± 1.33 h), IL-1 β (530.06 ± 2.51 h) or both factors (522.30 ± 5.57 h) did not

Figure 2. Gestational stress affected F0 pregnancy and maternal outcomes. F0 dams were exposed to psychological stress, indicated as Stress, and/or IL-1 β injections. (A) Gestational exposure to both Stress and IL-1 β led to an apparent increase in pregnancy complications and adverse pregnancy outcomes in the F0 generation, including preterm and post-term delivery. Dashed lines depict two standard deviations above and below the mean gestational length of N/Sa animals with confirmed gestational length, as shown by the dotted line. Half black/half colored symbols represent animals for which the gestational length was calculated based on day of delivery or complication as no video-confirmed time of delivery was available. Treatments administered are indicated below the graph. (B) Two-hit stress exposure expanded the variation in gestational length, as evident from a 4-, 6- and 13-fold increase in adjusted coefficient of variation (CV), respectively. (C) Maternal weight gain in the second trimester (GD11-18) was affected by the S*IL-1 β interaction. Post hoc tests with correction for multiple comparisons are shown. N = 7-8-6-8 dams, respectively. (D) By GD21, several animals had delivered or were euthanized. No interaction effect could be observed for weight gain between GD18 and GD21, only a main effect of IL-1 β remained (n = 6-8-6-5 dams). (E-G) Increased corticosterone levels (ng/mL) on GD18 were associated with lesser absolute weight gain (g) by GD18. The graphs show (E) all F0 mothers, (F) mothers exposed to any type of stressor (all N/Sa dams removed), and (G) S/IL-1 β dams only. The correlation coefficient R is indicated. Asterisks denote significant differences: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. CV: coefficient of variation; F: filial generation; GD: gestational day.

Figure 3. Prenatal stress impacted F1 offspring's weight. Female (red online and grey in print, A and B) and male (blue online and grey in print, C and D) offspring weights on P1 and P21 are shown. (A) An $S \times IL-1\beta$ interaction effect was seen in F1 females for their P1 weight. (B) This effect remained present until weaning on P21. $*P < 0.05$; $**P = 0.01$; $***P < 0.001$. (C) Stress and $IL-1\beta$ independently affected F1 male birth weight without interaction. (D) As of P7, only Stress on its own affected male offspring, which was consistent until P21. $*P < 0.05$; $**P < 0.01$. P1 offspring: $n = 44$ – 42 – 37 – 35 females/group and 35 – 40 – 41 – 43 males/group; P21 offspring: $n = 42$ – 40 – 35 – 26 females and 33 – 38 – 38 – 35 males/group, respectively. F: female; M: male; P: postnatal day. This figure is available in color at *Biology of Reproduction* online.

significantly impact pregnancy duration as compared to the N/Sa control group (527.03 ± 1.93 h) when analyzing only mothers with confirmed, video-recorded gestational length. There was an apparent rise in pregnancy complications and adverse pregnancy outcomes, including preterm and post-term delivery (Figure 2A). Two $S/IL-1\beta$ dams were euthanized during pregnancy due to complications, one N/ $IL-1\beta$ mother during labor because of dystocia. In addition, three dams (one each of N/Sa, S/Sa, and $S/IL-1\beta$) stopped gaining weight after the start of the psychological or immune stress treatments. These animals were not included in the analysis of gestational length as no accurate time of delivery was available. They are depicted in Figure 2A with half black/half colored symbols indicating that their gestational lengths were calculated based on the day of complication or weight loss. Despite the lack of significant difference in length of gestation between the groups, the interaction between Stress and $IL-1\beta$ did increase the variation in gestational length for the two-hit group when including all dams (adjusted coefficient of variation; $S \times IL-1\beta$ $F(1,25) = 10.906$, $P < 0.01$; Figure 2B).

