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Examining the joint contribution of placental *NR3C1* and *HSD11B2* methylation for infant neurobehavior

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Abstract

Infant neurobehavior, a potential sentinel of future mental and behavioral morbidity characterized in part by reflex symmetry, excitability and habituation to stimuli, is influenced by aspects of the intrauterine environment partially through epigenetic alterations of genes involved in the stress response. DNA methylation of two related cortisol response genes, the glucocorticoid receptor (*NR3C1),* a nuclear receptor to which cortisol binds, and 11-beta hydroxysteroid dehydrogenase (*HSD11B2),* the enzyme responsible for conversion of cortisol into inactive cortisone, independently associate with infant neurobehavior. Although these factors are part of a common cortisol regulation pathway, the combined effect of DNA methylation of these factors on infant neurobehavior has not been characterized. Therefore, we conducted an examination of the joint contribution of *NR3C1* and *HSD11B2* DNA methylation on infant neurobehavior. Among 372 healthy term newborns, we tested the interaction between placental *NR3C1* and *HSD11B2* DNA methylation in association with neurobehavior as assessed with the validated NICU Network Neurobehavioral Scales. Controlling for confounders, interactions between DNA methylation of these genes were detected for distinct domains of neurobehavior (habituation, excitability,

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

AAA conceived and designed the study, conducted data analyses, wrote and edited the manuscript. BML contributed to data analyses, reviewed and edited the manuscript. DAA and CL processed the samples, reviewed and edited the manuscript. CJM conceived and designed the study, contributed to data analyses, reviewed and edited the manuscript. All authors contributed to and approved the final manuscript.

asymmetrical reflexes). Moreover, different patterns of DNA methylation across the cortisol regulation pathway associated with different neurobehavioral phenotypes. Those with low *NR3C1* methylation but high *HSD11B2* methylation had lower excitability scores; those with high *NR3C1* methylation but low *HSD11B2* methylation had more asymmetrical reflexes; those with high DNA methylation across the entire pathway had higher habituation scores. These results suggest that epigenetic alterations across the cortisol regulation pathway may contribute to different neurobehavioral phenotypes, likely though varying degrees of glucocorticoid exposure during gestation. While the postnatal environment may continue to affect neurobehavioral risk, this study provides novel insights into the molecular basis for fetal origins of mental conditions.

Keywords

NR3C1; *HSD11B2*; DNA methylation; epigenetic; placenta; infant; neurobehavior; developmental origins of health and disease

INTRODUCTION

In order to better understand disease etiology and identify novel avenues for prevention and intervention, examining the developmental origins of mental health conditions is of increasing interest (Schlotz and Phillips, 2009). Many mental and behavioral health conditions are now known to be attributable in part to an adverse intrauterine environment (Barker, 1998; Raikkonen et al., 2012; Sandman and Davis, 2012; Schlotz and Phillips, 2009). Neurobehavior assessed in infancy (characterized in part by reflex symmetry, excitability and habituation to stimuli) has been shown to be a sentinel of future mental, neurological and behavioral morbidity (Liu et al., 2010; Stephens et al., 2010; Tronick and Lester, 2013), while also sensitive to a range of deleterious prenatal exposures (Bagner et al., 2009; Coyle et al., 2005; de Moraes Barros et al., 2006; de Moraes Barros et al., 2008a; de Moraes Barros et al., 2008b; Law et al., 2003; Lester et al., 2002; Napiorkowski et al., 1996; Salisbury et al., 2005; Salisbury et al., 2007; Smith et al., 2008; Stroud et al., 2009; Tronick and Lester, 2013). While perturbations in the developing stress-response system (the hypothalamic–pituitary–adrenal axis (HPA)) have been implicated in the pathophysiology of mental illness, the molecular mechanisms linking HPA dysregulation to infant neurobehavior have not been fully elucidated (Lester et al., 2014; Monk et al., 2012). Therefore, we examined variability in infant neurobehavior according to placental epigenetic alterations of HPA-related genes in order to provide novel mechanistic insights into the developmental origins of mental disease.

Epigenetics involves mechanisms that control patterns of gene expression without modification of the underlying nucleotide sequence of DNA. Epigenetic mechanisms, such as DNA methylation, are particularly relevant for understanding the early origins of mental disease as they are sensitive to environmental exposures and can be altered during critical periods of development like gestation (Robins et al., 2011) and early childhood (Meaney and Szyf, 2005), although epigenetic alterations can be influenced by a range of environmental exposures occurring in adulthood as well (Madrigano et al., 2012). The fetal environment is regulated by the placenta, which plays an active immune-endocrine functional role in

pregnancy, in addition to its role in nutrient, gas, and waste exchange (Marsit et al., 2012). The placenta is also involved in HPA development, including the development of the cortisol regulation pathway, through the activities of placentally expressed glucocorticoid receptor (NR3C1) and 11-β hydroxysteroid dehydrogenase type 2 (HSD11B2). NR3C1 is a nuclear receptor to which glucocorticoids like cortisol bind, and it facilitates cortisol's transcriptional activity, including regulation of *HSD11B2*. Placental HSD11B2 is responsible for converting cortisol into inactive cortisone, thereby protecting the developing fetus from overexposure to glucocorticoids during development. However, this protective mechanism has limits. If *NR3C1* is dysregulated potentially from significant prenatal stressors, the protective effect of placental HSD11B2 may be diminished, thereby allowing elevated levels of glucocorticoids into fetal circulation (Sarkar et al., 2001; Staud et al., 2006). Overexposure to glucocorticoids is associated with range of deleterious outcomes across the life course, including low birth weight, poor infant neurodevelopment, adult anxiety and cardiometabolic disorders (Cottrell and Seckl, 2009; Marsit et al., 2012; Wyrwoll et al., 2011).

