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Abstract:	Background Glucocorticoids and serotonin may mediate the link between maternal environment, fetal brain development and 'programming' of offspring behaviors. The placenta regulates fetal exposure to maternal hormonal signals in animal studies, but few data address this in humans. We measured prospectively maternal depressive symptoms during pregnancy and mRNAs encoding key gene products determining glucocorticoid and serotonin function in term human placenta and explored associations with infant regulatory behaviors. Methods Bi-weekly self-ratings of Center for Epidemiological Studies Depression Scale from 12-13th gestational week onwards and term placental mRNAs of 11beta-hydroxysteroid dehydrogenase type 2 (HSD2B11), 1 (HSD1B11), glucocorticoid (NR3C1), mineralocorticoid receptors (NR3C2) and serotonin transporter (SLC6A4) were obtained from 54 healthy mothers aged 32.2±5.3 years with singleton pregnancies and without pregnancy complications. Infant regulatory behaviors (crying, feeding, spitting, elimination, sleeping and predictability) were mother-rated at 15.6±4.2 days.

Results
Higher placental mRNA levels of HSD2B11 (0.41 standard deviation [SD] unit increase per SD unit increase; 95% Confidence Interval, 0.13-0.69, p = 0.005), HSD1B11 (0.30, 0.03-0.57, p = 0.03), NR3C1(0.44, 0.19-0.68, p = 0.001) and SLC6A4 (0.26, 0.00-0.53, p = 0.05) were associated with more regulatory behavioral challenges of the infant. Higher placental NR3C1 mRNA partly mediated the association between maternal depressive symptoms during pregnancy and infant regulatory behaviors ($p < 0.05$).

Conclusions
Higher placental expression of genes regulating feto-placental glucocorticoid and serotonin exposure is characteristic of infants with more regulatory behavioral challenges. Maternal depression acts, at least partly, via altering glucocorticoid action in the placenta to impact on offspring regulatory behaviors.

Maternal depressive symptoms during pregnancy, placental expression of genes regulating glucocorticoid and serotonin function and infant regulatory behaviors

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Background

Glucocorticoids and serotonin may mediate the link between maternal environment, fetal brain development and 'programming' of offspring behaviors. The placenta regulates fetal exposure to maternal hormonal signals in animal studies, but few data address this in humans. We measured prospectively maternal depressive symptoms during pregnancy and mRNAs encoding key gene products determining glucocorticoid and serotonin function in term human placenta and explored associations with infant regulatory behaviors.

Methods

Bi-weekly self-ratings of Center for Epidemiological Studies Depression Scale from 12-13th gestational week onwards and term placental mRNAs of 11beta-hydroxysteroid dehydrogenase type 2 (*HSD2B11*), 1 (*HSD1B11*), glucocorticoid (*NR3C1*), mineralocorticoid receptors (*NR3C2*) and serotonin transporter (*SLC6A4*) were obtained from 54 healthy mothers aged 32.2±5.3 years with singleton pregnancies and without pregnancy complications. Infant regulatory behaviors (crying, feeding, spitting, elimination, sleeping and predictability) were mother-rated at 15.6±4.2 days.

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Higher placental mRNA levels of *HSD2B11* (0.41 standard deviation [SD] unit increase per SD unit increase; 95% Confidence Interval, 0.13-0.69, $p = 0.005$), *HSD1B11* (0.30, 0.03-0.57, $p = 0.03$), *NR3C1*(0.44, 0.19-0.68, $p = 0.001$) and *SLC6A4* (0.26**,** 0.00-0.53, $p = 0.05$) were associated with more regulatory behavioral challenges of the infant. Higher placental *NR3C1* mRNA partly mediated the association between maternal depressive symptoms during pregnancy and infant regulatory behaviors ($p < 0.05$).

Conclusions

Higher placental expression of genes regulating feto-placental glucocorticoid and serotonin exposure is characteristic of infants with more regulatory behavioral challenges. Maternal depression acts, at least partly, via altering glucocorticoid action in the placenta to impact on offspring regulatory behaviors.

Key words: mother, placenta, infant, behaviour, pregnancy, mRNA, prospective

Introduction

A wealth of data suggests that exposure to an adverse maternal environment in prenatal life, such as obstetric complications, malnutrition, depressed or anxious mood, maternal treatment with synthetic glucocorticoids, may exert adverse consequences upon fetal brain development thereby altering neurobehavioral trajectories and increasing risk of neuropsychiatric disorders in later life (Cottrell and Seckl 2009, Glover 2011, Pesonen *et al* 2009, Räikkönen et al., 2010; Reynolds *et al* 2013, Roseboom *et al* 2006, Tuovinen *et al* 2012) . While the biological mechanisms underpinning this developmental plasticity phenomenon, dubbed 'programming', still remain elusive, data from experimental animal (Benediktsson *et al* 1997, Cottrell and Seckl 2009, de Vries *et al* 2007, Edwards *et al* 1993, Holmes *et al* 2006, Lindsay *et al* 1996, Lindsay *et al* 1996, Liu *et al* 2001, Moss *et al* 2001, Seckl 1998, Seckl and Meaney 2004, Seckl and Holmes 2007, Welberg *et al* 2000, Wyrwoll and Holmes 2012) and limited emerging human studies (Conradt *et al* 2013, Moisiadis and Matthews 2014, Oberlander *et al* 2008, O'Donnell *et al* 2012, Räikkönen *et al* 2014, Reynolds *et al* 2015) suggest that fetal overexposure to glucocorticoids may be a key underlying mechanism.

The placenta, which plays a vital role in mediating the maternal hormonal signals to the fetus, provides a barrier to fetal exposure to the much higher glucocorticoid levels in the maternal circulation (Seckl 1998, Seckl and Meaney 2004, Seckl and Holmes 2007). This is ensured by the placental enzyme 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which catalyzes metabolism of up to 80-90% of maternal active cortisol to inactive cortisone (Edwards *et al* 1993) . Among the other important placental regulators of feto-placental glucocorticoid overexposure are 11beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which catalyzes regeneration of active cortisol from inactive cortisone, and intracellular glucocorticoid (GR) and mineralocorticoid receptors (MR), which mediate glucocorticoid actions on gene transcription (Cottrell and Seckl 2009, Seckl 1998, Seckl and Meaney 2004, Seckl and Holmes 2007). Animal studies have shown

that increased feto-placental exposure to active glucocorticoids of maternal origins, alters offspring brain anatomy, functioning of the hypothalamic-pituitary-adrenal (HPA) axis, hinders learning and memory, and increases anxiety- and depression-like behaviors of the offspring (Holmes *et al* 2006, Welberg *et al* 2000, Wyrwoll and Holmes 2012) .

Fetal glucocorticoid exposure may also exert effects upon the development and subsequent function of the serotonergic nervous system of the offspring (Wyrwoll and Holmes 2012), a key system implicated in mood disorders (Ressler and Nemeroff 2000). Glucocorticoid and serotonergic systems are known to interact such that glucocorticoids regulate serotonin synthesis, transport, re-uptake and neuronal receptor expression, while serotonin controls glucocorticoid and mineralocorticoid receptor expression in the central nervous system (Wyrwoll and Holmes 2012). Recent data in the mouse show that placental deficiency of 11β-HSD2 is associated with increased serotonin synthesis and impairment in breakdown of serotonin in the brain of the offspring (Wyrwoll and Holmes 2012).