Absolute maternal gestational weight gain between GD11 and GD18 was reduced after exposure to two different stress hits, although litter sizes were not statistically different. An interaction effect was present between Stress and $IL-1\beta$ ($F(1,25) = 4.499$, $P < 0.05$), while neither stressor on its own had a similar influence (Figure 2C). On GD18, $S/IL-1\beta$ dams had gained on average 20 g less than their peers. However, by GD21 there was no interac-

tion visible anymore—only a main effect of $IL-1\beta$ ($F(1,21) = 6.237$, $P < 0.05$; Figure 2D).

Additionally, in spite of the absence of differences in prenatal glucose and corticosterone levels, a linear relationship between absolute maternal weight gain by GD18 and the serum corticosterone concentration on GD18 was found. Increased corticosterone levels were associated with less weight gain by the end of the second trimester. This was the case when including all F0 mothers (Pearson $R = 0.51$, $P < 0.01$), with exclusion of all N/Sa animals ($R = 0.53$, $P < 0.05$) and when analyzing the two-hit group alone, $R = 0.80$, $P < 0.05$ (Figures 2E–G).

Prenatal stress affected offspring growth trajectories in a gender-specific way

The effects of prenatal exposure to Stress and/or $IL-1\beta$ on body weight of the F1 generation were visible starting from P1. Female and male pups appeared to respond differently to both stressors, with male offspring also being heavier than their female counterparts at all times (three-way ANOVA, main effect of Sex on P1 ($F(1,309) = 44.576$, $P < 0.001$) and P21 with $F(1,279) = 4.840$, $P < 0.05$). In F1 females, an $S \times IL-1\beta$ interaction effect was seen ($F(1,154) = 6.645$, $P < 0.05$; Figure 3A). Female offspring of $S/IL-1\beta$ dams were 10–15% lighter than their peers on P1 (all $P < 0.001$), while N/ $IL-1\beta$ pups had a lower birth weight than

control animals as well ($F(1,154) = 4.285$, $P < 0.05$). This interaction effect remained present until after weaning on P21 ($F(1,139) = 7.488$, $P < 0.01$; Figure 3B).

Male pup weight on P1 on the other hand was independently affected by prenatal psychological stress ($F(1,155) = 5.285$, $P < 0.05$) and IL-1 β ($F(1,155) = 9.391$, $P < 0.01$) on their own, but without interaction between the factors (Figure 3C). As of P7, no effects of gestational IL-1 β injection were seen, whereas Stress on its own affected male progeny weight until P21 ($F(1,139) = 6.137$, $P < 0.05$; Figure 3D). Both male and female F1 offspring had caught up in weight by P30 (not shown).

Gestational stressors impacted uterine mRNA expression in a generation-dependent manner

We analyzed mRNA abundance in the uterus of F0 mothers, collected at weaning on LD21, as well as in F1 uterine tissues. Their uteri were retrieved on P160 as virgin adults. Barring few exceptions, IL-1 β exposure tended to upregulate the expression of the genes evaluated in maternal uteri, while Stress on its own hardly had any effect. Interestingly, most genes in F1 organs displayed an expression pattern reverse to the one in their mothers.

Stat5b, Zeb1, and Zeb2

The F0 uterine expression of the three genes analyzed, known to be involved in local progesterone metabolism and uterine contractility [48, 49], was unaffected by either stressor (Figure 4A–C). In their F1 daughters, maternal IL-1 β treatment induced a downregulation of uterine expression of *Stat5b* ($F(1,14) = 8.444$, $P < 0.05$; Figure 4D) and *Zeb2* ($F(1,14) = 4.894$, $P < 0.05$; Figure 4F). Stress on its own or in combination with IL-1 β had little to no effect. *Zeb1* expression in F1 uteri was not significantly impacted by maternal immune activation (Figure 4E).