There is emerging evidence to suggest that *NR3C1* and *HSD11B2* DNA methylation are each associated with infant neurobehavior. In previous work among 186 infants from the current sample, placental DNA methylation of *NR3C1* was marginally associated with infant neurobehavior in terms of quality of movement and attention regulation (Bromer et al., 2012); *HSD11B2* DNA methylation was associated with infant quality of movement and being born low birth weight (Marsit et al., 2012). Another study from this sample found greater *NR3C1* and *HSD11B2* placental methylation to be associated with worse neurobehavior among newborns whose mothers had either depression or anxiety during pregnancy (Conradt et al., 2013). These findings are congruent with work in other samples focused on stress-related gestational HPA programing that examined infant outcomes correlated with neurobehavior. One study of 82 infants found greater DNA methylation extent of cord blood *NR3C1* to predict dysregulated salivary cortisol response at 3 months (Oberlander et al., 2008), while another found that among 45 newborns studied, DNA methylation of placental *NR3C1* was associated with maternal smoking during pregnancy, and also with cortisol reactivity over the first month of life (Stroud et al., In Press). Another study of 25 infants whose mothers were exposed to high levels of stress (war trauma) during pregnancy found higher cord blood *NR3C1* DNA methylation to be associated with lower birth weight (Mulligan et al., 2012). Taken together, this emerging evidence suggests that gestational DNA methylation of *NR3C1* and *HSD11B2* is influenced by the intrauterine environment and their DNA methylation extent is associated with infant neurobehavior and related outcomes.

Although these factors are part of a common pathway, the neurobehavioral effects of *HSD11B2* and *NR3C1* DNA methylation have not been examined jointly. It is not known how neurobehavior may be affected if either or both of these gene promoters are simultaneously methylated. Moreover, because NR3C1 and HSD11B2 are not operating in isolation from one another, examining DNA methylation patterns across the cortisol regulation pathway may provide an enhanced representation of HPA dysregulation than when examining these factors separately. Therefore, we examined the joint contribution of

placental *NR3C1* and *HSD11B2* DNA methylation to infant neurobehavior. We hypothesized that (1) placental *NR3C1* and *HSD11B2* DNA methylation would interact to jointly influence infant neurobehavior, and (2) various patterns of DNA methylation across the gene promoters may associate with different domains of neurobehavior. We tested these hypotheses in a large population based sample of healthy term infants using a gold-standard assessment of infant neurobehavior predictive of mental health in later childhood (Liu et al., 2010). To our knowledge, this study is the first to examine the neurobehavioral impact of DNA methylation among two HPA-related genes simultaneously.

METHODS

Study Population

Study subjects were part of the Rhode Island Child Health Study, which enrolled healthy mother and infant pairs following delivery at the Women and Infants Hospital of Rhode Island (Providence, RI, USA). Term infants born small for gestational age (lowest 10^{th} percentile), or large for gestational age (highest 10^{th} percentile), based on birth weight and gestational age and calculated from the Fenton growth chart (Fenton, 2003), were selected; infants appropriate for gestational age matched on gender, gestational age (±3 days), and maternal age (±3 years) are also enrolled. Birth weight percentiles have been updated using the revised Fenton criteria (Fenton and Kim, 2013). Only singleton, viable infants were included in the study. Other exclusion criteria were maternal age (<18 or >40 years excluded), a life-threatening medical complication of the mother, and congenital or chromosomal abnormality of the infant. A structured chart review was used to collect information from the maternal inpatient medical record from delivery. While still in the hospital after delivery but prior to discharge, mothers participated in an interviewer-administered structured questionnaire to obtain information on demographics, prenatal health behaviors, and health and exposure histories. Infant neurobehavior was assessed after the first day of life but before hospital discharge.

Between September 1, 2009 and July 31, 2013, 1150 eligible infants were identified and 721 enrolled (63%). Of these, neurobehavior was assessed for 630 (87%). For this examination, 375 participants were examined for DNA methylation for both *NR3C1* and *HSD11B2*. There were no significant differences by maternal age, race, education, smoking during pregnancy and infant gender among those with and without DNA methylation information (*p*s>0.05; data not shown), although infants with DNA methylation data were on average 156 grams heavier at birth (t=−2.91, p<0.01). The analytic sample for the current study includes 372 infants who had both *NR3C1* and *HSD11B2* placental DNA methylation information available, as well as infant neurobehavior and covariate information. Analytic sample sizes for two neurobehavior subscales were smaller due to missing data related to requirements of the NNNS administration and scoring (e.g., infant must be in a sleep state at the beginning of the assessment, habituation n=212; require minimum number of completed items for scoring, attention n=332). Study protocols were approved by the Institutional Review Boards for Women and Infants' Hospital and Dartmouth College. Mothers provided written informed consent for participation and also for participation of her infant.