Glucocorticoid (Murphy *et al* 2006) and serotonin (Bonnin *et al* 2011) systems are both expressed in the placenta where interaction might occur and be relevant to exposure of the offspring's developing central nervous system. Yet, we are not aware of previous studies that have tested whether alterations in placental expression of key genes determining placental glucocorticoid and serotonin function play a role in programming of offspring behavioral outcomes and mediate the influence of prenatal maternal environmental adversity on these behavioral outcomes. Therefore, we tested if variations in placental mRNAs encoding 11β-HSD2 (*HSD11B2*), 11β-HSD1 (*HSD11B1*), GR (*NR3C1*), MR (*NR3C2*) and the serotonin transporter (*SLC6A4*) were associated with differences in regulatory behaviors of the offspring at a mean age of 15.6 days after birth. In the tests of mediation we focused on placental mRNA levels of *NR3C1* and *NR3C2* and on maternal

depressive symptoms during pregnancy as the prenatal maternal environmental adversity as we have recently shown in this sample that maternal depressive symptoms during pregnancy were significantly associated with higher placental mRNA levels of *NR3C1* and *NR3C2*, but were not significantly associated with the levels of *HSD11B2, HSD11B1* or *SLC6A4* (Reynolds *et al* 2015). Consequently, our new analyses reported here extends our previous study by testing if placental *NR3C1* and *NR3C2* mRNAs mediate the influence of maternal depressive symptoms during pregnancy on infant regulatory behaviors, providing the other criteria for mediation were also met (Hayes 2009), i.e. that placental *NR3C1* and *NR3C2* mRNAs and maternal depressive symptoms during pregnancy were also associated with infant regulatory behaviors.

Methods

Participants

The participants were 67 healthy pregnant women enrolled in the Prediction and Prevention of Preeclampsia (PREDO) Study as previously described (Räikkönen *et al* 2014, Reynolds *et al* 2015, Villa *et al* 2013) . The women did not report using glucocorticoid or antidepressant medication during pregnancy, had no obstetric complications and delivered term (37+0 - 41+6 weeks of gestation), singleton, healthy infants. Of these, 54 participated in a follow-up study at a mean infant age of 15.6 (standard deviation (SD) =4.3) days. Those who did not participate were similar to those who did ($p > 0.11$) except for a higher pre-pregnancy body mass index (mean (standard deviation) 27.1 (7.8) vs. 22.9 (3.5) kg/m^2, $p = 0.004$), placental weight (640.2 (102.2) vs. 573.8 (83.2) g, $p = 0.02$) and shorter length of gestation (275.9 (6.5) vs. 281.2 (8.6) days, $p = 0.04$) than participants. The study protocol was approved by the ethical committees at the Helsinki and Uusimaa Hospital district and written informed consent was obtained.

Maternal depressive symptoms

Center for Epidemiological Studies Depression Scale (Radloff 1977) for depressive symptoms was completed by the mothers bi-weekly during pregnancy from 12-13 gestational weeks onwards up to 14 times in total. The value at week 12 and mean values across weeks 14-26 and 28-38 were used as trimester-specific indices of depressive symptoms during pregnancy (Reynolds *et al* 2015) .

Placental tissue sampling and gene expression

Two sets of 9-site biopsies were collected from the decidual side of the placenta a maximum of 90 minutes after vaginal or caesarean delivery using standard protocols as previously described (Räikkönen *et al* 2014, Reynolds *et al* 2015) . The biopsies were put in RNA-later and stored at -20C. Total RNA was extracted from placental tissue sampled from a central site using QIAGEN RNeasy mini kits (Qiagen Ltd, West Sussex, UK). The RNA concentration and purity of all samples was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK) and the integrity of RNA confirmed by separating ribosomal RNA (rRNA) using electrophoresis in a 1% agarose/0.5xTBE (45mM Tris-borate, 1mM EDTA) gel with 0.1µl/ml Gel Red (Biotium, Hayward, CA, USA) at ~90V for 30 to 50 minutes. cDNA synthesis was carried out using the Access RT-PCR system (Promega, Southampton, UK). cDNA was incubated in triplicate with gene-specific primers and fluorescent probes either using the Universal Probe Library system from Roche Diagnostics Ltd (Burgess Hill, UK) for *HSD11B1* (forward primer: caatggaagcattgttgtcg, reverse primer: ggcagcaaccattggataag) and *NR3C2* (forward primer: tgggaattctgacttacttaacca, reverse primer: aatacaaaaagctgatgcagacc) or pre-designed assays from Applied Biosystems ((ABI), CA, USA) for *NR3C1* (Hs00230818_m1), *HSD11B2* (Hs00388669_m1) and *SLC6A4* (Hs00984349_m1) in RocheLightCycler 480 Probes mastermix. PCR cycling and detection of fluorescent signal was carried out using a Roche LightCycler 480. A standard curve was constructed for each primer-probe set using a serial dilution of cDNA pooled from all samples. Results were corrected to the control gene TATA-binding protein (*TBP*).

Infant regulatory behaviors

Mothers rated infant regulatory behaviors using the Neonatal Perception Inventory (Broussard and Hartner 1971). This inventory captures behaviors relating to infant's crying, feeding, spitting, elimination (bowel movements), sleeping and predictability. In order to diminish the potential bias that may result in too positive or negative perceptions of infant behaviors, the mother was first asked to rate concerns relating to regulatory behaviors she would expect an 'average' infant to display. She was then asked to rate concerns in relation to her own infant's regulatory behaviors. The ratings were made using a five-point scale ranging from no problems to a great amount of problems. A difference score between her own and the average infant's regulatory behaviors (own infant – average infant) reflects more regulatory challenges in her own infant's behavior. A principal components analysis revealed one factor (eigenvalue criterion > 1) explaining 40% of the total variance, which lends credence to unidimensional structure of the scale and gives support to construct validity. Cronbach's alpha for internal consistency was 0.71 which gives support for the scale's reliability.

Covariates and confounders

Sample time between placental birth and biopsy (min) was recorded. Mode of delivery (vaginal *vs*. elective/unplanned caesarean), parity (primiparous *vs*. multiparous), maternal pre-pregnancy body mass index (BMI) (kg/m^2), maternal age at delivery (yrs), smoking status during pregnancy (yes/no; number of cigarettes per day), infant sex (boy *vs*. girl), gestation length (days) and infant birth weight (g) were derived from hospital birth records and the Finnish National Birth Register. Alcohol consumption during pregnancy (number of drinks during the past four weeks) and maternal level of education (primary, secondary, tertiary) were self-reported. Center for Epidemiological

Studies Depression Scale (Radloff 1977) for depressive symptoms was completed by the mothers at the infant age of 15.6 days.