Crh and its receptors

In F0 uteri, the expression of *Crh* and its receptor *Crhr1* was respectively doubled ($F(1,20) = 7.090$, $P < 0.05$; Figure 5A) and tripled ($F(1,13) = 13.125$, $P < 0.01$; Figure 5B) after exposure to IL-1 β , with or without association with Stress. In F1 animals, a main downregulatory effect of Stress could be found for *Crh* and *Crhr1* ($F(1,14) = 5.754$, $P < 0.05$ and $F(1,14) = 5.961$, $P < 0.05$, respectively), with the low expression in the S/IL-1 β progeny contributing substantially to this. No significant interaction of Stress with IL-1 β was present (Figure 5D and E). *Crhr2* showed few consequences of administration of the stressors in maternal F0 uteri (Figure 5C), while a tendency towards downregulation was present in their daughters' uteri (S*IL-1 β $P = 0.074$; Figure 5F).

Glucocorticoid and mineralocorticoid receptors (Nr3c1/2) and 11 β -hydroxysteroid dehydrogenases (Hsd11bs)

Neither IL-1 β nor Stress significantly altered the mRNA expression of the glucocorticoid receptor *Nr3c1* or the enzyme *Hsd11b2* in either generation (F0 Figure 6A and B; F1 Figure 6C and D). There were no identifiable effects on the abundance of the mineralocorticoid receptor *Nr3c2* or enzyme *Hsd11b1* in F0 tissues (Figure 6E and F). The former did display a pattern similar to *Crh* and *Crhr1* in F1 uteri, although it did not reach significance (Stress $P = 0.077$). *Nr3c2* expression was the lowest after antenatal exposure to both Stress and IL-1 β (Figure 6G). As for *Hsd11b1*, the isoform of 11 β -hydroxysteroid dehydrogenase which reduces 11-dehydrocorticosterone back to its active form [50], the S*IL-1 β

interaction brought down expression in F1 adult virgin uteri to levels similar to those in the N/Sa animals as compared to either stressor alone ($F(1,11) = 19.147$, $P = 0.001$; Figure 6H).

The IL-1 receptor complex

The active receptor *Il1r1* and decoy *Il1r2* were not significantly influenced by either stressor in F0 mothers or their daughters (F0 Figure 7A and B; F1 Figure 7C and D). Maternal uterine expression of the IL-1 receptor accessory proteins was not different between the groups for *Il1rap* (Figure 7E), but showed a tendency toward an upregulatory IL-1 β effect for *Il1rapb* ($P = 0.066$; Figure 7F). On the contrary, in F1 uteri, Stress and IL-1 β interacted leading to a major decrease in abundance of *Il1rap* ($F(1,14) = 5.711$, $P < 0.05$) and *Il1rapb* ($F(1,14) = 4.617$, $P = 0.05$). For F1, *Il1rap* uterine mRNA expression in two-hit offspring was more than 60% lower than in control animals, while this amounted to 70% as compared to each stressor on its own (Figure 7G). As shown in Figure 7H, the S*IL-1 β interaction induced a downregulation of *Il1rapb* of 72% on average.

IL-1 β induces cytokine mRNA expression in F0 uteri at weaning, while S*IL-1 β interaction mediates the effects of single-stress hits in their daughters

