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Prenatal Cortisol Exposure Predicts Infant Cortisol Response to Acute Stress

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Summary

Experimental animal findings suggest that early stress and glucocorticoid exposure may program the function of the Hypothalamic-pituitary-adrenal (HPA) axis in the offspring. The extension of these findings to human development is not yet clear. A prospective longitudinal study was conducted on 125 mothers and their normally developing children. Amniotic fluid was obtained at, on average, 17.2 weeks gestation; infant behavior and cortisol response to a separation-reunion stress was assessed at 17 months. Amniotic fluid cortisol predicted infant cortisol response to separation-reunion stress: infants who were exposed to higher levels of cortisol *in utero* showed higher pre-stress cortisol values and blunted response to stress exposure. The association was independent of prenatal, obstetric, and socioeconomic factors and child-parent attachment. The findings provide some of the strongest data in humans that HPA axis functioning in the child may be predicted from prenatal cortisol exposure.

Keywords

Prenatal stress; amniotic fluid; cortisol; infant; fetal programming

Experimental animal evidence is now clear in linking prenatal stress with long-term behavioral, immunological, and neurophysiological alterations in the offspring (Coe, Kramer, Kirschbaum, Netter, & Fuchs, 2002; Maccari et al., 2003; Weinstock, 2008). A model emerging from the experimental animal studies is that prenatal maternal stress is associated with increased prenatal exposure to glucocorticoids (cortisol in primates) that alters the set point or programs the HPA axis function in the offspring, and that this may help shape behavioral, neuroendocrine, neurophysiological, and immunological outcomes. The application of these findings, or the underlying model, for human health is not yet clear, but has substantial potential clinical and public health significance. The aim of the current study was to translate and test a specific hypothesis from experimental animal studies to humans, namely, that prenatal exposure to cortisol would alter infant HPA axis function.

Maternal prenatal stress is emerging as a major component of human studies of abnormal and normal development. That is seen, for example, in the increasing interest in applying a developmental or fetal programming model to human health (Glover, O'Connor, &

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O'Donnell, 2010; P. Gluckman & Hanson, 2004; Huizink, Mulder, & Buitelaar, 2004; Talge, Neal, & Glover, 2007). According to this model, which has a strong evolutionary basis and a focus on reproductive fitness, the developing fetus adapts to early environmental (*in utero*) signals and responds accordingly to prepare for the subsequent environmental demands (Glover, 2011; P. D. Gluckman, Hanson, Beedle, & Spencer, 2008). Evidence supporting the model derives from many studies; a majority focus on endocrine, metabolic, and cardiovascular functioning. For example, studies link low birthweight to alteration in glucose metabolism in adulthood (Barker, 1999). In this case, the fetus/child's glucose metabolism is set by the poor early nutritional environment (e.g., the "thrifty phenotype"), with potentially adverse implications if the subsequent nutritional environment is not poor but nutritionally rich.

Experimental animal studies document that the HPA axis may be similarly programmed by early stress exposure. In some models animals exposed to prenatal maternal stress show, as adults, an amplified stress response to acute stress, although there is variation across (sub)species, according to the timing of exposure, and sex of the animal (Barbazanges, Piazza, Le Moal, & Maccari, 1996; Weinstock, 2008; Zuena et al., 2008). The implication is that early stress exposure, via the mediating causal factor of cortisol/corticosterone exposure, altersstress reactivity. Whether or not a similar pattern exists in humans is attracting considerable theoretical and clinical attention (Egliston, McMahon, & Austin, 2007).

The prenatal stress paradigm is now an active area for human research and may play an important role in the construction of other models of health and development in humans. That is because the programming of the HPA axis – a major focus of the prenatal stress paradigm – is implicated in a range of models linking stress and health. For example, the allostatic load model (Dowd, Simanek, & Aiello, 2009; Juster, McEwen, & Lupien, 2010; McEwen, 2000; Seeman, Epel, Gruenewald, Karlamangla, & McEwen, 2010) emphasizes HPA axis function and cortisol levels in particular as a bellwether of the organism's ability to respond to stress. Along with other biomarkers, cortisol may index the "wear and tear" on the organism from stress exposure.

Several studies suggest that prenatal maternal self-reports of stress are associated with child HPA axis function. This was shown in a subsample of the ALSPAC cohort in the UK, which linked prenatal anxiety to elevated waking cortisol in pre-adolescent children (O'Connor et al., 2005); in a subset of the Generation R study that linked parental stress to elevated cortisol in infants (Saridjan et al., 2010); an Australian study (Grant et al., 2009) that showed that prenatal anxiety was associated with elevated cortisol to the still-face procedure in infancy; a Dutch reporting linking prenatal anxiety to increased cortisol reactivity to stress throughout the first year (Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011); and a follow-up study that showed elevated cortisol levels in adolescents whose mothers experienced the stress of the Chernobyl nuclear disaster (Huizink et al., 2008). These studies do not identify what it is about prenatal anxiety or stress that may alter child HPA axis activity; in particular, the putative causal role of prenatal exposure to cortisol was not assessed.