Statistical analyses

Data were analyzed using SPSS Statistics version 22 for IBM. We first used linear regression analyses in testing associations between *HSD11B2, HSD11B1, NR3C1, NR3C2, SLC6A4* mRNAs in term placenta and infant regulatory behaviors at age 15.6 days. We then tested if maternal depressive symptoms during pregnancy acted via altering glucocorticoid action in and via the placenta to impact on offspring regulatory behaviors by using the Mediate macro for SPSS with 5000 bootstrapped samples (Hayes and Preacher 2014). For these analyses gene expression data were log transformed to account for non-normality (after transformation skewness index divided by standard error < 1.66 for all transformed variables) and thereafter the predictor and outcome variables were standardized to the mean of 0 and standard deviation of 1 to facilitate interpretation. Hence, unstandardized regression coefficients and 95% confidence intervals (95% CI) or t-statistics represent effect sizes, SD unit per SD unit. We present the findings as unadjusted effect sizes and effect sizes from linear regression analyses when adjusted simultaneously for sampling time between placental birth and biopsy, mode of delivery, parity, maternal pre-pregnancy BMI, maternal age at delivery, level of education, smoking status and alcohol consumption during pregnancy, gestation length, infant birth weight and sex (Model 1). In linear regression analyses we also made adjustments for maternal depressive symptoms measured at the infant age of 15.6 days, as maternal depressive symptoms may bias her perceptions (Model 2).

Results

Table 1 shows clinical characteristics of the sample. Table 2 shows associations between covariates and confounders and infant regulatory behaviors. Mothers with a lower level of education and those

who reported higher depressive symptoms in the third trimester and at infant age 15.6 days reported more infant regulatory behavioral challenges.

Placental mRNA levels and infant regulatory behaviors

Table 3 shows that higher placental mRNA levels of *HSD11B2, HSD11B1, NR3C1, SLC6A4*, but not *NR3C2*, were significantly associated with more regulatory behavioral challenges of the infant: the increase was 0.41, 0.30, 0.44 and 0.26 SD units per each SD unit increase in the respective mRNA level (P-values < 0.05). Figure 1 displays the regression lines and 95% CIs of the significant unadjusted associations and additionally shows that the proportion of variance in infant regulatory behaviors accounted for by placental *HSD11B2, HSD11B1, NR3C1* and *SLC6A4* mRNAs varied from 8.9 to 19.9 per cent.

Table 3 shows that when we made adjustments for sampling time between placental birth and biopsy, mode of delivery, parity, maternal pre-pregnancy BMI, maternal age at delivery, level of education, smoking status and alcohol consumption during pregnancy, gestation length, infant birth weight and sex (Model 1) the association with *HSD11B1* was rendered non-significant; When the associations were adjusted for maternal depressive symptoms at infant age 15.6 days (Model 2) the association with *SLC6A4* was rendered non-significant (Table 3).

Mediation analyses

As placental *NR3C2* mRNA level was not significantly associated with infant regulatory behaviors (Table 3) mediation via *NR3C2* mRNA expression was not tested. Similarly, since maternal first and second trimester depressive symptoms were not significantly associated with infant regulatory behaviors (Table 2) they were not included in the mediation analyses. As maternal depressive symptoms during the third pregnancy trimester, placental *NR3C1* mRNA level, and infant

regulatory behaviors were all significantly inter-related (Reynolds et al., 2015) (Tables 2 and 3), and hence the criteria for testing mediation were met (Hayes, 2009, Hayes and Preacher, 2014), we pursued in testing if *NR3C1* mRNA mediated the effect of maternal depressive symptoms during the third pregnancy trimester on infant regulatory behaviors. Indeed, the indirect path via placental *NR3C1* mRNA was statistically significant (indirect effect = 0.15, 95% CI 0.02-0.38, $p < 0.05$) (Figure 2). Yet, the association between maternal depressive symptoms and infant regulatory behaviors was not rendered to zero, but remained 'marginally' significant ($p = 0.08$) (Figure 2). This suggested that placental *NR3C1* mRNA partly mediated the effect of maternal depressive symptoms on infant regulatory behaviors (Figure 2). Figure 2 also shows that the proportion of variance in *NR3C1* mRNA accounted for by maternal depressive symptoms was 14.4% ($p = 0.006$) and the proportion of variance in infant regulatory behaviors accounted for by maternal depressive symptoms and placental *NR3C1* mRNA was 24.8% ($p = 0.0009$). Adjustment for covariates and confounders did not alter the significant paths (p-values < 0.05) or mediation (indirect effect = 0.13, 95% CI 0.01-0.34, $p < 0.05$ in Model 1; indirect effect = 0.10, 95% CI 0.01-0.30, $p < 0.05$ in Model 2).

Discussion

We show that variation in the mRNA levels of key gene products determining glucocorticoid and serotonin function in term human placenta is associated with more regulatory behavioral challenges of the infant at 15.6 days. More specifically, higher placental mRNA levels of *HSD11B2, HSD11B1, NR3C1* and *SLC6A4* were associated with infants who subsequently showed greater behavioral challenges in crying, sleeping, feeding, spitting and/or elimination. The findings with *NR3C1* and *HSD11B1* add increased placental sensitivity to glucocorticoids coupled with local amplification of glucocorticoid action within the placenta as a mechanism linking increased glucocorticoid exposure to adverse offspring outcomes. Yet, the finding of higher placental mRNA

level of *HSD11B2* was contrary to what we expected based on evidence from animal studies where fetal glucocorticoid overexposure has been secondary to down-regulation of placental *HSD11B2* gene expression and activity, and hence inhibition of the function of the placental glucocorticoid barrier (Benediktsson *et al* 1997, Edwards *et al* 1993, Holmes *et al* 2006, Lindsay *et al* 1996, Lindsay *et al* 1996, Seckl 1998, Seckl and Meaney 2004, Seckl and Holmes 2007, Welberg *et al* 2000, Wyrwoll and Holmes 2012) . As *HSD11B2* excludes glucocorticoids from the syncytiotrophoblast we can only speculate that our finding showing that the placental mRNA level of *HSD11B2* was higher in infants with more behavioral challenges may be an adaptive placental response to ameliorate the higher glucocorticoid availability and sensitivity in the placental compartment. Moreover the associations with *NR3C1* and *HSD11B2* remained significant when we made adjustments for several important factors that we have previously shown to relate to placental gene expression (Räikkönen *et al* 2014, Reynolds *et al* 2015) and/or infant regulatory behaviors, including mode of delivery, time difference between placental birth and biopsy, maternal age at delivery, parity, maternal pre-pregnancy BMI, smoking and alcohol consumption during pregnancy, maternal level of education, gestation length, infant birth weight and sex and maternal depressive symptoms measured in conjunction with infant behavioral ratings. In the models including maternal depressive symptoms at the time of making the infant ratings, *HSD11B1* remained significant while *SLC6A4* did not, and in the models including the other covariates *SLC6A4* remained significant while *HSD11B1* did not.