As was the case for the genes discussed above, expression patterns of the IL-1 receptor ligands differed between F0 and F1 uteri. While neither Stress nor IL-1 β exposure had any effect on the *Il1a* mRNA expression in F0 maternal uteri (Figure 8A), in offspring exposed to both factors *Il1a* expression was reduced to levels below those of the control group (S*IL-1 β $F(1,17) = 12.078$, $P < 0.01$; Figure 8B). With regard to *Il1b* expression, IL-1 β upregulated its own mRNA abundance in the F0 generation ($F(1,16) = 4.497$, $P = 0.05$; Figure 8C), but was not significantly altered in offspring uterine tissues ($P = 0.077$; Figure 8D). In F0 uteri, *Il1rn*, a member of the IL-1 cytokine family functioning as an endogenous receptor antagonist [58], was increased by both IL-1 β ($F(1,21) = 6.691$, $P < 0.05$) and Stress ($F(1,21) = 5.094$, $P < 0.01$) separately, with the highest expression in the S/IL-1 β animals (Figure 8E). On the contrary in the F1 generation, addition of IL-1 β to the antenatal Stress treatment mitigated the effects of both individual stressors (S*IL-1 β $F(1,15) = 17.350$, $P = 0.001$; Figure 8F). Stress and IL-1 β significantly amplified *Il1rn* on their own as compared to N/Sa uteri, an effect that was undone by the interaction between the factors. The expression of *Il6*, a cytokine known to be involved in the timing of parturition [59], was more than eight times higher in F0 IL-1 β treated uterine tissues, whether or not dams were subjected to psychological Stress ($F(1,18) = 13.804$, $P < 0.01$; Figure 8G). However, as depicted in Figure 8H, their offspring's uteri displayed an S*IL-1 β interaction effect, with the two-hit group having the lowest *Il6* expression levels ($F(1,16) = 8.190$, $P < 0.05$). The anti-inflammatory cytokine *Il10* was upregulated in postpartum uteri in the IL-1 β treated mothers as well ($F(1,19) = 8.428$, $P < 0.01$; Figure 8I). The expression pattern in F1 uterine tissues was rather opposite (Figure 8J).

Discussion

Using our two-hit model of maternal stress to explore the interaction between prenatal psychological and immune stress, we demonstrate here that exposure to two distinct prenatal stressors affect pregnancy, maternal, and offspring outcomes differently when combined as compared to either hit alone. Our findings further advocate that exposure to (a combination of) distinct prenatal stressors in a

Figure 4. Arbitrary *Stat5b*, *Zeb1*, and *Zeb2* mRNA levels in F0 and F1 uteri showed little changes after prenatal Stress and/or IL-1 β exposure. F0 uteri were collected at weaning on lactational day 21. F1 adult virgin females were sacrificed on postnatal day 160. Treatments are shown as “○” for Saline and “▲” for IL-1 β . Expression levels of (A) *Stat5b*, (B) *Zeb1*, and (C) *Zeb2* were not significantly affected by prenatal stressors in F0 uteri ($n = 6-7-6-6$ dams). Prenatal exposure to IL-1 β did reduce the abundance of (D) *Stat5b* and (F) *Zeb2* in F1 uterine tissues ($n = 3-6-4-5$ for both genes, *Zeb2* reciprocal transformation), whereas the abundance of (E) *Zeb1* was not significantly changed ($n = 3-6-3-5$). Data transformations prior to analyses are indicated on the Y-axis. Mean $\pm$ SEM or median for log-transformed data are shown. Asterisks indicate significant differences: * $P < 0.05$. 1/x: inverse transformation; F: filial generation; log₁₀: base 10 logarithmic transformation. This figure is available in color at *Biology of Reproduction* online.

Figure 5. Psychological stress and IL-1 β affected mRNA expression of genes in the uterine CRH system differently in F0 and F1. (A and B) Relative mRNA expression of *Crh* and *Crhr1* in F0 uteri at weaning ($n = 5-7-6-6$ and $n = 4-5-5-3$, respectively). IL-1 β exposure induced a two-fold increase in the abundance of (A) *Crh* and tripled the expression of (B) *Crhr1*. (C) The F0 mRNA expression profile of *Crhr2* showed little effects of gestational stress ($n = 6-7-6-5$). (D and E) In F1 tissues, Stress downregulated (D) *Crh* and (E) *Crhr1* mRNA. (F) A trend toward downregulation of *Crhr2* could be observed in the F1 generation ($P = 0.074$; $n = 3-6-4-5$ for all three genes). Mean $\pm$ SEM are indicated except for log₁₀ transformations, which are shown with the median value. * $P < 0.05$; ** $P < 0.01$. F: filial generation; log₁₀: base 10 logarithmic transformation. This figure is available in color at *Biology of Reproduction* online.