Placenta Sample Collection, Nucleic Acid Extraction and Bisulfite Modification

For each subject, 3 samples from each of the 4 quadrants (totaling approximately 10 g of tissue) were excised from the maternal side of the placenta, 2 cm from the umbilical cord insertion site, and free of maternal decidua. The samples were placed immediately in RNAlater (Life Technologies, Grand Island, NY) and stored at 4°C. At least 72 hours later, placenta samples were removed from RNAlater, blotted dry, snap-frozen in liquid nitrogen, homogenized by pulverization using a stainless steel cup and piston unit (Cellcrusher, Cork, Ireland) to create a uniform sample, and stored at −80°C until needed for examination. DNA was extracted from the placenta samples using the Qiagen DNAeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). Purified DNA was quantified using a ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and DNA samples (500 nanograms) were bisulfite-modified using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA.) and stored at −20°C. To prevent batch effects from bisulfite treatment interfering with the analysis, samples were randomized across conversion batches.

Bisulfite pyrosequencing DNA methylation analysis

The DNA methylation status for both the *NR3C1* exon 1F and *HSD11B2* promoter regions was assessed using quantitative bisulfite pyrosequencing as previously described (Bromer et al., 2012; Marsit et al., 2012). Percent DNA methylation at each CpG site (13 for *NR3C1*, 3 for *HSD11B2*) was quantified using the PyroMark MD instrument and the PyroMark Q-CpG software, version 1.0.11. (Qiagen). Bisulfite conversion controls were included on each sequencing read. In order for the sample's DNA methylation extent to be called, the bisulfite conversion rate must be >93%, and for all samples examined the conversion rate was >95%. All samples were sequenced in triplicates from the same bisulfite converted DNA template, and if the repeats differed by >10% the sample was repeated

NR3C1 and HSD11B2 DNA methylation extent

Since DNA methylation status of the individual CpG sites was highly correlated and as prior reports have linked average DNA methylation across the region sequenced with reduced expression (Bromer et al., 2012; Marsit et al., 2012), for both *NR3C1* and *HSD11B2*, DNA methylation across each of the CpG sites was averaged to obtain measures of overall methylation extent, one for each gene promoter. As the distributions of DNA methylation extent for *NR3C1* and *HSD11B2* were skewed, a $\log_{10}$ transformation was applied in order to approximate a normal distribution for each factor. Primary analyses considered *HSD11B2* and *NR3C1* continuously, and a continuous interaction term was constructed by multiplying these factors together. Additional analyses were conducted to understand the pattern of the *NR3C1* and *HSD11B2* interaction using a pooled categorical DNA methylation variable. To construct this variable, DNA methylation extent of the non-transformed *NR3C1* and *HSD11B2* were each dichotomized as high and low. Once dichotomized, a four-category variable was created characterizing the pattern of DNA methylation possible between the gene promoters: High DNA methylation in both *NR3C1* and *HSD11B2*, high methylation in *NR3C1* and low methylation in *HSD11B2*, low methylation in *NR3C1* and high methylation in *HSD11B2*, and low methylation in both factors. As there are no known cut points to signify a high degree of DNA methylation for *NR3C1* and *HSD11B2*, we classified the top

25% of the distributions for each as being highly methylated. Sensitivity analyses were conducted using more (15%) and less (33%) extreme cut points. As the pattern of associations were generally similar regardless of which cut point employed, we elected to use the 25% dichotomy as it was the most balanced between sensitivity to detect an effect and statistical power. Using this dichotomy, the cut points for high *NR3C1* and *HS11B2* DNA methylation were 3.3% and 15.2% methylated, respectively. High *NR3C1* DNA methylation included those with methylation values ranging from 3.3 to 9.5 (mean=5.20). Low *NR3C1* DNA methylation included those with methylation values ranging from 0.68 to 3.26 (mean=1.67). High *HSD11B2* DNA methylation included those with methylation values ranging from 15.2 to 24.4 (mean=17.48). Low *HSD11B2* DNA methylation included those with methylation values ranging from 4.75 to 15.16 (mean=12.13). Mean DNA methylation between high and low groups were statistically significant (*NR3C1* t=30.9, p<0.001; *HSD11B2* t=23.6, p<0.001). Finally, as past work has found inverse associations between *NR3C1*and *HSD11B2* DNA methylation with gene expression in this sample (Bromer et al., 2012; Marsit et al., 2012), we focus on methylation associations and do not repeat reported DNA methylation and gene expression associations.

Infant neurobehavior

Infant neurobehavior was assessed with the NICU Network Neurobehavioral Scale (NNNS), a validated assessment that comprehensively measures infant neurobehavior (Lester and Tronick, 2004) and prospectively predicts neurological problems, behavior problems, school function and IQ in early childhood (Liu et al., 2010; Stephens et al., 2010; Tronick and Lester, 2013). The NNNS was administered during the infant's inpatient stay but after the first 24 hours of life by certified psychometrists blinded to the study hypotheses and scored using established protocols (Lester and Tronick, 2004). Thirteen summary scores that reflect different domains of neurobehavioral functioning are derived from the exam and are described in Table 1.