Our study is also the first to show that higher placental *NRC31* mRNA level plays a role in the process that may partly mediate the effects of higher maternal depressive symptoms during the third trimester of pregnancy on more regulatory behavioral challenges of the infant. Statistical mediation via *NR3C1* mRNA was partial suggesting that other factors may also be involved. These factors may include other gene products, such as the corticotropin releasing hormone, or epigenetic

modifications involved in the signaling and interactions of glucocorticoids and serotonin between the mother, placenta and fetus. Indeed, a recent study demonstrated that higher maternal self-reported depression during pregnancy and higher placental methylation of the *NR3C1* were associated with poorer self-regulation, more hypotonia and more lethargy in the infant (Conradt *et al* 2013) . Yet, it remains unknown what the environmental exposures are that underlie the higher placental mRNA levels of *HSD11B1, HSD11B2* and *SLC6A4*. While in this study maternal depressive symptoms during pregnancy were not associated with mRNA levels of these genes and therefore we did not pursue testing them as mediators, one previous study has shown that higher maternal anxious but not depressed mood a day before an elective caesarean section was associated with lower placental *HSD11B2* mRNA level (O'Donnell *et al* 2012), and one other study has demonstrated that a history of maternal depressed mood during pregnancy, irrespective of the maternal antidepressant medication use, was associated with higher placental *SLC6A4* mRNA level, but not with *HSD11B2* (Ponder *et al* 2011) . Further studies are clearly warranted that unravel the maternal-placental-fetal pathways underpinning individual differences in early infant behaviors.

While we studied healthy term pregnancies, term placentas and healthy infants, our findings may have clinical relevance. Infant regulatory behaviors are among the earliest signs of neurobehavioral and neuropsychiatric problems in later life. Extensive data, including studies using the Neonatal Perception Inventory (the measure as used in our study), suggest that more regulatory behavioral challenges of the infant as reported by the mother are associated with later behavioral problems, including poorer cognitive functioning (Wolke *et al* 2009) and increased risk of externalizing behavior problems (Wolke *et al* 2002) and attention/deficit hyperactivity disorder (Hemmi *et al* 2011, Wolke *et al* 2002) in childhood. Extension of our findings into the prevalence of neurobehavioral and neuropsychiatric problems in childhood in this sample is subject to ongoing studies.

We used mother-reports of infant regulatory behavioral challenges. This may introduce a bias that results in too positive or negative perceptions of infant behaviors. To overcome any potential bias in reporting, the mothers were asked to rate regulatory behaviors she would expect an 'average' infant to display and then rate her own infant. In addition, we made adjustments for maternal depressive symptoms at the time of infant's assessment and her level of education that were expected to be associated with greater behavioral challenges of the infant. The majority of previous studies have, however, used mother- or parent-reports of infant regulatory behavioral challenges, as we did. Neurologists' assessments have been used in some studies, but these assessments usually concern oral-motor functioning in relation to feeding problems (Schmid *et al* 2010) . Hence, use of mother-reports remains a limitation, as long as golden standards do not exist on how to measure regulatory behavioral challenges in infancy.

We recognize that we lack data on several elements of the maternal-placental-fetal glucocorticoid and serotonin signaling pathway. As we lack data on fetal and intra-placental glucocorticoid and serotonin levels, we cannot determine the degree of maternal glucocorticoids and serotonin transmitted via the placenta to the fetus. However, while maternal and fetal cortisol levels are highly correlated, a recent study has demonstrated that during prenatal life, most fetal serotonin is of placental and fetal, not of maternal origins (Bonnin *et al* 2011) . We also have no information of any cell-specificity of actions and changes in the placenta itself, though the full thickness placental biopsies will principally reflect fetal characteristics. We only measured mRNA levels of the key genes of interest and lack data on levels of their respective protein levels. Further studies are needed to determine the mechanisms of altered gene expression, such as methylation of promoter and transcription factor binding sites and other epigenetic mechanisms, and whether these are modifiable, as are studies that focus on functional end products of the key genes of interest. Other

study limitations relate to external validity of the findings. As the studied sample comprised healthy term pregnancies, healthy babies and very few mothers reported depressive symptoms that were above the clinical cutoff of the CES-D scale, our findings may not generalize to samples with greater variation in adversity.

In conclusion, our findings suggest that higher placental expression of key genes determining glucocorticoid and serotonin function in term human placenta and regulating feto-placental glucocorticoid and serotonin exposure is characteristic of infants with more regulatory behavioral challenges, particularly challenges relating to crying, sleeping, feeding, spitting and elimination behaviors. Our findings also suggest that higher placental mRNA levels of the *NR3C1* plays a role in the process that may partly mediate the effects of maternal depressive symptoms during the third trimester of pregnancy on infant regulatory behavioral challenges. Our findings add placental glucocorticoid and serotonergic function as novel processes that may underpin developmental programming of early infant behaviors.

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Conflict of interest: None

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Figure legends

Figure 1. Unadjusted associations between mRNA levels of 11beta-hydroxysteroid dehydrogenase type 2 (*HSD11B2*; Panel A), 11beta-hydroxysteroid dehydrogenase type 1 (*HSD11B1*; Panel B), glucocorticoid receptor (*NR3C1*; Panel C) and serotonin transporter (*SLC6A4*; Panel D) in term placenta and infant regulatory behavioral challenges. The lines represent unadjusted unstandardized regression coefficients and 95% Confidence Intervals, and R^2 refers to the proportion of variance in infant regulatory behaviors accounted for by the placental mRNA levels.

Figure 2. Mediation analyses results showing that maternal depressive symptoms during the third trimester of pregnancy partly act via altering expression of glucocorticoid receptor (*NR3C1*) mRNA levels in term placenta to impact on infant regulatory behaviors. Numbers represent unadjusted unstandardized coefficients, 95% Confidence Intervals, P-values, and R^2 refers to the proportion of variance in infant regulatory behaviors accounted for by the placental mRNA levels of *NR3C1* and maternal depressive symptoms, and in placental mRNA levels of *NR3C1* by maternal depressive symptoms.

Table 1. Characteristics of the sample (N=54).