Figure 6. Prenatal stressors differentially altered F0 and F1 uterine gene expression related to glucocorticoid downstream signaling and metabolism. Expression levels of the glucocorticoid receptor *Nr3c1* (A and C) and *Hsd11b2* (B and D) were not affected by gestational stress in either generation. F0 $n = 6-7-6-6$ dams; F1 $n = 3-6-3-4$ and $3-6-4-5$, respectively. (E and F) Neither Stress nor IL-1 β significantly changed the mRNA abundance of (E) the mineralocorticoid receptor *Nr3c2* or (F) *Hsd11b1* in maternal tissues ($n = 6-7-6-6$ dams). (G) *Nr3c2* expression showed a tendency towards a Stress effect in F1 samples ($P = 0.077$; $n = 3-4-4-4$). (H) Two-hit maternal stress downregulated offspring *Hsd11b1* expression as compared to either stressor alone ($n = 3-3-4-5$ animals). Data shown after transformation when applicable with mean $\pm$ SEM or median (log-transformed values) indicated. ** $P < 0.01$; *** $P = 0.001$. 1/x: inverse transformation; F: filial generation; log₁₀: base 10 logarithmic transformation. This figure is available in color at *Biology of Reproduction* online.

Figure 7. Effects of prenatal two-hit stress on IL-1 receptor complex gene expression in F0 and F1 uteri were limited to the IL-1R accessory proteins. (A–D) mRNA expression patterns of *Il1r1* and *Il1r2* were not significantly affected in (A and B) F0 and (C and D) F1 uteri (F0 $n = 6-6-6-5$; F1 $n = 3-6-3-5$). (E) *Il1rap* abundance did not change after gestational stress in maternal tissues ($n = 6-7-6-5$). (F) A trend toward upregulation was found for F0 *Il1rapb* ($P = 0.066$; $n = 6-7-5-5$). (G and H) Interactions between Stress and IL-1 β more than halved F1 mRNA expression of (G) *Il1rap* and (H) *Il1rapb* while each stressor on its own did not have an effect (reciprocal transformation; $n = 3-6-4-5$). Graphs show transformed data when applicable, as indicated on the Y-axis. Mean $\pm$ SEM or median (log-transformation) are displayed. Asterisks indicate significant differences: * $P < 0.05$; ** $P < 0.01$ except *Il1rap* N/IL-1 β vs. S/IL-1 β ** $P = 0.01$. 1/x: inverse transformation; $\sqrt{\cdot}$: square root transformation; F: filial generation; log₁₀: base 10 logarithmic transformation. This figure is available in color at *Biology of Reproduction* online.

single generation has both immediate and long-lasting implications on the metabolic and uterine molecular level, affecting mRNA abundance of genes involved in local progesterone metabolism, stress processes as well as inflammation, and that these effects are transmitted in an intergenerational manner. Conversely, these effects are not straightforward but appear generation-specific, which is suggestive of additional, postnatal effects on offspring, potentially indicating epigenetic transmission of signatures linked to environmental stressors.

Models of cumulative stress

We developed the two-hit model inspired by our previous findings that effects of ancestral psychological stress start from the F1 or F2 generation [35]. The observation that no differences were seen in the stressed parental generation in our earlier model led to the hypothesis that in rats, similar to humans, a single stressor is insufficient for adverse outcomes. Additionally, although the experience of stress is inherent to life and pregnancy, a single stressor is hardly representative of day-to-day life. Moreover, while PTB can be induced in mice