Research suggesting a link between prenatal exposure to cortisol and infant HPA axis function has attracted much less attention. One study suggested that maternal salivary cortisol measured in the morning early in pregnancy was positively associated with elevated levels of child cortisol following inoculation (Gutteling, de Weerth, & Buitelaar, 2004). Davis (Davis, Glynn, Waffarn, & Sandman, 2010) found that maternal plasma cortisol measured in the afternoon was associated with elevated cortisol response to heel stick in newborns. The above studies differ in the manner of assessing child HPA axis function and

the source and timing of maternal measures, but generally imply that prenatal maternal stress may be associated with elevated cortisol output in the child. Significantly, no study has yet demonstrated that the prenatal stress association with child outcome is mediated by maternal cortisol (Bergman, Sarkar, Glover, & O'Connor, 2010; Davis et al., 2007; Gutteling, de Weerth, & Buitelaar, 2005).

This prospective longitudinal study extends this area of research in several ways. First, we include a measure of amniotic fluid cortisol. Amniotic fluid provides a more direct index of fetal exposure to cortisol *in utero* than does maternal saliva or plasma, and so offers greater leverage for testing the hypothesized effect of prenatal exposure. Second, we include a gold standard measure of parent-child relationship quality, attachment derived from the Strange Situation, as a potential modifier of the link between prenatal cortisol exposure and infant HPA axis function. Infant response to the Strange Situation also provided the context for assessing the infant's HPA response to a naturalistic stressor. Failing to consider quality of caregiving on prenatal stress or anxiety is a major limitation of some prior human studies because animal data demonstrate that postnatal rearing may eliminate the effects of prenatal stress (Maccari et al., 1995). The extent to which postnatal rearing moderates prenatal stress in humans is not yet clear, although positive instances have been reported (Bergman, et al., 2010). Third, in addition to assessing HPA axis past the newborn or neonatal stage, we include multiple possible factors that confound the link between prenatal exposure and infant HPA function, including socio-demographic risk and maternal postnatal stress and symptoms.

Methods

Participants

Mothers and babies were recruited as part of a prospective study on fetal hormone exposure and child development. Women were recruited sequentially from an amniocentesis clinic for karyotyping in a large urban maternity hospital. Written informed consent was obtained from mothers; the study was approved by the institutional research ethics committee at Imperial College London.

Of the 365 women who were recruited at amniocentesis, 109 were excluded because of clinical findings, prematurity, non-routine amniocentesis, or unknown birth outcome. The remaining 256 English-speaking mothers with full-term (≥37 weeks), healthy and singleton infants for whom prenatal biological samples were obtained were invited to return to the pediatric clinic when the child was between 14 and 19 months old. Of these, we were unable to locate 71 and a further 60 did not wish to participate or could not attend the clinic (e.g., because of moving away), resulting in a sample of n=125; of these, we obtained cortisol from 116 children pre-stress and 114 children post-stress. Failure to obtain a sample in these cases was due to insufficient sample or an uncooperative child. Mothers from whom we obtained child follow-up cortisol data did not differ from those whom we were not able to follow-up on key prenatal measures (e.g., amniotic fluid cortisol, prenatal stress and anxiety), but were about 1 year older on average (36.6 [SD=4.3] years compared with 35.3 [SD=5.2] years, respectively, $p < .05$).

Procedures

Mothers were explained research procedures upon arrival in the clinic lab. Following an explanation of the procedures and obtaining signed consent, a saliva sample was obtained from the child using a salivette. The Strange Situation (see below) was then carried out. Twenty minutes following the completion of the Strange Situation a second saliva sample

was obtained from the child. Samples were stored in pre-labelled containers and spun prior to assay.

Because of limitations of clinic availability, visits were conducted in the morning (usually starting at 9am) or in the afternoon (usually starting around 1pm). Given the known diurnal pattern of cortisol release, we investigated the role of visit timing in the analyses below. The variation in visit time also allowed us to test the robustness of the amniotic fluid exposure effect according to initial level (morning versus afternoon); this was a serendipitous rather than formally planned feature of the design.

