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Expression of stress-mediating genes is increased in term placentas of women with chronic self-perceived anxiety and depression

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Abstract: Anxiety, chronical stress and depression during pregnancy are considered to affect the offspring, presumably through placental dysregulation. We have studied the term placentae of pregnancies clinically monitored with the Beck's Anxiety Inventory (BAI) and Edinburgh Postnatal Depression Scale (EPDS). A cutoff threshold for BAI/EPDS of 10 classed patients into an Index group (>10, n=23) and a Control group (<10, n=23). Cortisol concentrations in hair (HCC) were periodically monitored throughout pregnancy and delivery. Expression differences of main glucocorticoid pathway genes: i.e. corticotropin-releasing hormone (CRH), 11β-hydroxysteroid dehydrogenase (HSD11B2), glucocorticoid receptor (NR3C1), as well as other key stress biomarkers (Arginine Vasopressin, AVP and O-GlcNAc transferase, OGT) were explored in medial placentae using real-time qPCR and western blotting. Moreover, gene expression changes were considered for their association with HCC, offspring, gender and birthweight. A significant dysregulation of gene expression for CRH, AVP and HSD11B2 genes was seen in the Index group, compared to controls, while OGT and NR3C1 expression remained similar between groups. Placental gene expression of the stress-modulating enzyme 11β-hydroxysteroid dehydrogenase (HSD11B2) was related to both hair cortisol levels (Rho= 0.54; $p<0.01$) and the sex of the newborn in pregnancies perceived as stressful (Index, $p<0.05$). Gene expression of CRH correlated with both AVP (Rho= 0.79; $p<0.001$) and HSD11B2 (Rho= 0.45; $p<0.03$), and also between AVP with both HSD11B2 (Rho= 0.6; $p<0.005$) and NR3C1 (Rho= 0.56; $p<0.03$) in the Control group but not in the Index group; suggesting a possible loss of interaction in the mechanisms of action of these genes under stress circumstances during pregnancy.

Keywords: antenatal stress; hair cortisol; term-placentae; RT-qPCR; human

1. Introduction

Antenatal maternal stress such as anxiety and depression have been widely associated with short- and long-term negative impact on the neurobiological and physiological functioning of the offspring [1–3]. Maternal distress during pregnancy and puerperium, which in Sweden has a 15% incidence [4–6], is apparently linked to newborns being at high risk of reduced birthweight, smaller head circumference and adverse neurodevelopmental outcomes; including increased hypothalamic–pituitary–adrenal axis (HPA) sensitivity, anxiety, depressive-like behaviors, attention deficit, hyperactivity, poor cognitive or emotional developmental disorder, among others [7–9]. Additionally, both the timing of action of these stressors along pregnancy and the sex of the offspring have been identified as key factors in the increased incidence of offspring disease development associated with maternal stress. Gerardin et al. (2011), reported that sons of mothers suffering from depression during pregnancy showed deficits in the regulation of motor skills and of behavior, whereas changes of the latter were not detected in daughters [10]. Also, children born from pregnancies where the mother was exposed to stressors during mid-gestation (months 3–5) were at higher risk for developing schizophrenia than those whose mothers were exposed to stressors during late pregnancy [11–13].

Stress factors during pregnancy lead to hypercortisolemia, poor reactivity and major circadian rhythm fluctuations of cortisol [14]. Maternal cortisol passes physiologically into the fetus yet being mostly inactivated by the placenta thus avoiding deleterious effects [15,16]. However, from week 20 of gestation, cortisol presence induces placental production of CRH which increases fetal cortisol production [17]. Of interest, it is during this period when the maternal HPA-system becomes increasingly refractory to stress, so the fetus may not be as prone to fluctuations of maternal cortisol. We have recently monitored maternal cortisol during pregnancy, in primiparous and multiparous women in hair (HCC), which better reflects its overall long-time biological activity. Cortisol levels were determined as ascending during pregnancy, to dip more strongly in multiparous 3 months prior to partum compared to primiparous, thus suggesting a quicker suppression of the hypothalamic CRH production by placental CRH in multiparous women (Marteinsdottir et al., manuscript). Under physiological conditions, the release of CRH is apparently regulating the timing of delivery, remodeling the fetal nervous system, and influencing developmental trajectories [18]. However, placental CRH seems to be significantly elevated in response to physical and behavioral maternal stress and may be an integrative marker of early adversity [19]. Nonetheless, the mechanisms underlying the manifestation of these maternal stressors in the placenta need to be further investigated.

Placental mechanisms underlying stress-induced elevations in maternal glucocorticoids as a key mechanism of stress transmission to the fetus [20] remain yet unclear, in the light of other maternal systems mediating stress responses beyond the HPA-system, i.e. the arginine vasopressin/oxytocin (AVP) or the autonomic nervous (ANS) systems. Maternal stress influencing placental function via glucocorticoid (GC) act via specific receptors (NR3C1) [21,22] and the modulatory enzyme 11b-hydroxysteroid dehydrogenase type 2 (11-β-HSD-2) [15,16]; altering expression of HPA-related genes which rule the stress-modulating enzyme O-LcNAc transferase (OGT) [23].

Here, we hypothesize that perceived maternal stress during pregnancy is capable of triggering stress-related molecular signaling in term placenta which could potentially affect fetal

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developmental outcomes, related to offspring gender/weight. To test this hypothesis, we have determined the term-placental expression of main glucocorticoid pathway genes: i.e. corticotropin-releasing hormone (*CRH*), 11β-hydroxysteroid dehydrogenase (*11β-HSD2*), glucocorticoid receptor (*NR3C1*), as well as other key stress biomarkers (Arginine Vasopressin, *AVP* and O-GlcNAc transferase, *OGT*) and their association with maternal and fetal HCC levels, offspring gender and birth weight.

2. Materials and Methods

2.1 Ethical considerations

The integrity of the patients has been granted by Ethical permits warranting full information prior to consent and full anonymity. Data was treated at group levels. No individual is to be identifiable in the publication. This is an established procedure in Swedish clinical investigations and it is fully described in the Ethical Perspectives in Neurology section (EPNs) permissions already obtained. All examinations and tests are harmless and have been used in several previous clinical studies. All data was treated coded and anonymously. The epidemiological surveys are already approved by the Human Research Ethics Committee Linköping (03-556,07-M66 08–08-M 233-8, 2017/513-31). The study was approved by the Regional Ethical Review Board in Linköping (nr 2011/499-31 & 2013/355-32).

2.2 Experimental design

A total of 390 pregnant women attending an antenatal care clinic in southeast Sweden were included in the study. The women completed anxiety and depression inventories and underwent hair cortisol collection on week 24-25, during childbirth and postpartum. Self-perceived symptoms of anxiety were assessed with the Beck's Anxiety Inventory (BAI) [24] and symptoms of depression were assessed with the Edinburgh Postnatal Depression Scale (EPDS) (Cox et al., 1987). Both inventories are well known, easy to use, validated in Sweden [25], and often used in research settings and as screening in clinical settings. As a measure of symptoms of depression and anxiety a cut off score of 10 was used for both the EPDS and the BAI.

A total of 23 women scored >10 at both EPDS/BAI – indicating symptoms of depression and anxiety are here referred to as index women. A total of 23 controls who scored <10 on both EPDS/BAI were randomly selected from the entire study population (n= 390). After childbirth, the placentae were immediately collected and stored at -80ºC until further analysis. Data on obstetric and neonatal outcomes were collected from standardized medical records.