	M (SD) / N (%)
Pregnancy and perinatal period:	
mRNA level in term placenta[1]	
HSD11B2[2]	0.70 (1.09)
HSD11B1[3]	0.74 (1.17)
NR3C1[4]	0.83 (1.23)
NR3C2[5]	0.80 (2.54)
SLC6A4[6]	0.65 (0.90)
Delivery mode, N (%)	
Vaginal	41 (75.9)
Elective section	5 (9.3)
Unplanned section	8 (14.8)
Time interval from placental birth to biopsy (min)	48.2 (21.6)
Weight of placenta (g)	573.8 (83.2)
Parity, primiparous, N (%)	21 (38.9)
Maternal age at delivery (yrs)	32.7 (5.2)
Maternal pre-pregnancy body mass index (kg/m^2)	22.9 (3.5)
Maternal education, tertiary (> 12 years), N (%)	34 (63.0)
Maternal smoking during pregnancy, N (%)	
Never smoker	49 (90.7)
Quit first trimester	2 (3.7)
Smoked throughout[7]	3 (5.6)
Maternal alcohol consumption during pregnancy, yes (0.5 – 4 drinks the past 4 weeks), N (%)[8]	9 (16.7)

Maternal Center of Epidemiological Studies	
Depression Scale score (Range 0-60)	
First trimester	9.1 (5.3)
Second trimester	8.9 (4.7)
Third trimester	9.7 (5.3)
Maternal Center of Epidemiological Studies	
Depression Scale score ≥ 16, yes, N (%)	
First trimester	8 (15.1)
Second trimester	6 (11.1)
Third trimester	7 (13.0)
Gestation length (days)	283.1 (8.4)
Birth weight (g)	3605 (422)
Birth length (cm)	51.0 (2.0)
Ponderal index at birth (kg/m^3)	27.7 (2.3)
Follow-up after birth:	
Infant age (days)	15.6 (4.3)
Maternal Center of Epidemiological Studies	9.2 (7.2)
Depression Scale score (Range 0-60)	
Maternal Center of Epidemiological Studies	10 (19.6)
Depression Scale score ≥ 16, yes, N (%)	
Infant regulatory behaviors[9]	
Average infant (Range 1-30)	18.0 (1.8)
Own infant (Range 1-30)	19.7 (3.0)
Difference score (Own infant – Average infant)	-1.8 (3.3)

Note. [1]mRNA values are median (interquartile range); [2]*HSD11B2* refers to 11beta-hydroxysteroid dehydrogenase type 2; [3]*HSD11B1* refers to 11beta-hydroxysteroid dehydrogenase type 1;[4] *NR3C1* refers to glucocorticoid receptor, [5]*NR3C2* refers to mineralocorticoid receptor: [6] *SLC6A4* refers *to* serotonin transporter; [7] < 10 cigarettes per day, n = 1; < 10 cigarettes per week or less often, n = 2; [8] One drink refers to 33 cl of beer or cider (3.5-4.7% vol alcohol), 12 cl of mild wine (7-16% vol alcohol), 8 cl of strong wine (15-22% vol), 4 cl of liquor (>22% vol);

[9] Higher values indicate more regulatory behavioral challenges

Table 2. Association between covariates and confounders and infant regulatory behaviors.

	More regulatory behavioral challenges of the infant	
Covariates and confounders:	Effect size in SD units[1] (95% CI)	P
Vaginal delivery *vs.*		
Elective section	-0.08 (-1.05-0.89)	0.87
Unplanned section	-0.12 (-0.91-0.67)	0.77
Time interval between birth and placental sampling	-0.19 (-0.46-0.08)	0.16
Weight of placenta	0.11 (-0.19-0.41)	0.45
Primiparous *vs.* multiparous	0.13 (-0.44-0.69)	0.66
Maternal age at delivery	0.02 (-0.27-0.30)	0.90
Maternal primary/secondary *vs.* tertiary education	-0.87 (-1.38- -0.35)	0.001
Maternal pre-pregnancy body mass index	-0.13 (-0.51-0.26)	0.52
Never smoker *vs.*		
Quit first trimester	-0.38 (-1.85-1.09)	0.61
Smoked throughout	-0.07 (-1.29-1.14)	0.91
No alcohol consumption *vs*. yes (0.5-4 drinks during past 4 weeks)[2]	-0.47 (-1.21-0.25)	0.19
Infant sex (boy *vs*. girl)	-0.30 (-0.85-0.28)	0.28
Gestation length (SD units)	0.08 (-0.20-0.35)	0.59
Birth weight (SD units by sex)	0.01 (-0.26-0.28)	0.94

Maternal Center of Epidemiological		
Studies Depression Scale score		
First trimester	0.18 (-0.10-0.45)	0.20
Second trimester	0.20 (-0.09-0.48)	0.18
Third trimester	0.37 (0.09-0.64)	0.01
At infant age of 15 days	0.27 (0.01-0.52)	0.04

Note. [1] SD units, Standard deviation units; 95% CI, 95% Confidence Interval,

[2] One drink of alcohol refers to 33 cl of beer or cider (3.5-4.7% vol alcohol), 12 cl of mild wine (7-16% vol alcohol), 8 cl of strong wine (15-22% vol), 4 cl of liquor (>22% vol).

Table 3. Associations between mRNA levels of 11beta-hydroxysteroid dehydrogenase type 2 (*HSD11B2*), 11beta-hydroxysteroid dehydrogenase type 1 (*HSD11B1*), glucocorticoid receptor (*NR3C1*), mineralocorticoid receptor (*NR3C2*) and serotonin transporter (*SLC6A4*) in term placenta with infant regulatory behaviors.

	More regulatory behavioral challenges of the infant					
	Unadjusted		Model 1[3]		Model 2[4]	
mRNA level in term placenta:	Effect size in SD units[1] (95% CI)[2]	P	Effect size in SD units[1] (95% CI)[2]	P	Effect size in SD units[1] (95% CI)[2]	P
HSD11B2	0.41 (0.13-0.69)	0.005	0.30 (0.01-0.58)	0.04	0.31 (0.03-0.58)	0.03
HSD11B1	0.30 (0.03-0.57)	0.03	0.20 (-0.10-0.50)	0.20	0.25 (0.14-0.61)	0.002
NR3C1	0.44 (0.19-0.68)	0.001	0.44 (0.17-0.71)	0.002	0.27 (0.01-0.54)	0.045
NR3C2	0.14 (-0.16-0.44)	0.34	0.22 (-0.09-0.52)	0.16	0.03 (-0.24-0.30)	0.81
SLC6A4	0.26 (0.00-0.53)	0.047	0.30 (0.01-0.58)	0.04	0.23 (-0.06-0.48)	0.13

Note. [1] SD units, Standard deviation units: [2] 95% CI, 95% Confidence Interval; [3] Model 1 refers to adjustments made for mode of delivery, time difference between placental birth and biopsy, maternal age at delivery, maternal education, parity, maternal pre-pregnancy body mass index, smoking and alcohol consumption during pregnancy, gestation length, birth weight by sex and infant sex; [4] Model 2 refers to additional adjustments made for maternal depressive symptoms (Center of Epidemiological Studies Depression Scale score) at infant age of 15.6 days.

Figure(s)

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Figure 1.