Covariates

Maternal age, race, education, prenatal tobacco use, depression during pregnancy, infant gender and birth weight percentile were included as covariates in multivariable models based on their potential to confound placental DNA methylation and infant neurodevelopment associations (Rothman and Greenland, 1998). Race was dichotomized as white and not white based on the distribution of race/ethnicity in the sample. Education was dichotomized according to the highest level attained (high school or less versus more than high school). Prenatal tobacco use was assessed dichotomously (yes/no) as recorded in the medical record. Maternal depression during pregnancy was recorded as present or absent in the medical record and treated dichotomously in analysis. Maternal age and birth weight percentile were treated continuously.

Statistical analysis

Descriptive statistics were calculated, and bivariate relations among DNA methylation and study covariates were examined via Pearson's correlations and independent t-tests. Bivariate correlations between *NR3C1* and *HSD11B2* and NNNS summary scales were examined.

Correlations among the 13 NNNS summary scales were also examined. Next, we tested whether *NR3C1* and *HSD11B2* jointly affected infant neurobehavior following standard methods for testing continuous interactions (Aiken and West, 1991; Quinn and Keough, 2002). We first test the null hypothesis, H_0, of no interaction by fitting a set of linear regression models that included the main effects and interaction terms for *NR3C1* and *HSD11B2*. Because examination of the interaction between *HSD11B2* and *NR3C1* DNA methylation was the underlying motivation for this analysis, we focus on interpreting the nature of the interaction with additional analyses (Aiken and West, 1991; Quinn and Keough, 2002). For the interactions with *p*'s<0.10, additional models were fit using the four category combined methylation variable as the primary predictor to assess how *NR3C1* and *HSD11B2* DNA methylation jointly affected infant neurobehavior. As it is possible to have conditional relationships in the absence of significant main effects, we examined the pattern of the interaction in the additional models even if one or more of the main effects were not significant (Aiken and West, 1991; Quinn and Keough, 2002). Statistical significance was determined by p-values < 0.05. Additionally, to correct for multiple comparisons among the 13 tests of interaction, we implemented the Benjamini-Hochberg procedure for controlling for the false discovery rate by estimating q-values and the expected proportion of false discovery for the interactions with uncorrected p-values < 0.10 (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001; Thissen et al., 2002).

RESULTS

Participant characteristics and bivariate associations between study covariates and DNA methylation variables are listed in Table 2. Mothers were on average 29.4 years old, 27% were not white, 27% had a high school education or less, 4.8% smoked during pregnancy and 14.5% were depressed during pregnancy. Half of the infants were male and birth weight was on average at the 56th percentile. None of the study covariates were associated with *NR3C1* DNA methylation. Maternal age, education, and birth weight were each marginally associated with *HSD11B2* DNA methylation. While other covariates were not associated with *NR3C1* or *HSD11B2* DNA methylation, all were included in multivariable models to be conservative.

Table 3 lists descriptive information for *NR3C1* and *HSD11B2*. The average DNA methylation extents across *NR3C1*and *HSD11B2* CpG loci were 2.1% and 13.8% respectively. Such a relatively low degree of DNA methylation was expected in this healthy cohort of term births and is similar to what has been previously reported (Appleton et al., 2013; Bromer et al., 2012; Conradt et al., 2013; Marsit et al., 2012; Stroud et al., In Press). Overall, the pattern of methylation across CpG sites was similar for each gene promoter. Nearly 4% had high (top 25th percentile) DNA methylation of *NR3C1* and *HSD11B2*, 22% had high *NR3C1* but low *HSD11B2* methylation, 22% had low *NR3C1* but high *HSD11B2* methylation, and 53% had low methylation of both gene promoters. *NR3C1*and *HSD11B2* were inversely associated with one another (r=−0.26, p<0.001).

Table 4 lists correlations among the NNNS summary scales. While some neurobehavioral domains are significantly associated in the expected directions (e.g., higher attention associated with greater self-regulation, less stress and less arousal), some domains were not

correlated (e.g., asymmetrical reflexes not correlated with habituation, excitability, or arousal), which underscores the ability of the NNNS assessment to capture distinct domains of neurobehavior. Of all the NNNS domains, *NR3C1* and *HSD11B2* were correlated with stress (r_{NR3C1}= −0.18, p<0.001; $r_{HSD11B2}$=−0.11, p<0.05) and asymmetrical reflexes (r_{NR3C1}= −0.16, p<0.01; $r_{HSD11B2}$=−0.12, p<0.05).