2.3 Hair cortisol measurements

Hair cortisol concentrations (HCC) were measured and expressed as pg/mg using a competitive radioimmunoassay in methanol extracts of pulverized hair. An approximately 3 mm thick hair and 3 cm long sample was cut close to the scalp from the posterior vertex area of the head. The hair samples were further cut into 1,25 cm to reflect the cortisol accumulations for each month, based on an assumption of an average hair growth rate of 1 cm per month (Wennig et al., 2000). The hair samples analyzed in this study weighted between 5 mg and 6 mg. In the laboratory, each sample was put into a 2 mL QiaGenRB sample tube together with a 0.5 mm QiaGen stainless steel

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bead and weighed on a Sartorius MC 210p microscale (Qiagen, Hilden, Germany). The samples were put in specially made aluminium cylinders accommodating five 2 mL Eppendorf tubes and frozen in liquid nitrogen for 2 minutes. This was followed by mincing in a Retch Tissue Lyser II at 23 Hz for 2 minutes to produce a fine hair powder. The cortisol was extracted by adding 1 mL of methanol (Chromasolv, Sigma-Aldrich, Darmstadt, Germany) to each tube and placing the tubes in a metal holder on a plate with a 5-degree inclination on a horizontal shaker at room temperature, keeping the steel beads in constant gentle motion within the tubes for a minimum of 10 hours. Finally, the tubes were centrifuged for 1 minute at 13000 g at 4°C in a microcentrifuge (Thermo Scientific, Waltham, MA, USA), and 800 µL of the supernatant were moved to another plastic sample tube for lyophilization in a SpeedVac Plus SC210A (Savant, Coral Springs, FL, USA) using an Edwards XDS 5 vacuum pump for at least 3 hours. The samples were dissolved in radioimmunoassay buffer and analysed as described by Morelius et al 2004 (Morelius et al., 2004). A hair sample of 3–10 mg is needed to maintain a total inter-assay coefficient of variation below 8% for hair extraction and measurement of cortisol by the radioimmunoassay. The intra-assay coefficient of variation for the radioimmunoassay itself was 7% at 10 nmol/L. The antiserum cross-reacts 137% with 5α-dihydroxycortisol, 35.9% with 21-deoxycortisol, and 35.9% with prednisolone, but less than 1% with endogenous steroids (Karlén et al., 2011).

2.4 Tissue collection and preparation

Placenta samples were obtained immediately after childbirth, placed on ice and dissected into one gram of tissue per sample. The umbilical cord sample was cut ≈4 cm away from its site of insertion at the placental disk. The villus parenchyma sections were obtained by dissecting a 1.5-cm square-shaped segment through the entire ≈2.5-cm thickness of the placental disk (≈5 cm away from site of cord insertion) and then splitting it into the following three equal parts: maternal (includes thin basal plate), middle, and fetal (includes the chorionic plate). The amnion and chorion from the reflected membranes were isolated by peeling apart the incompletely fused membranes. We chose an area of the membranes at least 4 cm away from the junction with placenta disk and from the site of rupture of the amniotic bag. The peeled chorion was processed along with its atrophied villi and interdigitated decidua. The samples were snap frozen, packed for final storage at -80°C.

2.5 RNA Extraction

Total RNA was isolated from pools of 4 different segments of placenta samples retrieved from the fetal side using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, tissue samples were mechanically disrupted in 1 mL Trizol reagent using a TissueLyser II (Qiagen, Hilden, Germany). The homogenized tissues were centrifuged at 12,000 × g at 4 °C for 10 min. Then, supernatants were incubated with bromochloropropane (100 µL/mL homogenized) for 5 min at room temperature. Samples were then centrifuged at 12,000 × g at 4 °C for 15 min. The aqueous phases obtained were mixed with isopropanol and RNA precipitation solution (1.2 M NaCl and 0.8 M Na2C6H6O7) and incubated at room temperature for 10 min. Then, samples were centrifuged at 12,000 × g at 4 °C for 10 min. After discarding the supernatant, 1 mL of 75% ethanol was added to the pellet fraction and centrifuged at 7,500 × g at 4 °C for 5 min. The RNA pellets obtained were air-dried for 30 min and mixed with 30 µL of RNase free water. The quantity of the obtained total RNA was measured with a NanoDrop

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ND-1000 (Thermo Fisher Scientific, Fremont, CA, USA). The quality of the samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA-integrity number (RIN) values obtained ranged 8–10.

2.6 Protein extraction

Proteins from placental samples were isolated as previously described [26]. Briefly, 200 µL of RIPA buffer (Sigma-Aldrich, Darmstadt, Germany) mixed with 2 µL of protein cocktail inhibitor (Thermofisher Scientific, Fremont, CA, USA) was added to each sample prior to sonication (Amplitude 50 W, 140). Then, samples were incubated at 4ºC for 60 minutes in rotation and later centrifugated at 13,000 × g at 4ºC for 10 min. After centrifugation, the supernatants were collected and proteins were quantified using a DC Protein assay kit (Bio Rad, Hercules, CA, USA), following manufacturer's instructions. Protein suspensions were denatured by heating at 70 ºC for 10 min and kept at -20ºC until analyses.

2.7 Relative quantitative RT-PCR

First, total RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with 25mM dNTPs Mix, RT random primers, 20U of RNase inhibitor and MultiScribe Reverse Transcriptase according to the manufacturer's protocol. qPCR was performed in 10-µL reactions with 5 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, CA, USA), 50 nM for each set of primers, 2 µL of synthetized cDNA and water to a final volume of 10 µL. All reactions were carried out using the Real-Time PCR Detection System (CFX96™; Bio-Rad Laboratories, Inc; CA, USA). The thermal cycling profile was 50◦C for 2 min, 95◦C for 10 min, 40 cycles at 95◦C for 15 s, and 60◦C for 1 min. Melt curve analysis was carried out to evaluate the specificity of each PCR reaction by detection of one single peak on the dissociation curve profile. The gene relative expression levels were quantified using the $2^{(-\Delta\Delta ct)}$ [27] method and *GAPDH* as a reference gene for cDNA normalization.

2.8 Western blotting (WB)

To prepare Western Blots, 10 µL (5 µg) of each protein suspension were loaded into 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (BIORAD) and transferred to polyvinyldifluoride (PVDF) membranes (BioRad, Richmond, CA). Membranes were then incubated for 1 hour in Odyssey Blocking solution (LI-COR Biosciences, Lincoln, NE, USA) and washed 3x10 minutes in washing buffer (Tris-phosphate-buffered saline) containing 0.1% Tween-20 (Sigma-Aldrich). Then, membranes were incubated at 4 ºC overnight with the primary antibodies (anti-CRH; rabbit polyclonal antibody (LSBio-B11889, LSBio, WA, USA), anti-AVP polyclonal antibody (MBS9205129, MyBiosources, CA, USA) and anti-HSD11B2 polyclonal antibody (ab80317, abcam, Cambridge, UK) at 1:1000, 1:500 and 1:1000 dilution rate, respectively. The day after, the membranes were washed 3x10 minutes and incubated for 1 h with the reference anti-GAPDH rabbit polyclonal primary antibody (ab181602, abcam, Cambridge, UK) at 1:10,000 dilution rate, washed again 3x10 minutes and finally incubated with a secondary antibody (goat anti-rabbit IRDye 800 CW, LI-COR Biosciences, Lincoln, NE, USA) at a 1:20,000 dilution on blocking buffer. After extensive washing, the membranes were scanned using the Odyssey CLx (LI-COR Biosciences, Lincoln, NE, USA), and

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images of the blots were obtained using the Image Studio 4.0 software (LI-COR Biosciences, Lincoln, NE, USA). Raw data comparisons were made only within each blot.

2.9 Statistical analysis

Statistical analyses were conducted using SPSS statistical software (version 24.0; SPSS Inc., Chicago, IL, USA). Gene expression data were analyzed for normality of residuals using the Kolgomorov-Smirnov test. Since data were not normally distributed, the Mann-Whitney U-test was used to analyze the data. Differences were considered significant at $p < 0.05$. Associations of maternal cortisol levels with placental gene expression and associations among gene expression patterns between groups, were analyzed using Spearman´s rho. Gene expression values were represented as log FC ($2^{-\Delta\Delta ct}$) (violin plots) or FC ($2^{-\Delta\Delta ct}$) (graphs).