Measures

Amniotic fluid cortisol—During amniocentesis an aliquot of up to 4ml of amniotic fluid surplus to clinical requirement was drawn for the study and stored at −80°C until assay. Time of collection, to the nearest 15 minutes, was recorded. Total cortisol in amniotic fluid was assayed by radio-immunoassay (Coat-A-Count, DPC, Los Angeles,CA), cortisol having been extracted by dichloromethane and reconstituted prior to assay. Intra- and inter-assay coefficients of variation for the amniotic fluid cortisol assay were 4.4% and 6.5% respectively. There was some variation in the time of day of assessment and gestational age of amniotic fluid collection; these factors are considered as covariates. In addition, maternal plasma was sampled at the same prenatal assessment; it is included for exploratory purposes. Maternal blood samples were collected immediately before the amniocentesis procedure, centrifuged, and supernatant plasma was stored at −80°C until batch assay. Total cortisol was assayed by radio-immunoassay using Coat-a-Count (DPC,Los Angeles,CA). Intra- and inter-assay coefficients of variation for the cortisol assay were 5.4% and 4.1%, respectively.

Infant cortisol in response to stress—Saliva samples obtained from the child pre- and 20 minutes following the termination of the separation-reunion procedure were stored at −20C until assay. Samples were thawed and analyzed using commercially available ELISA assay kits (Salimetrics,UK). Intra- and inter-assay variation were 7.9% and 8.9%, respectively.

Maternal stress, anxiety, and depressive symptoms—Maternal psychological stress and anxiety in the prenatal period were included as adjuncts to the prenatal cortisol exposure data. The Spielberger State-Trait Anxiety Inventory (STAI) (Spielberger, Gorusch, & Lushene, 1983)) was completed at both prenatal and postnatal visits. The STAI is a widely used index of anxiety symptoms and has considerable validity, reliability, and clinical utility; we report state anxiety because of our interest in change from the pre- to post-natal periods. Mothers also completed a 26-item Stressful Life Events Questionnaire (SLEQ), adapted from Barnett et al.(Barnett, Hanna, & Parker, 1983), at the postnatal visit, and reported if the event occurred and, if it did, whether the event "affected me a little" or "affected me a lot." Mothers reported if the event occurred antenatally or postnatally or both. In addition, mothers completed the Edinburgh Postnatal Depression Scale (EPDS) (Cox, Holden, & Sagovsky, 1987)), a widely-used index of maternal depression with considerable validity; the EPDS was assessed at the postnatal visit only.

Infant-mother attachment—Ainsworth's Strange Situation (Ainsworth, Blehar, Waters, & Wall, 1978) is an extensively researched 7-episode laboratory assessment that capitalizes on the mild stress of the separation-reunion paradigm to assess the extent to which the child uses the parent as a secure base for exploration. Coding was made using established procedures for classifying dyads as Insecure-Avoidant, Secure, Insecure-Ambivalent/ Resistant, and Insecure-disorganized. The Strange Situation is arguably the most extensively studied and valid index of parent-child relationship quality in infancy. Securely attached

children experience significantly more sensitive and response parenting than those rated as having an Insecure pattern (Beebe et al., 2010; De Wolff & van Ijzendoorn, 1997), and Secure attachment predicts optimal behavioral and social development (Sroufe, 2005). Ratings from the Strange Situation index current relationship quality, but the relative stability in attachment (in)Security in infancy implies that it indexes the predominant pattern of caregiving quality in the child's early months of life. The primary coder received standard training at the Institute of Child Development, University of Minnesota, and achieved > 80% agreement on a reliability test of 35 cases.

Covariates—Information on maternal age, parity, ethnicity (categorized according to UK National Health Service ethnic codes), smoking (cigarettes per day; because of a restricted range, this was re-coded to no smoking versus any smoking), alcohol use in the week prior to the prenatal assessment (number of units per week, ranging from 0 = none to 3 = > 14 units/week), and prescription drug use during pregnancy was collected at recruitment. Also recorded was the gestational age at amniocentesis visit and the time of day the samples were drawn. Information regarding birth outcomes and child sex was collected from the child's hospital notes. Standard deviation score of birth weight adjusted for gestational age and sex was calculated using software based upon 1990 British Growth Reference data. Gestational age at birth was assessed to the nearest day by ultrasound-determined fetal biometry. Crown-rump-length(CRL) was used at and before 13 weeks and biparietal diameter(BPD) after 13 weeks to establish gestation using Hadlock's charts installed in the reporting software.