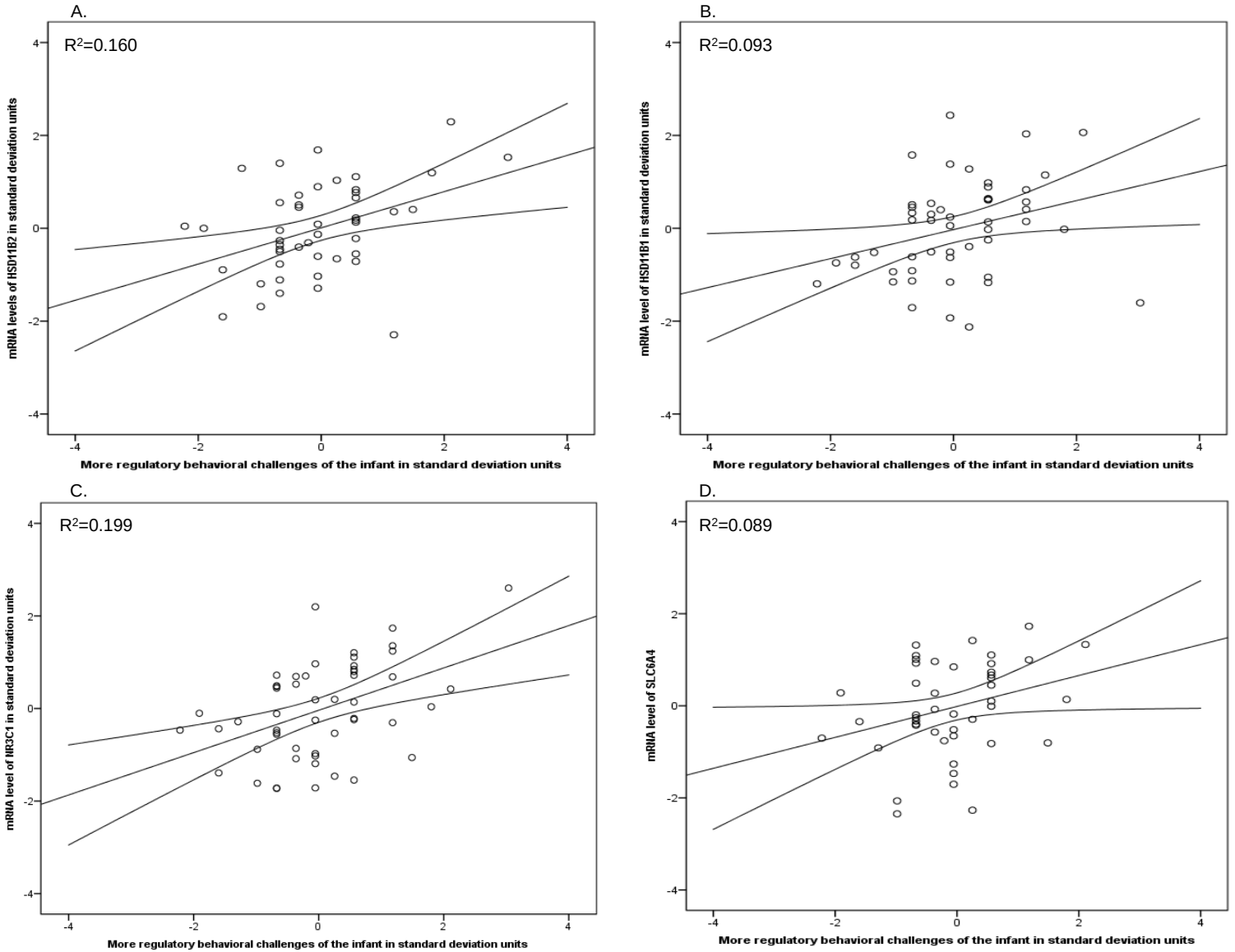

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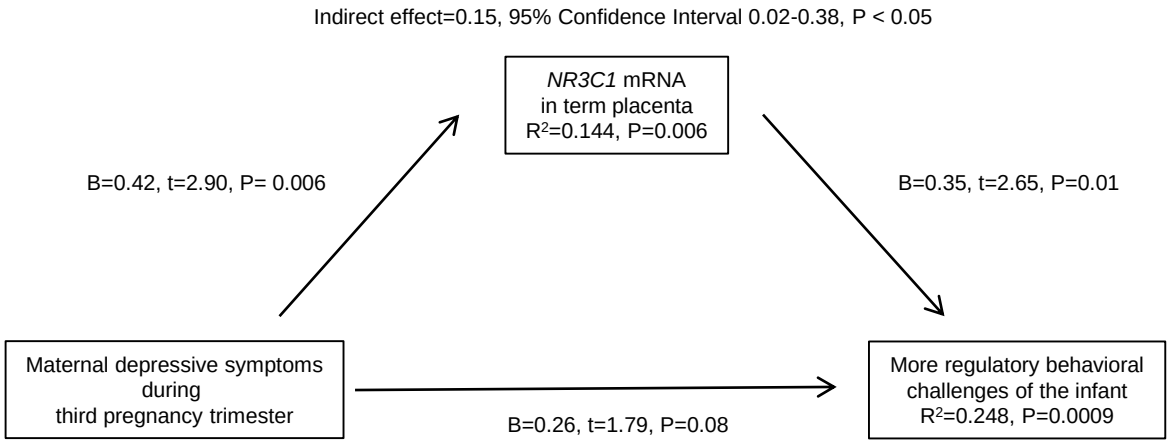
Figure 2.



Katri Räikkönen PhD
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Helsinki 16th May 2015

RE: Ref. Ms. No. PSM-D-15-00153

Dear Editor Professor Carmine M. Pariante

Many thanks for your interest in our manuscript that has been re-titled according to suggestions of reviewer #1 **"Maternal depressive symptoms during pregnancy, placental expression of genes regulating glucocorticoid and serotonin function and infant regulatory behaviors"** and for giving us the opportunity to revise this. We detail below how we have addressed the concerns by the reviewer.

We believe that our paper has improved considerably and hope that this version of the manuscript is now acceptable for publication in Psychological Medicine.

We are looking forwards to hearing your response.

Sincerely,

Katri Räikkönen PhD
Academy professor

Reviewers' and editor's comments:

PLEASE NOTE: Figures, which should be uploaded as a separate file, should be produced using size 8 point Arial font for the legend. Any wording within a figure should ideally be in Arial - 8 point size is standard, but this may vary depending on space limitations within individual figures.

RESPONSE: All text and numbers in Figure 1 and 2 are in Arial 8.

Comments by Reviewer #1

Reviewer #1: The focus of this study is to assess the expression of genes regulating glucocorticoid and serotonin function in placenta and to determine the association gene expression with maternal depressed mood during pregnancy and mother-rated infant behaviour (crying, feeding, spitting, elimination, sleeping) at 15.6 days of age. The data on the association of placental gene and maternal depressed mood during pregnancy has been published. The study subjects were women enrolled in the Prediction and Prevention of Preeclampsia (PREDO) study (n=54 women participated in the current study). This is a well written study assessing a well-desinged cohort. However, there are serious concerns regarding the over interpretation of the data provided in this manuscript.

1. The title should reflect the proper title of genes that were assessed for expression. For example, NR3C1 for glucocorticoid receptor and SLC6A4 for serotonin transporter. Glucocorticoid and serotonin mRNA levels were not quantified. Alternatively it could not be so specific (as in the abstract) and include for example, as statement such as: expression of genes regulating serotonin and glucocorticoid function were determined.

RESPONSE: We thank the reviewer for this comment and have changed the title as suggested as being less specific. Our paper is now entitled "Maternal depressive symptoms during pregnancy, **placental expression of genes regulating glucocorticoid and serotonin function** and infant regulatory behaviors" Please see p 1.