3. Results

3.1 Prenatal stress influenced gene expression of term-placentas

After qPCR analyses, the data obtained was visualized by a Principal Component Analysis (PCA) plot, in which each data point represents an individual placenta, and each color represents a different group (Index vs Control) (**Figure 1A**). The closer the data points are to each other, the more closely related the transcriptional responses are. Moreover, to gain insight into similarities among replicates, the set of genes tested by qPCR was subjected to a hierarchical clustering procedure and presented as heatmaps (**Figure 1B**). The heatmap indicates that the selected differential gene set associates the biological samples correctly into two groups each representing one of the two conditions (Index vs Control). The heat map reveals that on average, mRNA expression within placental tissues of mothers clinically diagnosed as stressed (Index) was generally higher than the control group.

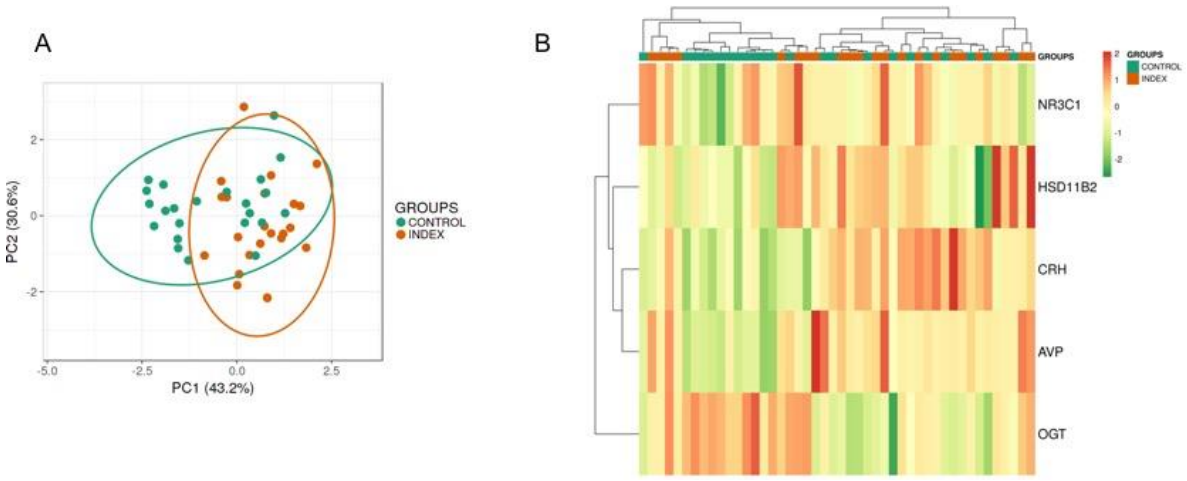

Figure 1A-B. Principal component analysis (PCA) plot of gene expression data of term-placenta of patients indicating symptoms of depression and anxiety (Index; n=23) and patients with no symptoms of depression or anxiety (Control, n=23). A: SVD with imputation is used to calculate principal components. X and Y axis shows principal component 1 and principal component 2 that explain 43.2% and 30.6% of the total variance, respectively. B: Heat map plot of gene expression data of term-placenta samples of patients indicating symptoms of depression and anxiety (Index; n=23) and patients with no

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symptoms of depression or anxiety (Control, n=23). Both rows and columns are clustered using correlation distance and average linkage. Colors represent mRNA levels (red: higher, green: lower).

The qPCR analyses revealed differential gene expression between groups. Women with perceived symptoms of anxiety and depression during pregnancy had altered expression patterns for *CRH, HSD11B2* and *AVP* genes in the placentas, compared to the control population. An upregulation of CRH ($p<0.05$), *HSD11B2* ($p<0.05$), and *AVP* ($p<0.001$) gene expression was found in the Index group compared to the control, while *OGT* and *NR3C1* gene expression appeared similar between groups (**Figure 2**).

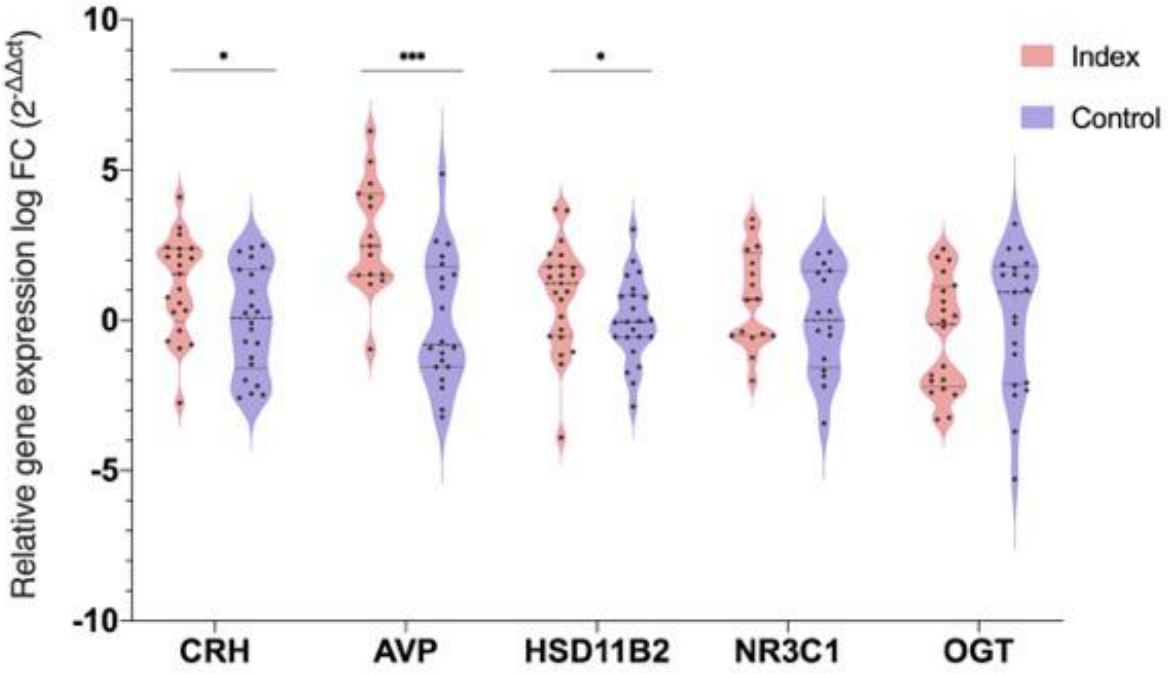

Figure 2. Differential gene expression of stress-related genes (corticotropin-releasing hormone; *CRH,* Arginine Vasopressin; *AVP*, 11β-hydroxysteroid dehydrogenase; *HSD11B2*, glucocorticoid receptor; *NR3C1* and O-GlcNAc transferase; *OGT*) in term-placenta samples of patients indicating symptoms of depression and anxiety (Index) and patients with no symptoms of depression or anxiety. The gene names are indicated in the X axle and the value on Y axle represents the gene expression level in the binary logarithm (log 2) value. Data are presented by violin plot showing median and inter-quartile range (Q1-Q3). Asterisks indicate significant differences among groups (*$p<0.05$; ***$p<0.001$).

Additionally, Spearman correlation analyses was performed to investigate the association among the expression of all genes tested in this study (**Table 1**). Interestingly, a significant positive correlation between *CRH* with both *AVP* (Rho= 0.79; $p<0.001$) and *HSD11B2* (Rho= 0.45; $p<0.03$), and also between *AVP* with both *HSD11B2* (Rho= 0.6; $p<0.005$) and NR3C1 (Rho= 0.56; $p<0.03$) gene

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expression in the Control group was observed. Such correlations were not evident in the Index group, suggesting a possible loss of interaction in the mechanisms of action of these genes under stress circumstances.