Table 5 displays the linear regression models that include the main effects (Model 1) and interactions (Model 2) of placental *NR3C1* and *HSD11B2* DNA methylation in association with infant neurobehavior. P-values listed in this table are uncorrected for multiple comparisons. After adjusting for covariates (Model 2), interactions between *NR3C1* and *HSD11B2* were observed for habituation (β=9.64, *SE*=3.97, uncorrected *p*=0.02), excitability (β=10.50, *SE*=5.71, uncorrected *p*=0.06), and asymmetrical reflexes (β= −5.21, *SE*=2.74, uncorrected *p*=0.05). Maternal age (β=0.04, *SE*=0.18, *p*=0.03) and infant gender (β= −0.60, *SE*=0.30, *p*=0.05) were associated with excitability in adjusted models. No other covariates were associated with other domains of infant neurobehavior in adjusted models.

The q-values for habituation, excitability and asymmetrical reflexes were each 0.06. This indicates that 6% of the 3 observed interactions (habituation, excitability, asymmetrical reflexes) were false discoveries. As such, we could expect less than one of the tests to be a false discovery (i.e., 0.06*3=0.18 false discovery, or less than 1 one false discovery).

Table 6 displays the adjusted linear regression models examining how the interactions for habituation, excitability and asymmetrical reflexes were patterned using the combined categorical DNA methylation variable. Those who had low *NR3C1* methylation and high *HSD11B2* methylation scored nearly a full point lower in excitability compared to those with low levels of DNA methylation across both gene promoters (excitability was measured on a scale of 0–13; higher scores indicate greater excitability). Those who had high *NR3C1* but low *HSD11B2* DNA methylation scored about a half a point higher for asymmetrical reflexes compared to those with low levels of methylation across both gene promoters (asymmetrical reflexes was measured on a scale from 0–7; higher scores indicate a greater number of asymmetric reflexes). There was no significant association between the combined DNA methylation variable and infant habituation, although a non-significant trend was evident towards higher habituation scores among those with high degrees of both *NR3C1* and *HSD11B2* methylation (*p*=0.14).

DISCUSSION

The results of this study suggest that placental DNA methylation of *NR3C1* and *HSD11B2* may jointly influence distinct domains of infant neurobehavior. The patterning of the interactions suggests that there may be various ways in which the cortisol regulation pathway can be modulated to potentially influence infant neurobehavior. Moreover, this study illustrates that methylation of this pathway may be associated with orthogonal neurobehavioral domains as there was no correlation between habituation, excitability, and asymmetrical reflexes in this study. These findings are particularly noteworthy as this study was the first to consider the simultaneous epigenetic contribution of two prospectively assessed HPA-related genes in association with infant neurobehavioral phenotype. Also,

infant neurobehavior was measured with a valid assessment that elicits a mild cortisol response (Stroud et al., In Press) and predicts significant mental, behavioral and cognitive morbidity in later childhood (Liu et al., 2010). Therefore, this study provides evidence that gestational programming of the cortisol regulation pathway during fetal development may contribute to variations in neurobehavior identifiable at birth, which may potentially influence mental health trajectories of children as they grow up.

The patterning of the interactions suggests some potential mechanisms for how placental epigenetic alterations to *NR3C1* and *HSD11B2* may jointly affect different domains of neurobehavior. For example, infant excitability reflects physiologic and motor reactivity to stimuli; lower scores suggest better control over body systems in the presence of stimuli and potential stressors. We found that lower excitability scores were associated with low *NR3C1* and high *HSD11B2* methylation. In pilot work in this sample, we found an inverse association between DNA methylation and expression of *NR3C1* and *HSD11B2* (i.e., higher methylation associated with lower expression) (Bromer et al., 2012; Marsit et al., 2012). Although we were unable to examine gene expression among the full sample in this study, the pattern of DNA methylation we observed for excitability may suggest reduced expression of *HSD11B2* and greater *NR3C1* expression, which could in turn be associated with increased fetal exposure and response to cortisol. In such a scenario, the placenta may adaptively utilize the active cortisol, which could work to protect the fetus from the exposure. Though we cannot determine the levels of gene expression associated with DNA methylation in this study, we find that among healthy children, those who have this particular pattern of DNA methylation at birth seem to show less neurobehavioral impairment in terms of reactivity to stimuli than other groups.

A slightly different but related set of processes may help explain the *NR3C1* and *HSD11B2* interaction association for asymmetrical reflexes. Higher asymmetrical reflex scores can suggest neurological problems as reflexes are normally elicited on both the left and right side of the infant. Those who had high *NR3C1* but low *HSD11B2* DNA methylation (which may suggest low *NR3C1* expression and high *HSD11B2* expression) had significantly higher asymmetrical reflex scores. Though we did not directly assess gene expression in this study, this pattern of DNA methylation observed in our sample may possibly suggest that the low degree of glucocorticoid expression that follows *NRC31* DNA methylation, particularly in the presence of functioning glucocorticoid barriers, may deleteriously associate with this domain of neurobehavior as some glucocorticoid exposure and response is necessary for appropriate fetal development (Cottrell and Seckl, 2009). Thus, among healthy children, those who have this particular pattern of DNA methylation may exhibit some asymmetry in neurological function compared to other groups.