2. Including further experiments, such as protein expression of the relevant genes, etc, would strengthen the study. It is unclear what the biological relevance is of the associations between gene expression (mRNA) and infant regulatory behaviour.

RESPONSE: We agree with the reviewer that further experiments that would include measurement of functional end products of the key genes of interest would strengthen the study. Unfortunately we do not have these data available and therefore cannot include the suggested protein expression data in the manuscript. We have added lack of these data in the discussion as a study limitation and state that "We only measured mRNA levels of the key genes of interest **and lack data on levels of their respective protein levels**. Further studies are needed to determine the mechanisms of altered gene expression, such as methylation of promoter and transcription factor binding sites and other epigenetic mechanisms, and whether these are modifiable **as are studies that focus on functional end products of the key genes of interest**." Please see p 15.

3. It would be useful to include some analysis in the children eg cord blood.

RESPONSE: This would indeed be interesting. The sample size is however small and this would add a level of complexity to the findings reported here. Apart of phenotypic data, further studies are clearly warranted that include analyses of biological samples of the children as well. In the discussion we have stated that "We recognize that we lack data on several elements of the maternal-placental-fetal glucocorticoid and serotonin signaling pathway." Please see p 15.

4. Given the numerous studies assessing methylation of the genes the study would be strengthened by including analyses of gene promoter methylation and assessing the association with gene expression.

RESPONSE: This would be interesting too. We have discussed lack of these data as a study limitation. “Further studies are needed to determine the mechanisms of altered gene expression, **such as methylation of promoter and transcription factor binding sites and other epigenetic mechanisms**, and whether these are modifiable, as are studies that focus on functional end products of the key genes of interest. “ Please see p 15.

5. It would be useful to see what the RNA data looks like rather than just providing the effect size in the regression models.

RESPONSE: We have now reported median values and interquartile ranges, rather than mean values and standard deviations because of the skewed distributions, of the mRNA levels of the key genes of interest in Table 1. For analyses these variables were log-transformed and we now report in the statistical analysis section that this transformation was successful as requested by reviewer #3. Please see Table 1.

6. Please use proper gene names for the RNA data. Without this, it is difficult to gage the biological relevance of the findings.

RESPONSE: As suggested by the reviewer, we have used proper gene names throughout the manuscript.

7. The scatter plots are difficult to read. Are adjusted or unadjusted values plotted?

RESPONSE: We are plotting unadjusted values and have now clarified this in the results section as well as in the figure legend. We have clarified this in the figure legends and in the results section. Please see p 11, 12 and 22.

8. Discussion. Data must not be over interpreted. Only mRNA levels were assessed, which really does not tell anything regarding protein or activity. For example, a statement such as 1st sentence, paragraph 2, page 13, … `higher placental GR mRNA level may mediate the effects of higher maternal depressive symptoms during third trimester of pregnancy on more regulatory behavioural challenges of the infant.' It's unclear how biologically NR3C1 mRNA expression in placenta could affect infant behaviour at 15 days of age without additional experiments.

RESPONSE: We agree with the reviewer and have now stated that “Our study is also the first to show that higher placental *NRC31* mRNA level **plays a role in the process that may mediate** the effects of higher maternal depressive symptoms during the third trimester of pregnancy on more regulatory behavioral challenges of the infant.” Please see p 13 and 16.

Comments by Reviewer #2

Reviewer #2: RE: Manuscript number PSM-D-15-00153, "Maternal depressive symptoms during pregnancy, placental glucocorticoid and serotonin mRNA levels and infant regulatory behaviors." The premise of this manuscript was to identify associations between prenatal exposure to maternal depressive symptoms, mRNA levels, indicating gene expression, of genes involved in the newborn response to stress, and newborn regulatory behaviors. This is a very exciting and generally methodologically sound study. Most of the epigenetic literature in humans testing prenatal programming hypotheses are related to prenatal exposures and DNA methylation, but

very few look at gene expression or relate gene expression to infant behavioral outcomes. While there are some limitations, I think they can be addressed or at the very least noted in the discussion. If this paper gets published (and I think it should) I think it will have a very high impact.

1. Introduction: I think the significance and innovation of this study would be strengthened with a discussion about how very few (if any) studies relate placental gene expression to functional behavioral outcomes in infancy. Discuss why such studies are important (e.g., tests of prenatal programming hypotheses need to demonstrate that prenatal experiences relate to behavioral outcomes).

RESPONSE: We thank the reviewer for this suggestion. We have modified the text in the introduction to point out this novel aspect of our study. Please see p 6 and 7.

2. These ideas are not clear, "We have previously shown in this sample that maternal depressive symptoms during pregnancy were significantly associated with higher placental mRNA levels of GR and MR, but were not significantly associated with the levels of 11β-HSD2, 11β-HSD1 and SERT (Reynolds et al 2015). Consequently, placental GR and MR mRNAs could be tested as potential mediators providing the other criteria for mediation were met (Hayes 2009): namely, that placental GR and MR mRNAs and maternal depressive symptoms during pregnancy were also associated with infant regulatory behaviors. Hence, our secondary objective was to examine if maternal depressive symptoms acted via altering glucocorticoid action in and via the placenta to impact on infant regulatory behaviors." I think the authors are saying there were no main effects of depressive symptoms on mRNA 11β-HSD2, 11β-HSD1 and SERT but that does not rule out that these genes may serve as mediators of infant behavioral outcomes. Thus, the authors include these genes in their analysis since there is evidence that they may mediate the effect of depressive symptom exposure on newborn outcomes. If I am misunderstanding, though, please clarify.

RESPONSE: We have edited this part of the introduction and hope that the rationale for including *NR3C1* and *NR3C2* in the mediation analyses is now made clearer. Please see p 6 and 7.

3. Please include assay design information for GR, 11β-HSD2 and SERT (this could be in an appendix or in online supplementary material).

RESPONSE: These were pre-designed assays but the reference number has been added. Please see p 8.

4. I think the biggest limitation of this study is that the measure of infant regulatory behaviors is not particularly strong. It is maternal report, though I appreciate that the authors tried to limit maternal bias. For instance, it is well known that mothers with depression rate their infant as temperamentally more difficult. Including an objective measure of newborn neurobehavior, such as the NNNS, would increase the reliability and validity of this construct. Given that this field is in it's infancy, however, I do not think their measure of infant regulatory behavior is woefully inadequate, and the authors note the limitations of their instrument in the discussion.

RESPONSE: Unfortunately we did not include the NNNS in our study protocol. We have, as the reviewer points out, discussed the limitations related to the measure of infant regulatory behaviors.

5. It would be helpful if the authors included their rational behind assessing prenatal maternal depression during the first, second, and third trimesters. What were the correlations between CESD scores during each trimester? Previous work shows that maternal anxiety during

2nd trimester (but not 3rd) is related to child behavioral outcomes. Why would only 3rd trimester depression be significantly associated with newborn behavior?