Table 1: Spearman correlations between gene expression data of stress-related genes (corticotropin-releasing hormone; *CRH*, Arginine Vasopressin; *AVP*, 11β-hydroxysteroid dehydrogenase; *HSD11B2*, glucocorticoid receptor; *NR3C1* and O-GlcNAc transferase; *OGT*) in term-placentas of patients indicating symptoms of depression and anxiety (Index), patients with no symptoms of depression nor anxiety (Control) and all patients examined in this study (All). Comparisons showing statistical significance are marked in bold font.

	AVP			*HSD11B2*			*OGT*			*NR3C1*		
	INDEX	CONTROL	ALL	INDEX	CONTROL	ALL	INDEX	CONTROL	ALL	INDEX	CONTROL	ALL
CRH	Rho = 0.29 P= 0.3 n=15	**Rho = 0.79 P<0.001 n=20**	**Rho = 0.6 P<0.001 n=35**	Rho = -0.23 P= 0.31 n=21	**Rho = 0.45 P= 0.03 n=22**	Rho = 0.2 P= 0.22 n=43	Rho = -0.36 P= 0.12 n=19	Rho = -0.38 P= 0.09 n=20	**Rho = -0.46 P= 0.003 n=39**	Rho = -0.09 P= 0.75 n=14	Rho = 0.23 P= 0.37 n=16	Rho = 0.12 P= 0.52 n=30
AVP				Rho = 0.08 P= 0.76 n=15	**Rho = 0.6 P= 0.005 n= 20**	**Rho = 0.56 P<0.001 n=35**	Rho = -0.16 P= 0.57 n=15	Rho = -0.41 P= 0.09 n=18	Rho = -0.33 P= 0.06 n=33	Rho = 0.007 P= 0.98 n=12	**Rho = 0.56 P= 0.03 n=15**	Rho = 0.36 P= 0.07 n=27
HSD11B2							Rho = 0.29 P= 0.21 n=20	Rho = 0.09 P= 0.69 n=21	Rho = 0.11 P= 0.5 n=41	Rho = 0.12 P= 0.64 n=16	Rho = 0.34 P= 0.2 n=16	Rho = 0.28 P= 0.12 n=32
OGT										Rho = 0.09 P= 0.73 n=15	Rho = 0.009 P= 0.97 n=16	Rho = 0.008 P= 0.96 n=31

3.2 Prenatal stress influence on stress-like placental protein expression

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The expression of proteins related to the significantly altered genes found in this study was analyzed by western blotting. CRH and AVP proteins were clearly detected (**Figure 3A-C**) with bands of 22 kDa and 17 kDa found in placental tissue of either Index or Control groups corresponding to CRH (anti-CRH; rabbit polyclonal antibody; LSBio-B11889) and AVP (anti-AVP polyclonal antibody; MBS9205129) proteins, respectively (**Figure 3A and 3C**). Significant changes in the expression of these proteins was not observed between groups. However, a trend for higher CRH protein levels in female placental samples compared to males was found following the line of CRH gene expression analyses (**Figure 3B**).

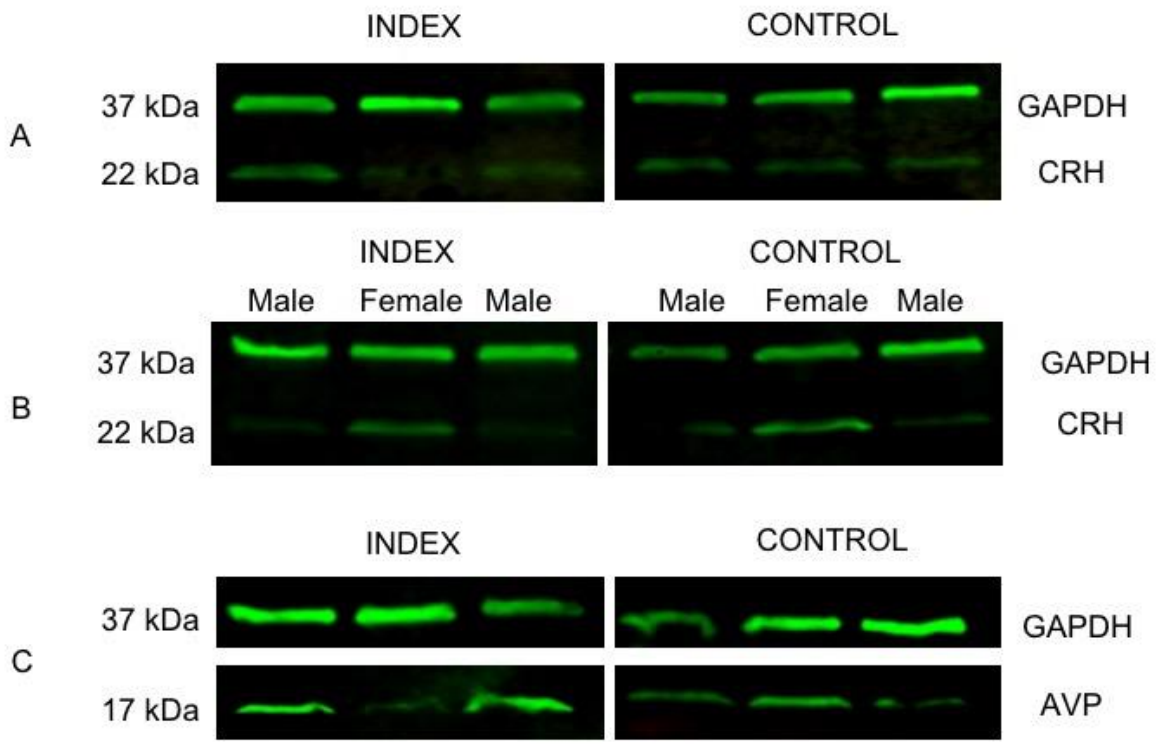

Figure 3A-C. Western Blot (WB) detection of the corticotropin-releasing hormone (CRH) and Arginine Vasopressin (AVP) proteins in term-placentas of patients indicating symptoms of depression and anxiety (Index) and patients with no symptoms of depression or anxiety (Control). **A**: Human anti-CRH polyclonal antibody (LS-B11889) tested in index and control group and **B**: between males and females identified expected bands at 22 kDa. In **C**: the human anti-AVP polyclonal antibody (MBS9205129) tested in index and control groups identified expected bands at 17 kDA.

3.3 Prenatal stress influences the association of placental HSD11B2 gene expression with hair cortisol levels.

A highly significant positive correlation between *HSD11B2* (Rho=0.54; p<0.001) term-placenta gene expression and HCC-levels in the index group at parturition was found (**Table 2**), despite significant differences in maternal HCC-levels between Index and Control group were not detected at any tested time point. The finding indicates a clear positive feedback between cortisol levels during labor and this cortisol-modulatory enzyme.

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Table 2. Spearman correlations between maternal hair cortisol levels (at week 24-25 of pregnancy, parturition and 8 weeks postpartum), and gene expression data of stress-related genes (corticotropin-releasing hormone; *CRH*, Arginine Vasopressin; *AVP*, 11β-hydroxysteroid dehydrogenase; *HSD11B2*, glucocorticoid receptor; *NR3C1* and O-GlcNAc transferase; *OGT*) in term-placentas of patients indicating symptoms of depression and anxiety (Index), patients with no symptoms of depression nor anxiety (Control) and all patients examined in this study (All). Comparisons showing statistical significance are marked in bold font.