Figure 8. Relative gene expression of cytokines in F0 and F1 uterus was affected by prenatal two-hit stress in a stressor- and generation-dependent manner. (A) Whereas neither stressor affected maternal gene expression ($n = 6-7-6-6$ dams), (B) two-hit stress cancelled out the individual effects of Stress and IL-1 β on the expression of *Il1a* mRNA in the F1 generation ($n = 5-6-4-5$). (C) IL-1 β stimulated its own expression in parental F0 uterus ($n = 6-6-3-5$; $*P < 0.05$). (D) No significant difference was found for *Il1b* in F1 tissues ($n = 5-4-4-5$). (E) Uterine *Il1m* expression in F0 tissues was upregulated by Stress and IL-1 β on their own ($n = 6-7-6-6$). (F) In F1 uteri, the S*IL-1 β interaction negatively affected *Il1m* mRNA levels as compared to each stressor on its own ($n = 5-4-4-5$). (G) While Stress had no effect on *Il6* abundance in F0 uteri, IL-1 β led to an 8-fold increase of expression (reciprocal transformation; $n = 6-7-4-5$). (H) Similar to the expression of *Il1a* and *Il1b*, in F1 samples S*IL-1 β inverted the effects of each stressor on its own on *Il6* ($n = 5-5-4-5$). (I and J) Direct and indirect exposure to IL-1 β appeared to have inverse effects on uterine *Il10* expression. (I) Abundance of the anti-inflammatory cytokine *Il10* was increased by IL-1 β injections in F0 uteri ($n = 4-7-6-6$). (J) An opposite expression pattern was found in their F1 offspring uterine tissues ($n = 4-5-4-4$). Data are shown after transformations as indicated on the Y-axis. Mean $\pm$ SEM or median for log₁₀-transformed data are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. 1/x: inverse transformation; $\sqrt{}$: square root transformation; F: filial generation; log₁₀: base 10 logarithmic transformation. This figure is available in color at *Biology of Reproduction* online.

by exposure to endotoxins or IL-1 β alone [21], this is not the case in rats [51]. The latter is in accordance with the cumulative stress or multiple-hit hypothesis [60, 61] and the concept of allostatic load [18, 62, 63]. As per these frameworks, a single or low number of hits is not enough to cause harm. When evaluated within the realm of stress, the physiological and behavioral changes that ensue are necessary to adapt to the changing environment. As long as these responses are limited in time and to the stressor, they are beneficial. However, in the event of repeated or chronic stress one develops a stress or allostatic load, representing the “wear and tear” of these stressors on the mind and the body [62, 63]. When the body’s ability to cope is surpassed and the otherwise normal adaptive processes become harmful, adverse outcomes can develop. These concepts can also be applied to pregnancy and maternal stress load, with (early) activation of the parturition cascade, adverse pregnancy outcomes, and perinatal programming occurring once this threshold is crossed [18]. Indeed, adverse outcomes in our studies only occur after piling up of stressors, including multigenerational stress or distinct prenatal stressors, as demonstrated here [35, 64, 65].

When interpreting the presented data, however, it is imperative to keep in mind the inherent differences between mammals with

regard to development. This is especially true for the brain, including the HPA axis and its controlling brain regions [66] and the immune system [67]. In fact, although humans are conventionally considered to be immature when born, the brain is fairly well developed at birth, relative to rodents [66, 68, 69]. Consequently, maternal postpartum behavior in rodents should most likely be taken into account when assessing perinatal programming effects on the brain, the immune system, and potentially peripheral neuroendocrine systems. Maternal behavior is impacted by both gestational [70, 71] and ancestral stress [35, 47], which can lead to suboptimal maternal care; therefore, altered maternal behavior could function as a separate stressor, adding to the allostatic load. The transgenerational transmission of a single stressor, as we demonstrated in our previous study, is in line with this hypothesis [35].

Biological effects of maternal two-hit stress

Despite the presence of increased variation in F0 gestational length as well as higher occurrence of adverse events including early delivery, resorption, and post-term birth after exposure to both stressors, we were not able to show that maternal stress, whether caused by

psychological stress, IL-1 β or both, resulted in shorter gestations. This is consistent with other research in rodents as well as our previous multigenerational model where shorter pregnancy duration was only visible as of the F1 pregnancies [35, 72]. In contrast, other studies demonstrated that prenatal restraint or immune stress reduces pregnancy duration in the parental generation [73, 74]. It is possible that the stressors applied are not intense enough and in order to deliver early, these animals would need an additional hit, for instance by having the added effect of altered maternal behavior on pregnancy outcomes, as we have seen in prior studies [35, 47]. Furthermore, several dams in the S/IL-1 β group experienced severe complications and were euthanized; therefore, the exact timing of the end of pregnancy was not recorded and the dams were not included in the final analysis.