The patterning in *NR3C1* and *HSD11B* DNA methylation in association with habituation may possibly suggest that a high degree of DNA methylation of both genes may interfere with the infant's ability to adapt and acclimate to environmental stimuli, such as blocking out noises to sleep. As high *HSD11B2* DNA methylation may suggest deterioration of the protective glucocorticoid barrier function, increased *NR3C1* DNA methylation and associated possible low glucocorticoid expression may potentially suggest a compensatory response to protect the developing fetus from overexposure to glucocorticoids. However,

such dysregulation of the entire cortisol regulation pathway may work to exacerbate rather than promote neurobehavior. While we did not measure gene expression directly in the current study, our data suggest that among healthy children, those with this particular pattern of DNA methylation may have greater neurobehavioral difficulty in processing stimuli than other groups. However, the association for habituation with the pooled categorical DNA methylation variable was underpowered and did not reach statistical significance. As such, we encourage others to replicate this finding using larger samples in order to examine how DNA methylation patterning across the cortisol regulation may affect this domain of neurobehavior.

Epigenetic changes in the intrauterine environment are increasingly being examined in relation to infant neurobehavior (Lester et al., 2014). This emerging body of research can provide molecular insights into the individual differences in behavior and mental functioning seen in later childhood. Findings from this and other studies may ultimately help clarify why mental and behavioral vulnerabilities in childhood emerge, why some children are more or less susceptible to poor outcomes and why children differently respond to neurobehavioral intervention. Moreover, this area of inquiry has begun to identify programming mechanisms sensitive to a range of environmental stimuli that occur in utero, which may one day offer novel avenues for intervention to safeguard the neurodevelopmental health of babies.

This study has some limitations. Like other work in this area, we cannot conclusively identify the mechanisms linking the intrauterine environment to the observed HPA-related epigenetic alterations and interactions, and with infant neurodevelopment. First, as the study population is comprised of healthy, term infants, we may have observed a lower degree of DNA methylation than would be observed in more heterogeneous samples. Also, we tested interaction associations among 13 neurobehavioral domains and as such, some of the observed associations may be due to chance. While results from the false discovery rate analyses help to mitigate this concern, the possibility of type 1 error remains. We encourage future work to replicate these associations among a targeted set of neurobehavioral outcomes. Additionally, as there are no established or biologically meaningful cut points to indicate high and low levels of placental *NR3C1* and *HSD11B2* DNA methylation, we dichotomized these factors according to variable distributions to assess relative differences in DNA methylation in relation to phenotype. While this approach reliably identified a group that has a higher degree of HPA-related DNA methylation, we could have misclassified some participants. This approach should be considered a preliminary categorization scheme to understand how variations in patterning of DNA methylation across multiple HPA-related gene promoters may yield differential effects on neurodevelopment. We encourage future work to build off this approach and consider this and alternative techniques to assess the combined influence of DNA methylation of HPA-related factors.

These limitations notwithstanding, this study has a number of strengths. This study is the first to consider the joint contribution of placental *NR3C1* and *HSD11B2* DNA methylation for infant neurodevelopment. Also, we used a validated neurobehavioral assessment that past work has found to prospectively predict mental, behavioral and cognitive problems in

later childhood (Liu et al., 2010). Moreover, as neurodevelopmental programming can extend well into the postnatal period (Meaney and Szyf, 2005), we assessed neurobehavior at birth so as to not confound associations with postnatal programming effects. Also, we focused on a healthy population of infants from uncomplicated pregnancies, thereby mitigating concerns that associations could be confounded by maternal or infant morbidity. Moreover, the sample is large and sufficiently powered to detect statistical interactions between placental *NR3C1* and *HSD11B2*.

Evidence is accumulating that epigenetic alterations occurring *in utero* to genes involved in the stress response can influence infant neurobehavior. Our study adds to this emerging evidence base and examined two HPA-related gene promoters involved in the regulation of cortisol as jointly influencing neurobehavior in infancy. Our findings revealed distinct patterns of placental DNA methylation across the cortisol regulation pathway which associated with different infant neurobehavioral phenotypes. While we acknowledge that the programming of mental conditions extends well into childhood, these associations provide novel insights into the molecular basis for the gestational origins of mental conditions. Moreover, this work underscores the utility of examining DNA methylation across multiple genes simultaneously. We encourage future work to adopt this approach in examining DNA methylation across relevant groupings of genes. Doing so may enable a broader understanding of how HPA dysregulation may affect mental health in infancy and across the life course.

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Highlights

- We examine placental epigenetic contributions of HPA genes for infant neurobehavior
- *NR3C1* and *HSD11B2* methylation interact to predict different neurobehavior domains
- Various methylation patterns across genes led to diverse neurobehavioral phenotypes

Table 1

NNNS summary domain definitions

NNNS domain	Description
Attention	Ability to localize and track auditory and visual stimuli
Quality of movement	Attributes of motor control, including smoothness, lack of tremors, maturity
Self-regulation	Capacity to modulate arousal and organize motor activity, physiology and state in response to stimulation
Habituation	Response decrement to repeated stimuli during sleep
Stress/abstinence	Number of stress and abstinence signs observed during the exam
Arousal	Level of state and motor arousal maintained through the exam
Handling	Extent to which handling strategies were used during the attention assessment to maintain alertness
Excitability	High levels of motor, state and physiologic reactivity
Lethargy	Low levels of motor, state and physiologic reactivity
Hypertonicity	Hypertonic responses in trunk, arms, legs or general tone
Hypotonicity	Hypotonic responses in trunk, arms, legs or general tone
Non-optimal reflexes	Number of poor scores to elicited reflexes
Asymmetric reflexes	Number of asymmetric responses to elicited reflexes