GROUP	CORTISOL MEASUREMENTS	CRH	*OGT*	*HSD11B2*	*AVP*	*NR3C1*
INDEX	Week 24-25 of pregnancy	Rho = 0.37 P= 0.09 n=21	Rho = 0.2 P= 0.38 n=20	Rho = 0.021 P= 0.92 n=23	Rho = -0.35 P= 0.19 n=15	Rho = 0.31 P= 0.24 n=16
	Parturition	Rho = 0.34 P= 0.12 n=21	Rho = 0.02 P= 0.99 n=20	**Rho = 0.54 P= 0.007 n=23**	Rho = 0.37 P= 0.17 n=15	Rho = 0.29 P= 0.27 n=16
	8 weeks Postparturition	Rho = 0.45 P= 0.06 n=17	Rho = 0.09 P= 0.72 n=17	Rho = 0.29 P= 0.22 n=19	Rho = -0.12 P= 0.68 n=13	Rho = -0.24 P= 0.27 n=15
CONTROL	Week 24-25 of pregnancy	Rho = -0.04 P= 0.84 n=22	Rho = -0.19 P= 0.4 n=21	Rho = -0.17 P= 0.44 n=23	Rho = 0.21 P= 0.36 n=20	Rho = 0.171 P= 0.52 n=16
	Parturition	Rho = 0.03 P= 0.88 n=22	Rho = -0.08 P= 0.71 n=20	Rho = 0.03 P= 0.87 n=22	Rho = -0.017 P= 0.94 n=20	Rho = 0.46 P= 0.06 n=16
	8 weeks Postparturition	Rho = -0.14 P= 0.5 n=19	Rho = -0.32 P= 0.18 n=19	Rho = -0.23 P= 0.32 n=20	Rho = -0.27 P= 0.28 n=17	Rho = -0.15 P= 0.6 n=14
ALL	Week 24-25 of pregnancy	Rho = 0.15 P= 0.33 n=43	Rho =- 0.05 P= 0.75 n=41	Rho = -0.006 P= 0.96 n=46	Rho = 0.019 P= 0.91 n=35	Rho = 0.24 P= 0.19 n=32
	Parturition	Rho = 0.17 P= 0.25 n=43	Rho = -0.09 P= 0.57 n=40	Rho = 0.28 P= 0.054 n=45	Rho = 0.044 P= 0.8 n=35	**Rho = 0.4 P= 0.023 n=32**
	8 weeks Postparturition	Rho = 0.04 P= 0.8 n=36	Rho = -0.065 P= 0.71 n=36	Rho = 0.01 P= 0.94 n=39	Rho = -0.35 P= 0.052 n=30	Rho = -0.16 P= 0.4 n=29
	Parturition	Rho = 0.28 P= 0.07 n=42	Rho = -0.05 P= 0.74 n=40	Rho = -0.11 P= 0.45 n=45	Rho = 0.08 P= 0.65 n=34	Rho = 0.26 P= 0.16 n=31
	8 weeks Postparturition	Rho = 0.23 P= 0.19 n=31	Rho = -0.12 P= 0.49 n=32	Rho = 0.07 P= 0.66 n=34	Rho = -0.14 P= 0.47 n=27	Rho = 0.17 P= 0.38 n=26

3.4 Placental sex depicts differences in the gene expression of HSD11B2

The impact of offspring sex on stress-like gene expression among placental samples harvested from Index and Control women at term was further evaluated. These analyses demonstrated different responses on gene expression levels between male and female placentas (Fig. 4A-E). Overall, *CRH*, *HSD11B2* and *OGT* showed greater (although not significant) levels of expression in females than males in the total of patients evaluated, (**Figure 4A, 4C and 4E**, respectively). However, this pattern shifted for *HSD11B2* gene expression in the index group, being significantly downregulated in females compared to males ($p< 0.05$) (**Figure 4C**). In contrast, maternal stressors did not significantly influence a downregulation of *AVP* and *NR3C1* gene expression levels in female when compared to male placentas, despite a trend being present (**Figure 4B and 4D**, respectively).

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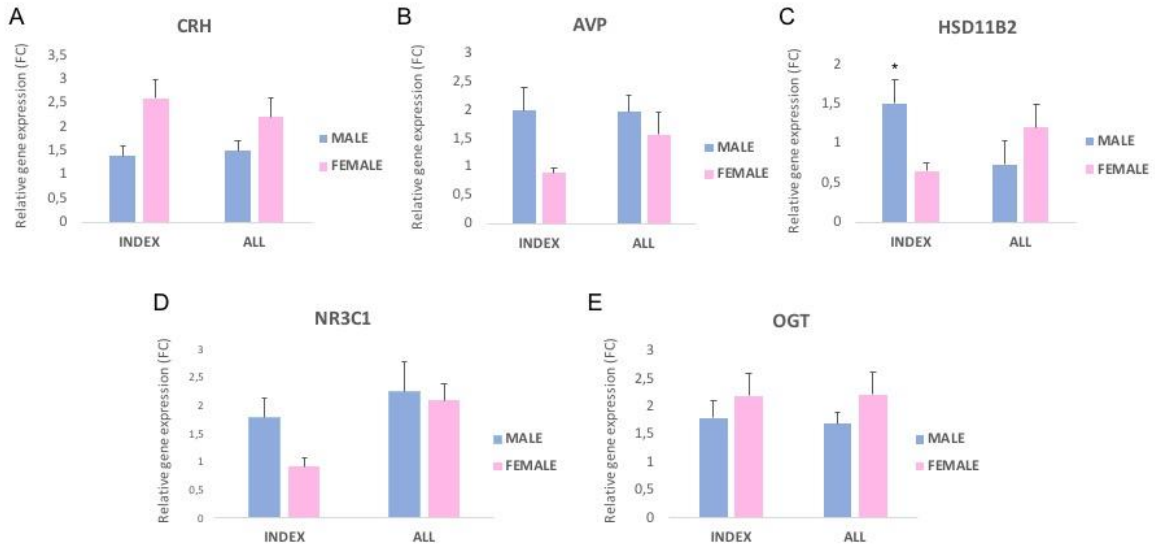

Figure 4. Differential gene expression of stress-related genes (corticotropin-releasing hormone; *CRH,* Arginine Vasopressin; *AVP,* 11β-hydroxysteroid dehydrogenase; *HSD11B2,* glucocorticoid receptor; *NR3C1* and O-GlcNAc transferase; *OGT*) in male and female term-placentas of patients indicating symptoms of depression and anxiety (Index) and all patients examined. Asterisks indicate significant differences among groups (*p<0.05).

4. Discussion

In the current study, we report a robust and tissue-specific effect of maternal stress during pregnancy on gene and protein expression levels of stress-related molecules. Amongst the most interesting results from this study, we found that *CRH* gene doubled its expression in placenta samples from index women compared to the control group. It is known that, during pregnancy, CRH is responsible of preparing the environment for childbirth, thus influencing developmental trajectories towards this event. However, less is known about the gene encoding this stress-related hormone. Although it has been reported as expressed in human placenta under physiological circumstances, this is, to the best of our knowledge, the first study to report an overexpression of this gene in term-placentas under maternal stress influence. CRH is considered the central upstream mediator of stress pathway activation [28,29], and has been associated with concentration-dependent effects upon the immune system [30]. In the context of psychological stressors, CRH triggers cytokine release and associated fever response prior to a rise in glucocorticoid levels [31]. Additionally, prenatal maternal stress is positively correlated with higher circulating levels of the pro-inflammatory cytokines IL-6 and TNFα, particularly during the first trimester; potentially linking stress and maternal immune activation that could affect fetal programing [32,33]. These effects may be mediated in part by CRH at peripheral sites of the immune system, serving to promote pro-inflammatory signaling in addition to effects of the downstream glucocorticoids [34]. In contrast, a body of evidence support the concept that CRH is capable of downregulating the immune system by decreasing T cell proliferation and natural killer (NK) cell cytotoxicity [35]. Peripherally, CRH can also act as an anti-inflammatory molecule reducing

inflammatory exudate volume in various disease models [36]. Despite these apparent immunostimulant or immunosuppressive actions, the effects of CRH on the immune system are complex, and time- and tissue-specific [37,38]. It is generally accepted that glucocorticoids suppress immune responses in the acute phase on any inflammatory process. Yet, if they are present for up to a week prior to an immune challenge, glucocorticoids may serve to enhance pro-inflammatory responses. Glucocorticoids also have tissue specificity in their effects. For example, while chronic elevations of glucocorticoids suppress the peripheral immune system, they can promote a pro-inflammatory state on the immune cells in the brain [39]. Our findings support the notion that gene levels of placental *CRH* increase under prenatal stress conditions and that might have an impact on subsequent immune system process in the child, thus affecting further systematic development. However, follow-up studies of relevant immune-related genes in term-placentas are required.