In like manner, maternal stress quite consistently leads to decreased weight gain during pregnancy in most animal models with respect to the metabolic effects of antenatal adversity [35, 74, 75]. Gestation however is considered a state of physiological hyperphagia to meet the increased energy demands of both mother and fetus [76]. In the present study, we found that psychological stress or IL-1 β on their own did not affect gestational weight gain between GD11 and 18, but the interaction did. Interestingly, when evaluating weight accumulation between GD11 to GD21, only the IL-1 β effect remained. A possible explanation is the loss of S/IL-1 β dams due to complications as discussed above. It is however known that restraint and other types of stress result in a negative energy balance in nonpregnant animals [77, 78], with longer term weight loss primarily caused by increased thermogenesis through stimulation of the sympathetic nervous system after initial activation of the CRH system [79]. However, we were unable to demonstrate any changes in glycaemia or corticosterone levels as measures of stress between the groups on G18 or LD1. These findings in maternal blood are somewhat consistent with the studies by the group of Brunton [80, 81]. They showed that social defeat stress induces an increase in corticosterone levels in F0 pregnant animals; however, only on the first 4 days of stress exposure and at lower levels than virgin animals [80]. Conversely, our prior work demonstrated that these metabolic changes were not detected until pregnancy in F2 dams after multigenerational stress [35]. Taken together, our results emphasize the impact of stressor-related parameters, including amount, nature, and intensity as well as duration, timing of exposure and previous stressful experiences on the downstream consequences of prenatal stress.

Molecular consequences of maternal two-hit stress

A main finding of the present study is that prenatal stress appears to affect the uterine mRNA expression of genes considered part of the local neuroendocrine and immune systems and progesterone metabolism. Interactions between these systems both on a central and local level are known to play an essential role in maintenance of pregnancy and regulation of parturition [17, 82]. CRH and IL-1 β , produced by reproductive tissues and inflammatory cells alike, fulfill key roles in those interactions on both levels, and interrelate with prostaglandins and steroid hormones [83, 84]. The stimulatory effects of IL-1 and CRH on cytokine and chemokine production [17, 19, 85] and the effects of CRH during pregnancy on myometrium [17, 86, 87], placenta [88, 89] and fetal membranes [90], are widely recognized. CRH and its analogs as well as their receptors play important roles in the relative quiescence of the uterus during pregnancy as well as myometrial contractility and interact with cytokines for both processes [17, 52, 86]. Thus, it should come as

no surprise that increases in maternal circulating IL-1 β upregulated the F0 mRNA expression of *Crb* and its receptor *Crb1*, as well as the abundance of *Il1b* itself, *Il1rn*, *Il6*, and the anti-inflammatory cytokine *Il10*.

In opposition to our findings in F0 samples, F1 perinatal exposure to both stressors led to a major downregulation of abundance of *Hsd11b1*, which interconverts corticosterone to its inactive form and back [50]. The expression of both *Il1rap* and *Il1rapb* as well as the pro-inflammatory cytokines *Il1a*, *Il1rn*, and *Il6* was extensively reduced as well. Also, a trend toward interaction effects of Stress and IL-1 β with regard to the mRNA expression of *Crb2* was observed. Furthermore, the expression patterns in IL-1 β -treated progeny were consistently opposite to those in their mothers, with F1 displaying downregulation of abundance.