RESPONSE: We have reported these correlations in our recently published paper by Reynolds RM, Pesonen A-, O'Reilly J, Tuovinen S, Lahti M, Kajantie E, Laivuori H, Villa P, Hämäläinen E, Seckl JR, Räikkönen K (2015). Maternal depressive symptoms throughout pregnancy are associated with increased placental glucocorticoid sensitivity. *Psychological Medicine.* Advance Online Publication. DOI: http://dx.doi.org/10.1017/S003329171400316X).

In the paper cited above we have reported that the mean CES-D scores at each time point were significantly correlated: r= 0.66, 0.67 and 0.83 (all p-values <0.001) between first trimester value and second trimester mean value, first trimester value and third trimester mean value, and second and third trimester mean values, respectively.

In the paper cited above we have also discussed why depression at different time points during gestation may have different effects: It is recognized that there are critical windows during gestation when the developing fetus may be particularly vulnerable to in utero exposures (Räikkönen et al., 2012). For example, the third trimester has been shown to be a vulnerable time when maternal mental state and cortisol levels are linked to later offspring outcome (Yehuda et al., 2005). These findings suggest that maternal mood earlier in pregnancy can also influence fetal glucocorticoid exposure and outcome.

6. Is the regulatory measure comprised of different scales? Could the authors use factor analysis to determine if different scales emerge? Otherwise, could the authors justify their choice of examining whether certain genes are related to specific regulatory behaviors? Do the authors have hypotheses regarding why the expression of certain genes should be related to, for example, crying but not spitting up? I don't think this section of the manuscript is necessary unless the authors have a clear theoretical rational.

RESPONSE: We have included the results of a principal components analysis to the revised version of the manuscript. This analysis supports unidimensionality of the scale. Please see p 9. Further, as the reviewer suggests we have deleted the findings in relation to 'different' regulatory behaviors from the results section as the scale is clearly unidimensional.

7. Could the authors please clarify heading, "Placental GR mRNA levels as a mediator." A mediator of what?

RESPONSE: We have edited the heading and in the revised version it is 'Mediation analyses'. Please see p 11.

8. Another limitation is that most women seemed to have lower depressive symptoms. How many fell in clinical range (CESD of 16 or above?) Again, this is not justification for not accepting the paper, but a limitation that should be addressed, since most of the epigenetic literature on maternal depression includes relatively well-off, "low risk" samples.

RESPONSE: In Table 1 we have reported the number of women who scored CES-D of 16 or above at each trimester and have noted that the health of the sample may limit generalizability from these findings in the discussion. Please see Table 1 and p 16.

Comments by Reviewer #3

Reviewer #3: Comments for Author (s)

Title: Maternal depressive symptoms during pregnancy, placental glucocorticoid and serotonin mRNA levels and infant regulatory behaviors
MS: PSM-D-00153

This paper tests mediation of mRNA levels in the association between maternal depressive symptoms during pregnancy and infant regulatory behaviors. mRNA levels in placenta for 11B-HSD2, 11B-HSD1, GR, and SERT were associated with greater "regulatory behavioral challenges" during infancy in models that adjusted for multiple variables. GR mRNA mediated the association between depressive symptoms and regulatory behaviors. Overall, this is an interesting paper and likely one of the few examples linking mRNA from placental tissue to maternal ratings of infant behavior. There are some concerns, outlined below, that deserve further clarification.

1) It's not completely clear how the 2015 paper and the current paper diverge in their presentation. From my reading, depressive symptoms and their relation to mRNA levels was the focus of the first paper whereas this second is focused on 1) depressive symptoms and mRNA levels and their relation to child outcomes, and 2) potential mediation of mRNA in the pathway from maternal depression to child outcomes. The set-up for this paper and how it builds from or is different from the previous paper needs to be made clearer in the last paragraph of the introduction. The description that is currently there needs to be more explicit.

RESPONSE: We have edited the last paragraph of the introduction to be more explicit on how this work differs from what we previously reported. We hope that the edited text is clearer. Please see p 6 and 7.

2) Since child regulatory behaviors is a critical piece of this paper, it is important to describe the validity of this measure in the methods section. Some of this information comes later in the discussion, but should be also in the methods section. More information in the methods section is needed to confirm for the reader that this measure of regulatory behaviors is indeed meaningful. Most of the items seem to reflect normal behavior for a 1 year old infant. The alpha level is moderate. Further, the method for assessment (a difference score), while interesting, may or may not be valid.

RESPONSE: We have included results of the principal components analyses to support the validity of this scale. We have however not presented data on criterion validity in the methods section and then repeated these data in the discussion, as we would like to present the reader a more thorough discussion related to different aspects of the measure including its' limitations. Please see p 9.

3) The data were transformed, but the results of this transformation were not reported. Was the transformation effective? Are the regression residuals normally distributed after transformation?

RESPONSE: We used log-transformation for the variables and then standardized the variables to mean of 0 and standard deviation of 1. We have included a sentence in the statistical analyses indicating that the log-transformation was successful. Please see p 10.

4) The sample size is fairly small and there are several covariates in the analysis. Power to detect associations, as well as mediation effects, may be compromised. Post-hoc power analysis could help in this respect.

RESPONSE: We agree that the sample size is small. However, if we did not detect significant associations, then we think that post hoc power analysis would be in place, though post hoc power calculations are not recommended in general (because actual relative estimate and its

variance are being ignored, which by the time of analysis are already known) (e.g. Smith and Bates (1992) Confidence limit analyses should replace power calculations in the interpretation of epidemiologic studies. Epidemiology 3 449-450). Since we detected significant associations, our sample provides sufficient power to detect at least robust significant associations as reported in Tables and Figures. Also, inspection of the confidence intervals supports detection of robust associations (if sample size is too small, this is reflected in wide CIs, Bates and Smith (1992)).

5) In general, it would be useful to interpret the effect sizes for the reader. Most of the effects are fairly small. The proportion of the mediated effect could be calculated and reported. Specifically, what percent of variance is accounted for by the mediated effect? Also, in the mediation analyses it is not clear if maternal depression at the time of her rating of her child was in the model. This should be clarified.

RESPONSE: This is a good point. We have included the proportion of variance accounted for in mRNA of *NR3C1* by maternal depressive symptoms and in regulatory behaviors by *NR3C1* and depressive symptoms. Please see Figure 2 and text reporting the mediation analyses on p 12.

6) Given the small sample effects, what else besides maternal depression drives mRNA levels of these genes? Could be useful in the discussion to comment on this and why this was not analyzed in the models.

RESPONSE: We have included a number of covariates and confounders that may either drive mRNA levels or ratings of infant regulatory behaviors. We have also discussed the findings in the context of previous research. We have however left out discussion of common pregnancy disorders, pre-eclampsia and gestational diabetes, as women in our sample did not suffer from these complications.

7) In general, it could be made clearer what is actually being measured from placental expression of key genes. Is this a mix of maternal and infant blood - so are the expression levels characteristic of mothers or offspring, or does it matter?

RESPONSE: We acknowledge that a limitation of the study is that we used full thickness placental biopsies and so while predominantly reflecting fetal characteristics there may be mixture of maternal and fetal tissue. We have included this limitation in the discussion. Please see p 15.