Moreover, the action of CRH can be potentiated by vasopressin, oxytocin, epinephrine, norepinephrine, and angiotensin II as previously reported [40,41]. We studied the Arginine-Vasopressin stress-hormone (*AVP)* gene expression and its association with *CRH* levels in at term-placental samples exposed to prenatal stress. AVP plays a major role in the homeostasis of fluid balance, vascular tonus, and the regulation of the endocrine stress response [42]. There are at least three distinct pathways by which AVP exerts its functions. Firstly, AVP regulates water absorption via the posterior pituitary. Secondly, AVP is critically involved in the hypothalamic-pituitary-adrenal (HPA) stress axis via the posterior pituitary and thirdly AVP, by remaining in the central nervous system, contributes to behavior and cognitive functions [43]. Furthermore, AVP is important in the control of fetoplacental blood pressure and facilitates the transition of the newborn to air breathing, cardiovascular adaptation, thermogenesis, glucose and water homeostasis [44]. It is well known that human fetuses actively secrete AVP in response to acute hypoxia and simultaneously redistribute fetal ventricular output towards the placenta [45]. Consistent with our results, AVP shows a pick of expression under fetal “stress” circumstances, such as heat stress, leading to widespread effects on fetal cardiovascular, renal and lung functions [46]. Moreover, Copeptin, a marker of AVP secretion, is elevated throughout human and mice pregnancies complicated by preeclampsia [47]. Also, AVP infusion into pregnant mice resulted in hypertension, renal glomerular endotheliosis, intrauterine growth restriction, decreased placental growth factor (PGF), altered placental morphology, placental oxidative stress, and placental gene expression consistent with human preeclampsia [48]. In the present study, we found an upregulation of placental *AVP* expression in index women compared to the control group, suggesting that not only direct fetal stress but also maternal stress during pregnancy may be capable of inducing higher levels of this stress-like hormone probably leading to placental hypoxia and future adverse physiological functions. Furthermore, we observed a positive significant correlation in the expression of *CRH* and *AVP* genes in the control group but not in the index group. Thus, our data support the notion that there is a positive feedback between the expression of these two genes in the placenta under physiological pregnancies which is altered under maternal-induced stress. Additionally, we found a significant increment in the gene expression of the 11-beta hydroxysteroid dehydrogenase (*HSD11B2*), that encodes 11ß–HSD2, the enzyme responsible for conversion of cortisol into inactive cortisone, in index placentas compared to the control group. Placental 11ß–HSD2 buffers the impact of maternal glucocorticoid exposure by converting

cortisol/corticosterone into inactive metabolites [21,49], thus preventing the activation of glucocorticoid receptors [21,22]. However, previous studies indicate that maternal adversity including stress during pregnancy, can lead to a misfunction of this enzyme [50]. In mice, mutation of the *HSD11B2* gene leads to hypertension, and increased anxiety-like behavior in adulthood [51]. *HSD11B2* placental methylation has been associated with worse neurobehavior among newborns whose mothers had either depression or anxiety during pregnancy [52]. These studies suggest that the regulation of placental 11ß–HSD2 levels may be a mechanistic link between the experience of maternal gestational stress and long-term health outcomes in offspring. Moreover, we observed an upregulation of this gene as the levels of maternal cortisol raised up during parturition in index women, but not in the control group, suggesting that higher levels of this enzyme are needed under stress condition in an attempt to block the transfer of cortisol within the fetal compartment.

Interestingly, *HSD11B2* gene expression was positively correlated with *CRH* and *AVP*, as well as *AVP* gene expression was also positively correlated to the glucocorticoid receptor (*NR3C1*), a nuclear receptor to which cortisol binds, in the control group but not in the index group, suggesting a clear dysregulation of stress-related gene interactions under prenatal stress circumstances.

Additionally, several diseases associated with prenatal stress exhibit sex bias [53], however, very little is known regarding the effects of maternal stress and the influence of fetal sex on placental function. To further investigate potential mechanisms underlying the sex-specific effect of maternal stressors on placental function, we examined weather our target genes and proteins were differently expressed depending on the sex of the offspring. Among these, *CRH*, *HSD11B2* (except for index group) and *OGT* gene expression and CRH protein expression were higher in female placentas compared to male when all the samples were analyzed. However, *HSD11B2* showed a significant increase in expression in male compared to female placentas in the index group. Previous studies in rodents and humans, reported that, the activity and sensitivity of placental 11βHSD2 to maternal stimuli are sex-specific [54,55]. A wealth of data supports that, stress-like disorders are sex-biased, being more common in women than in men [56–58]. Hyperarousal, which is a dysregulated state that contributes to increased agitation, restlessness, lack of concentration, and sleep disruption, contributing to symptoms of stress, anxiety and depression [59], is more pronounced in females than males [60,61]. In particular, a major brain arousal center, the noradrenergic locus coeruleus (LC), appears to be more activated in female than male during emotion-evoking tasks, reinforcing the statement that a stressful event may elicit a greater LC- mediated arousal response in women than in men [62]. We here suggest that, besides the evidence that biological factors can increase female vulnerability to stress and stress-related pathologies [63,64], also, females are more sensitive to the transmission of maternal stress through the placenta under the regulation of certain stress-related genes. Particularly, in normal pregnancies, placental 11β-HSD2 activity is significantly higher in female than male fetuses [55]. However, we found a significant downregulation of the expression of this gene in female compared to male placentas in the index group, which indicates that females might be subjected to a misfunction of the cortisol-blocking potential of this gene, thus being more exposed to a cortisol outbreak with potential developmental risks for the newborn.

In contrast, expression of *AVP and NR3C1* was slightly downregulated in female placentas compared with male placentas. Although the mechanisms underlying this sex-specific pattern in

maternal stress transmission are not clear, our results support the idea that males are more capable to circumvent the effects of maternal stressors by strengthening the maintenance of gene expression levels under physiological concentrations.

Overall, these findings provide novel evidence for the association of perceived maternal anxiety and depression during pregnancy and the dysregulation of placental gene expression of CRH, AVP and HSD11B2 as potential mechanisms underlying adverse physiological and neurodevelopmental consequences for the newborn ultimately contributing to disease risk. Moreover, the sex-specificity of stress-related HSD11B2 gene regulation found in this study may provide new insights by which sex biases in neurodevelopmental programing occurs, leading to the identification of novel targets for therapeutic development.