A possible explanation for this surprising phenomenon is that the S*IL-1 β interactions observed in the F1 samples as well as the reversal of the mRNA expression patterns between the generations are an example of fetal programming and fit within the match/mismatch framework or predictive adaptive response concept [61, 91, 92]. These hypotheses refer to a form of developmental plasticity in which the fetus or infant incorporates early life events, thereby developing a phenotype that is optimally adapted to the conditions it expects to encounter later in life. In the current study, this foreseen environment containing similar adversities would be one in which the individual will experience a high stress and inflammatory load, “matching” the early life milieu. Therefore, the major downregulation of mRNA abundance of the aforementioned genes in the S*IL-1 β offspring would indicate a lowering of the baseline expression to accommodate future exposure to stressors and would not necessarily be a maladaptation. Yet, in the event of a mismatch between the expected and experienced environment during adulthood, the perinatal programming effects would turn the resultant physiologic state harmful and result in increased vulnerability for pathology, for instance increases in adverse pregnancy outcomes. This would be in accordance with the association between early life adverse events and reduced HPA axis reactivity to acute stressors [93], although in general prenatal stress is thought to sensitize the HPA axis (for a review on early life stress effects on the HPA axis, see Ref. 94).

On that note, it is possible that local neuroendocrine and immune systems are regulated in a different manner than the central stress response system, and therefore perinatal programming results in differential gene expression between both levels. It is generally accepted that differences in epigenetic regulation of transcription are at the root of this phenomenon [95]. In this respect, perinatal stress programming could result in different end results depending on the organ system or tissue analyzed. Most evidence regarding the influence of prenatal and transgenerational stress on the epigenome points toward a central role of both the maternal and fetal/neonatal HPA axes accompanied by increased glucocorticoid exposure [96] as well as direct effects of in utero and postnatal environmental exposure, such as maternal care [97, 98]. While an abundance of literature is available regarding effects of stress on the central stress response system, to the best of our knowledge programming of the uterine transcriptome by prenatal stress has only been shown for environmental toxicants including endocrine disruptors [99–101] and in our previous work [35]. Nevertheless, our data are sufficient to strongly suggest intergenerational programming effects of maternal stress in uterus as well, possibly creating an opportunity to elucidate the epigenetic mechanisms underlying these findings as well as extend the scope of epigenome research beyond the more obvious and extensively studied organs and genes.

Conclusions

In summary, the two-hit stress model presented here was successful in demonstrating the cumulative effects of distinct prenatal stressors on pregnancy outcomes. Effects of maternal stress are also visible on the molecular level on local uterine progesterone, neuroendocrine and immune systems, involved in numerous aspects of uterine physiology, especially regulation of uterine quiescence and contractility. While the findings of the present study strengthen the hypothesis that multiple-hit stress has different effects than single stressors, these outcomes are not straightforward and appear dependent on the nature of the stressor(s), timing since exposure and the generation. Indeed, prenatal exposure to both stressors led to dampening of uterine gene expression in F1 progeny, which could fit within either the cumulative stress framework or the match/mismatch concept, although these hypotheses are not necessarily mutually exclusive. Altogether, this is suggestive of epigenetic mechanisms mediating the programming effects of prenatal stressors on the uterus. For a better understanding of the mechanisms behind these results and in order to elucidate which framework our model fits into, the F1 generation should be exposed to the vulnerable situation of pregnancy. Such studies could yield valuable information indicating whether these findings are a form of maladaptation or a sign of resilience. Furthermore, additional research may point towards interventions to mediate maternal stress and potentially reverse these epigenetic effects, both in rats and humans.

Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1093/biolre/btzy001) online.

Supplemental Table S1. Overview of the genes analyzed, their respective proteins, and specific roles in the uterus.

Supplemental Table S2. Quantitative RT-PCR forward and reverse primer sequences. The annealing temperature T_A is indicated per primer pair.

Supplemental Figure S1. Quantitative RT-PCR threshold cycles (C_T) of the housekeeping gene Cyclophilin A (*Ppia*). Graphs depict data from two different qPCR runs of (A-B) F0 and (C-D) F1 tissue samples with F0 n = 6-7-6-6 and F1 n = 3-6-4-6 animals. No significant differences were observed. Mean $\pm$ SEM is indicated. F: filial generation.

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