Table 2

Participant characteristics and bivariate associations with *NR3C1* and *HSD11B2* DNA methylation*

		NR3C1		*HSD11B2*	
Characteristic	**Mean ± SD or % (n)**	***r* or *t***	**p-value**	***r* or *t***	**p-value**
Maternal age, years	29.37 ± 5.65	−0.02	0.69	0.10	0.05
Maternal race, not white	27.15 (101)	0.26	0.79	0.76	0.45
Maternal race, white	72.85 (271)				
Maternal education, high school or less	27.15 (101)	1.14	0.24	1.92	0.06
Maternal education, more than high school	72.85 (271)				
Tobacco use during pregnancy, yes	4.84 (18)	−0.13	0.90	1.48	0.16
Tobacco use during pregnancy, no	95.16 (354)				
Depressed during pregnancy, yes	14.52 (54)	0.73	0.46	0.61	0.54
Depressed during pregnancy, no	85.5 (318)				
Infant sex, male	50.27 (187)	1.13	0.26	0.08	0.42
Infant sex, female	49.73 (185)				
Birth weight, percentile	56.49 ± 35.35	−0.07	0.18	0.10	0.07

*Bivariate associations were assessed via Pearson's correlations (*r*) and independent t-tests (*t*) for continuous and dichotomous covariates respectively.

Table 3

Descriptive information for *NR3C1* and *HSD11B2* DNA methylation variables

Individual gene promoters	Mean % methylated ± SD	Chromosomal Location
NR3C1		
CpG1	1.42 ± 2.42	
CpG2	1.13 ± 1.99	
CpG3	1.51 ± 2.29	
CpG4	1.07 ± 1.72	Chr 5: 142783592
CpG5	1.40 ± 2.26	Chr 5: 142783599
CpG6	1.62 ± 2.10	Chr 5: 142783602
CpG7	4.50 ± 2.37	Chr 5: 142783608
CpG8	2.28 ± 2.57	Chr 5: 142783611
CpG9	3.59 ± 3.45	Chr 5: 142783501
CpG10	3.76 ± 2.81	Chr 5: 142783503
CpG11	3.36 ± 3.66	Chr 5: 142783513
CpG12	4.11 ± 3.97	Chr 5: 142783519
CpG13	3.54 ± 3.81	Chr 5: 142783533
Overall	2.09 ± 1.91	Chr 5: 142783555
HSD11B2		Chr 5: 142783570
CpG1	8.94 ± 2.91	Chr 5: 142783573
CpG2	19.35 ± 3.81	
CpG3	9.39 ± 2.73	Chr 16: 67464389
CpG4	16.29 ± 3.77	Chr 16: 67464395
Overall	13.18 ± 1.26	Chr 16: 67464399
		Chr 16: 67464412
Combined DNA methylation variable	% (n)	
High *NR3C1* and high *HSD11B2*	3.76 (14)	
High *NR3C1* and low *HSD11B2*	21.51 (80)	
Low *NR3C1* and high *HSD11B2*	21.77 (81)	
Low *NR3C1* and low *HSD11B2*	52.96 (197)	

Table 4

Means (SD) and correlations among NNNS summary scores

	Mean (SD)	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Attention	4.01 (1.26)	1.0												
2. Movement quality	4.07 (0.66)	0.13*	1.0											
3. Self-regulation	4.76 (0.92)	0.31***	0.52***	1.0										
4. Habitation	7.29 (1.29)	0.06	0.06	−0.03	1.0									
5. Stress	0.18 (0.07)	−0.17**	−0.37***	−0.50***	−0.12	1.0								
6. Arousal	4.14 (0.79)	−0.17**	−0.31***	−0.67***	0.03	0.37***	1.0							
7. Handling	0.35 (0.24)	−0.35***	−0.23***	−0.38***	−0.02	0.28***	0.47***	1.0						
8. Excitability	4.73 (2.88)	−0.26***	−0.56***	−0.82***	0.05	0.46***	0.83***	0.45***	1.0					
9. Lethargy	6.56 (2.50)	−0.71***	0.002	0.05	−0.02	−0.03	−0.29***	−0.05	−0.15***	1.0				
10. Hypertonia	0.44 (0.82)	−0.08	−0.16**	−0.16**	−0.001	0.15**	0.16**	0.13*	0.20***	−0.01	1.0			
11. Hypotonia	0.61 (0.84)	−0.02	−0.06	−0.09	0.001	0.02	−0.16**	−0.09	−0.08	0.25***	−0.03	1.0		
12. Non-opt. reflexes	6.06 (2.11)	0.09	−0.03	0.06	−0.17*	0.17***	−0.27***	−0.11*	−0.16**	0.22***	−0.02	0.21***	1.0	
13. Asym. reflexes	1.81 (1.38)	0.14*	−0.04	0.05	−0.01	0.14**	−0.02	−0.01	−0.05	−0.09	−0.06	0.01	0.14***	1.0