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References

1. Money, K.M.; Barke, T.L.; Serezani, A.; Gannon, M.; Garbett, K.A.; Aronoff, D.M.; Mirnics, K. Gestational diabetes exacerbates maternal immune activation effects in the developing brain. *Mol. Psychiatry* **2018**, *23*, 1920–1928.
2. Beversdorf, D.Q.; Manning, S.E.; Hillier, A.; Anderson, S.L.; Nordgren, R.E.; Walters, S.E.; Nagaraja, H.N.; Cooley, W.C.; Gaelic, S.E.; Bauman, M.L. Timing of prenatal stressors and autism. *J. Autism Dev. Disord.* **2005**, *35*, 471–478.
3. Black, S.R.; Goldstein, B.L.; Klein, D.N. Parental depression moderates the relationships of cortisol and testosterone with children's symptoms. *J. Affect. Disord.* **2019**, *251*, 42–51.
4. Sydsjo, G.; Agnafors, S.; Bladh, M.; Josefsson, A. Anxiety in women - a Swedish national three-generational cohort study. *BMC Psychiatry* **2018**, *18*, 168.
5. Josefsson, A.; Vikstrom, J.; Bladh, M.; Sydsjo, G. Major depressive disorder in women and risk for future generations: population-based three-generation study. *BJPsych open* **2019**, *5*, e8.
6. Lilliecreutz, C.; Laren, J.; Sydsjo, G.; Josefsson, A. Effect of maternal stress during pregnancy on the risk for preterm birth. *BMC Pregnancy Childbirth* **2016**, *16*, 5.
7. Emack, J.; Kostaki, A.; Walker, C.-D.; Matthews, S.G. Chronic maternal stress affects growth, behaviour and hypothalamo-pituitary-adrenal function in juvenile offspring. *Horm. Behav.* **2008**, *54*, 514–520.
8. Li, J.; Yang, H.; Guldin, M.-B.; Vedsted, P.; Vestergaard, M. Increased utilisation of primary healthcare

in persons exposed to severe stress in prenatal life: a national population-based study in Denmark. *BMJ Open* **2015**, *5*, e005657.

9. Grizenko, N.; Shayan, Y.R.; Polotskaia, A.; Ter-Stepanian, M.; Joober, R. Relation of maternal stress during pregnancy to symptom severity and response to treatment in children with ADHD. *J. Psychiatry Neurosci.* **2008**, *33*, 10–16.
10. Gerardin, P.; Wendland, J.; Bodeau, N.; Galin, A.; Bialobos, S.; Tordjman, S.; Mazet, P.; Darbois, Y.; Nizard, J.; Dommergues, M.; et al. Depression during pregnancy: is the developmental impact earlier in boys? A prospective case-control study. *J. Clin. Psychiatry* **2011**, *72*, 378–387.
11. Huttunen, M.O.; Niskanen, P. Prenatal loss of father and psychiatric disorders. *Arch. Gen. Psychiatry* **1978**, *35*, 429–431.
12. Gao, Y.; Hyttel, P.; Hall, V.J. Regulation of H3K27me3 and H3K4me3 during early porcine embryonic development. *Mol. Reprod. Dev.* **2010**, *77*, 540–549.
13. Ellman, L.M.; Schetter, C.D.; Hobel, C.J.; Chicz-Demet, A.; Glynn, L.M.; Sandman, C.A. Timing of fetal exposure to stress hormones: effects on newborn physical and neuromuscular maturation. *Dev. Psychobiol.* **2008**, *50*, 232–241.
14. Spiga, F.; Zhao, Z.; Lightman, S.L. Prolonged treatment with the synthetic glucocorticoid methylprednisolone affects adrenal steroidogenic function and response to inflammatory stress in the rat. *Brain. Behav. Immun.* **2020**.
15. Florio, P.; Severi, F.M.; Ciarmela, P.; Fiore, G.; Calonaci, G.; Merola, A.; De Felice, C.; Palumbo, M.; Petraglia, F. Placental stress factors and maternal-fetal adaptive response: the corticotropin-releasing factor family. *Endocrine* **2002**, *19*, 91–102.
16. Sandman, C.A.; Glynn, L.; Wadhwa, P.D.; Chicz-DeMet, A.; Porto, M.; Garite, T. Maternal hypothalamic-pituitary-adrenal disregulation during the third trimester influences human fetal responses. *Dev. Neurosci.* **2003**, *25*, 41–49.
17. Cheng, Y.H.; Nicholson, R.C.; King, B.; Chan, E.C.; Fitter, J.T.; Smith, R. Corticotropin-releasing hormone gene expression in primary placental cells is modulated by cyclic adenosine 3′,5′-monophosphate. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 1239–1244.
18. Vannuccini, S.; Bocchi, C.; Severi, F.M.; Challis, J.R.; Petraglia, F. Endocrinology of human parturition. *Ann. Endocrinol. (Paris).* **2016**, *77*, 105–113.
19. Sandman, C.A. Prenatal CRH: An integrating signal of fetal distress. *Dev. Psychopathol.* **2018**, *30*, 941–952.
20. Ghaemmaghami, P.; Dainese, S.M.; La Marca, R.; Zimmermann, R.; Ehlert, U. The association between the acute psychobiological stress response in second trimester pregnant women, amniotic fluid glucocorticoids, and neonatal birth outcome. *Dev. Psychobiol.* **2014**, *56*, 734–747.
21. Benediktsson, R.; Calder, A.A.; Edwards, C.R.; Seckl, J.R. Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. *Clin. Endocrinol. (Oxf).* **1997**, *46*, 161–166.
22. Seckl, J.R.; Meaney, M.J. Glucocorticoid programming. *Ann. N. Y. Acad. Sci.* **2004**, *1032*, 63–84.
23. Howerton, C.L.; Morgan, C.P.; Fischer, D.B.; Bale, T.L. O-GlcNAc transferase (OGT) as a placental

biomarker of maternal stress and reprogramming of CNS gene transcription in development. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 5169–5174.

24. Beck, A.T.; Epstein, N.; Brown, G.; Steer, R.A. An inventory for measuring clinical anxiety: psychometric properties. *J. Consult. Clin. Psychol.* **1988**, *56*, 893–897.

25. Rubertsson, C.; Borjesson, K.; Berglund, A.; Josefsson, A.; Sydsjo, G. The Swedish validation of Edinburgh Postnatal Depression Scale (EPDS) during pregnancy. *Nord. J. Psychiatry* **2011**, *65*, 414–418.

26. Alvarez-Rodriguez, M.; Vicente-Carrillo, A.; Rodriguez-Martinez, H. Hyaluronan improves neither the long-term storage nor the cryosurvival of liquid-stored CD44-bearing AI boar spermatozoa. *J. Reprod. Dev.* **2018**, *64*, 351–360.

27. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402–408.

28. Bale, T.L.; Vale, W.W. CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 525–557.

29. Tan, H.; Zhong, P.; Yan, Z. Corticotropin-releasing factor and acute stress prolongs serotonergic regulation of GABA transmission in prefrontal cortical pyramidal neurons. *J. Neurosci.* **2004**, *24*, 5000–5008.

30. Black, P.H. Central nervous system-immune system interactions: psychoneuroendocrinology of stress and its immune consequences. *Antimicrob. Agents Chemother.* **1994**, *38*, 1–6.

31. Morrow, L.E.; McClellan, J.L.; Conn, C.A.; Kluger, M.J. Glucocorticoids alter fever and IL-6 responses to psychological stress and to lipopolysaccharide. *Am. J. Physiol.* **1993**, *264*, R1010-6.

32. Coussons-Read, M.E.; Okun, M.L.; Schmitt, M.P.; Giese, S. Prenatal stress alters cytokine levels in a manner that may endanger human pregnancy. *Psychosom. Med.* **2005**, *67*, 625–631.

33. Coussons-Read, M.E.; Okun, M.L.; Nettles, C.D. Psychosocial stress increases inflammatory markers and alters cytokine production across pregnancy. *Brain. Behav. Immun.* **2007**, *21*, 343–350.

34. Cao, J.; Cetrulo, C.L.; Theoharides, T.C. Corticotropin-releasing hormone induces vascular endothelial growth factor release from human mast cells via the cAMP/protein kinase A/p38 mitogen-activated protein kinase pathway. *Mol. Pharmacol.* **2006**, *69*, 998–1006.

35. Pawlikowski, M.; Zelazowski, P.; Dohler, K.; Stepien, H. Effects of two neuropeptides, somatoliberin (GRF) and corticoliberin (CRF), on human lymphocyte natural killer activity. *Brain. Behav. Immun.* **1988**, *2*, 50–56.