* p<0.05,

** p<0.01,

*** p<0.001

Table 5

Linear regression models for the main effects and interactions between placental *NR3C1* and *HSD11B2* methylation for infant neurobehavior

	Model 1		Model 2	
	β (SE)	p	β (SE)	p
Attention				
NR3C1	−0.33 (0.27)	0.22	−1.73 (2.99)	0.52
HSD11B2	−0.33 (0.74)	0.66	−0.70 (1.10)	0.52
*NR3C1*HSD11B2*			1.27 (2.71)	0.64
Quality of movement				
NR3C1	0.25 (0.13)	0.05	1.86 (1.45)	0.20
HSD11B2	0.43 (0.37)	0.25	0.88 (0.55)	0.11
*NR3C1*HSD11B2*			−1.46 (1.32)	0.27
Self-regulation				
NR3C1	0.30 (0.18)	0.10	1.53 (2.03)	0.45
HSD11B2	0.75 (0.52)	0.15	1.10 (0.77)	0.16
*NR3C1*HSD11B2*			−1.12 (1.85)	0.54
Habituation				
NR3C1	−0.03 (0.34)	0.92	−10.56 (4.35)	0.02
HSD11B2	1.15 (0.99)	0.25	−1.54 (1.48)	0.30
*NR3C1*HSD11B2*			9.64 (3.97)	0.02
Stress				
NR3C1	0.04 (0.01)	0.002	−0.005 (0.16)	0.98
HSD11B2	−0.05 (0.04)	0.18	−0.07 (0.06)	0.27
*NR3C1*HSD11B2*			0.04 (0.14)	0.77
Arousal				
NR3C1	−0.18 (0.15)	0.25	−2.90 (1.71)	0.09
HSD11B2	−0.37 (0.43)	0.39	−1.14 (0.64)	0.08
*NR3C1*HSD11B2*			2.48 (1.55)	0.11
Handling				
NR3C1	0.03 (0.05)	0.50	−0.01 (0.53)	0.99
HSD11B2	−0.13 (0.14)	0.32	−0.14 (0.20)	0.47
*NR3C1*HSD11B2*			0.06 (0.49)	0.94
Excitability				
NR3C1	−0.96 (0.56)	0.09	−12.48 (6.29)	0.04
HSD11B2	−2.16 (1.59)	0.18	−5.39 (2.37)	0.02
*NR3C1*HSD11B2*			10.50 (5.71)	0.06
Lethargy				
NR3C1	−0.16 (0.49)	0.74	6.30 (5.52)	0.24
HSD11B2	1.01 (1.39)	0.47	2.83 (2.08)	0.17
*NR3C1*HSD11B2*			−5.89 (5.01)	0.25
Hypotonia				

	Model 1		Model 2	
	β (SE)	**p**	**β (SE)**	**p**
NR3C1	0.20 (0.16)	0.22	2.56 (1.78)	0.15
HSD11B2	0.33 (0.45)	0.46	1.00 (0.67)	0.14
*NR3C1*HSD11B2*			−2.55 (1.62)	0.18
Hypertonia				
NR3C1	−0.14 (0.16)	0.37	1.30 (1.80)	0.42
HSD11B2	−0.04 (0.45)	0.93	0.37 (0.68)	0.59
*NR3C1*HSD11B2*			−1.32 (1.63)	0.42
Non-optimal reflexes				
NR3C1	0.78 (0.41)	0.06	2.73 (4.64)	0.56
HSD11B2	0.85 (1.17)	0.46	1.40 (1.75)	0.42
*NR3C1*HSD11B2*			−1.80 (4.21)	0.67
Asymmetrical reflexes				
NR3C1	0.67 (0.27)	0.01	6.39 (3.02)	0.03
HSD11B2	−1.14 (0.76)	0.14	0.46 (1.14)	0.68
*NR3C1*HSD11B2*			−5.21 (2.74)	0.05

β coefficients represent change in neurobehavior per one unit change in DNA methylation (log_{10} scale); SE (standard error); p (p-value). All models adjusted for maternal age, race, education, tobacco use, depression, infant sex and birth weight percentile. P-values are uncorrected for multiple comparisons.

Table 6

Linear regression models examining the patterning of placental *NR3C1* and *HSD11B2* methylation interactions for infant neurobehavior

Combined methylation variable	Habituation	Excitability	Asymmetrical reflexes
High *NR3C1* and high *HSD11B2*	0.68 (0.59)	−0.33 (0.79)	0.20 (0.38)
High *NR3C1* and low *HSD11B2*	−0.23 (0.22)	−0.61 (0.38)	0.42 (0.18)*
Low *NR3C1* and high *HSD11B2*	−0.10 (0.22)	−0.90 (0.38)*	−0.18 (18)
Low *NR3C1* and low *HSD11B2*	*reference*	*reference*	*Reference*

Cell entries are β (SE). Models control for maternal age, race, education, tobacco use, depression, infant sex and birth weight percentile.

* $p<0.05$, uncorrected for multiple comparisons.