36. Karalis, K.; Crofford, L.; Wilder, R.L.; Chrousos, G.P. Glucocorticoid and/or glucocorticoid antagonist effects in inflammatory disease-susceptible Lewis rats and inflammatory disease-resistant Fischer rats. *Endocrinology* **1995**, *136*, 3107–3112.

37. Karalis, K.; Muglia, L.J.; Bae, D.; Hilderbrand, H.; Majzoub, J.A. CRH and the immune system. *J. Neuroimmunol.* **1997**, *72*, 131–136.

38. You, X.; Liu, J.; Xu, C.; Liu, W.; Zhu, X.; Li, Y.; Sun, Q.; Gu, H.; Ni, X. Corticotropin-releasing hormone (CRH) promotes inflammation in human pregnant myometrium: the evidence of CRH initiating parturition? *J. Clin. Endocrinol. Metab.* **2014**, *99*, E199-208.

39. Sorrells, S.F.; Sapolsky, R.M. An inflammatory review of glucocorticoid actions in the CNS. *Brain. Behav.*

Immun. **2007**, *21*, 259–272.

40. Bilezikjian, L.M.; Vale, W.W. Regulation of ACTH secretion from corticotrophs: the interaction of vasopressin and CRF. *Ann. N. Y. Acad. Sci.* **1987**, *512*, 85–96.
41. Faucher, D.J.; Laptook, A.R.; Parker, C.R.; Porter, J.C.; Rosenfeld, C.R. Increased fetal secretion of ACTH and cortisol by arginine vasopressin. *Am. J. Physiol.* **1988**, *254*, R410-6.
42. Christ-Crain, M.; Fenske, W. Copeptin in the diagnosis of vasopressin-dependent disorders of fluid homeostasis. *Nat. Rev. Endocrinol.* **2016**, *12*, 168–176.
43. Evers, K.S.; Wellmann, S. Arginine Vasopressin and Copeptin in Perinatology. *Front. Pediatr.* **2016**, *4*, 75.
44. Chard, T.; Hudson, C.N.; Edwards, C.R.; Boyd, N.R. Release of oxytocin and vasopressin by the human foetus during labour. *Nature* **1971**, *234*, 352–354.
45. Oosterbaan, H.P.; Swaab, D.F. Amniotic oxytocin and vasopressin in relation to human fetal development and labour. *Early Hum. Dev.* **1989**, *19*, 253–262.
46. Smith, J.; Halse, K.G.; Damm, P.; Lindegaard, M.L.; Amer-Wahlin, I.; Hertel, S.; Johansen, M.; Mathiesen, E.R.; Nielsen, L.B.; Goetze, J.P. Copeptin and MR-proADM in umbilical cord plasma reflect perinatal stress in neonates born to mothers with diabetes and MR-proANP reflects maternal diabetes. *Biomark. Med.* **2013**, *7*, 139–146.
47. Sandgren, J.A.; Deng, G.; Linggonegoro, D.W.; Scroggins, S.M.; Perschbacher, K.J.; Nair, A.R.; Nishimura, T.E.; Zhang, S.Y.; Agbor, L.N.; Wu, J.; et al. Arginine vasopressin infusion is sufficient to model clinical features of preeclampsia in mice. *JCI insight* **2018**, *3*.
48. Scroggins, S.M.; Santillan, D.A.; Lund, J.M.; Sandgren, J.A.; Krotz, L.K.; Hamilton, W.S.; Devor, E.J.; Davis, H.A.; Pierce, G.L.; Gibson-Corley, K.N.; et al. Elevated vasopressin in pregnant mice induces T-helper subset alterations consistent with human preeclampsia. *Clin. Sci. (Lond).* **2018**, *132*, 419–436.
49. Staud, F.; Mazancova, K.; Miksik, I.; Pavek, P.; Fendrich, Z.; Pacha, J. Corticosterone transfer and metabolism in the dually perfused rat placenta: effect of 11beta-hydroxysteroid dehydrogenase type 2. *Placenta* **2006**, *27*, 171–180.
50. Sarkar, S.; Tsai, S.W.; Nguyen, T.T.; Plevyak, M.; Padbury, J.F.; Rubin, L.P. Inhibition of placental 11beta-hydroxysteroid dehydrogenase type 2 by catecholamines via alpha-adrenergic signaling. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2001**, *281*, R1966-74.
51. Marsit, C.J.; Maccani, M.A.; Padbury, J.F.; Lester, B.M. Placental 11-beta hydroxysteroid dehydrogenase methylation is associated with newborn growth and a measure of neurobehavioral outcome. *PLoS One* **2012**, *7*, e33794.
52. Conradt, E.; Lester, B.M.; Appleton, A.A.; Armstrong, D.A.; Marsit, C.J. The roles of DNA methylation of NR3C1 and 11beta-HSD2 and exposure to maternal mood disorder in utero on newborn neurobehavior. *Epigenetics* **2013**, *8*, 1321–1329.
53. Barke, T.L.; Money, K.M.; Du, L.; Serezani, A.; Gannon, M.; Mirnics, K.; Aronoff, D.M. Sex modifies placental gene expression in response to metabolic and inflammatory stress. *Placenta* **2019**, *78*, 1–9.
54. Wilcoxon, J.S.; Schwartz, J.; Aird, F.; Redei, E.E. Sexually dimorphic effects of maternal alcohol intake and adrenalectomy on left ventricular hypertrophy in rat offspring. *Am. J. Physiol. Endocrinol. Metab.* **2003**, *285*, E31-9.

55. Murphy, V.E.; Gibson, P.G.; Giles, W.B.; Zakar, T.; Smith, R.; Bisits, A.M.; Kessell, C.G.; Clifton, V.L. Maternal asthma is associated with reduced female fetal growth. *Am. J. Respir. Crit. Care Med.* **2003**, *168*, 1317–1323.

56. Sheikh, J.I.; Leskin, G.A.; Klein, D.F. Gender differences in panic disorder: findings from the National Comorbidity Survey. *Am. J. Psychiatry* **2002**, *159*, 55–58.

57. Kessler, R.C.; Petukhova, M.; Sampson, N.A.; Zaslavsky, A.M.; Wittchen, H.-U. Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. *Int. J. Methods Psychiatr. Res.* **2012**, *21*, 169–184.

58. Breslau, N. Gender differences in trauma and posttraumatic stress disorder. *J. Gend. Specif. Med.* **2002**, *5*, 34–40.

59. Palagini, L.; Moretto, U.; Novi, M.; Masci, I.; Caruso, D.; Drake, C.L.; Riemann, D. Lack of Resilience Is Related to Stress-Related Sleep Reactivity, Hyperarousal, and Emotion Dysregulation in Insomnia Disorder. *J. Clin. sleep Med. JCSM Off. Publ. Am. Acad. Sleep Med.* **2018**, *14*, 759–766.

60. Plante, D.T.; Landsness, E.C.; Peterson, M.J.; Goldstein, M.R.; Riedner, B.A.; Wanger, T.; Guokas, J.J.; Tononi, G.; Benca, R.M. Sex-related differences in sleep slow wave activity in major depressive disorder: a high-density EEG investigation. *BMC Psychiatry* **2012**, *12*, 146.

61. Kokras, N.; Dalla, C. Preclinical sex differences in depression and antidepressant response: Implications for clinical research. *J. Neurosci. Res.* **2017**, *95*, 731–736.

62. Kobayashi, I.; Mellman, T.A. Gender differences in sleep during the aftermath of trauma and the development of posttraumatic stress disorder. *Behav. Sleep Med.* **2012**, *10*, 180–190.

63. Bangasser, D.A.; Valentino, R.J. Sex differences in stress-related psychiatric disorders: neurobiological perspectives. *Front. Neuroendocrinol.* **2014**, *35*, 303–319.

64. Bangasser, D.A.; Eck, S.R.; Telenson, A.M.; Salvatore, M. Sex differences in stress regulation of arousal and cognition. *Physiol. Behav.* **2018**, *187*, 42–50.