Data analysis

We first report sample characteristics and background correlations between key study variables. We then test the hypothesis that infant HPA axis activity is predicted from amniotic fluid cortisol exposure using a repeated measures multivariate analysis of variance, with pre- vs. post-stress as a within-subjects factor and amniotic fluid cortisol as a between-subjects factor; we also examine this association using the area under the curve. We include several covariates on an *a priori* basis because of their known or suspected influence on amniotic fluid or infant cortisol (Sarkar, Bergman, Fisk, O'Connor, & Glover, 2007a), specifically, gestational age of amniocentesis, time of amniocentesis sampling, maternal age and education, smoking and alcohol use in pregnancy, infant birthweight adjusted for gestational age, time of infant cortisol assessment, and child sex. A parallel analytic approach was used to test the hypothesis that the link between amniotic fluid cortisol and infant cortisol response to stress was moderated by the quality of the parent-child relationship. Amniotic fluid and infant cortisol levels were log transformed to address non-normative distributions (although findings are substantively identical with non-transformed values). Our primary focus is on amniotic fluid cortisol because that is the most direct index of fetal exposure, but we also consider maternal plasma and maternal-reported prenatal stress as predictors in supplementary analyses. Supplementary analyses are also carried out to test the robustness of the amniotic fluid prediction of infant cortisol. In addition, we examined other potential between-subjects factors as potential predictors and moderators of amniotic fluid cortisol, notably, child sex, and pre- and postnatal maternal mood.

Results

Descriptive and preliminary analyses

Demographic data (Table 1) show that the sample was diverse on multiple psychosocial and demographic indicators. As other studies have found, we obtained a small number of extremely high (> 3 SD above the mean) infant cortisol values (6 of 116 pre-stress values and 4 of 114 post-stress values), which were replicated in every case in lab analysis. In some

cases the explanation of these high values was discerned (use of cortisone cream). Because of concerns about the interpretability of extremely high scores (e.g., not detecting a medical or medication account for very high scores), we excluded these high values pre- and post-stress assessments (the key findings reported below are essentially identical with and without the excluded cases). This resulted in a sample of 110 infants on whom we had both pre- and post-stress cortisol data; however, Strange Situation classifications were not available for two infants because of procedural problems, leaving n=108 with both Strange Situation data and pre- and post-Strange Situation cortisol. The range of gestational age at amniocentesis was 15-37 weeks, but 90% of procedures were conducted within a relatively narrow window of 15-20 weeks; we include timing of amniocentesis as a covariate (in analyses below) and also note that the findings are substantially the same when we eliminated the small group of women from whom we obtained amniotic fluid outside of the relatively narrow period of 15-20 weeks gestation.

The distribution of attachment patterns among the 108 infants on whom we had pre- and post-Strange Situation cortisol data was typical for a normative sample: 59% Secure (n=64), 14% Insecure-Avoidant (n=15), 12% Insecure-Ambivalent (n=13), and 15% Insecure-Disorganized (n=16). We found no sample-wide increase in cortisol in response to the Strange Situation for the n=108 children for whom we had valid pre- and post-Strange Situation cortisol data ($t(108) = .05$, ns; ln means [SD] were, respectively, 1.23 [.74] and 1.23 [.73]). Stability of individual differences in cortisol levels from the pre- to the post-Strange Situation assessment was moderate-high $r(108) = .55$, $p < .001$).

Because of the impracticality of requiring all families to attend the hospital clinic lab at the same time of day, we arranged for morning and afternoon slots. That meant that we needed to account for variation in time of cortisol sampling in the analyses because of the diurnal pattern in cortisol. The typical time of initial cortisol assessment in the lab visit was late morning (the average was approximately 11.30am); less than 10% of the infant assessments were conducted after 2pm. Correlation analyses indicated that variation in time of initial assessment was not associated with pre-stress cortisol ($r(108) = -.09$); however, a more detailed repeated measures multivariate analysis of variance indicated that there was a significant effect of time of day on infant cortisol levels ($F(1,106) = 4.95$, $p < .05$) but that this differed significantly for pre- and post-Strange Situation assessments (pre-/post-Strange Situation X time of day interaction ($F(1,106) = 4.90$, $p < .05$). Follow-up analyses indicated that, for children assessed prior to noon, there was a non-significant increase in cortisol ($t(72) = -1.17$, ns; ln values: 1.28 [.78] to 1.38 [.69]), but that children assessed in the afternoon showed a significant decrease in cortisol levels from pre- to post-Strange Situation ($t(36) = 2.12$, $p < .05$; ln values: 1.14 [.63] to .93 [.75]). Accordingly, time of initial assessment was included as a covariate (continuous and categorical approaches for assessing time of day yielded parallel results). In addition, we re-analyzed the data separately for the infants assessed in the morning and obtained substantively identical results; see below (too few infants were assessed in the afternoon to assess them separately).

Amniotic fluid cortisol is the most direct, readily accessible, index of fetal exposure to the putative mediating mechanism, cortisol. We did, nevertheless, obtain measures of other indicators of prenatal stress used in the literature, namely, maternal report of state anxiety and stressful life events as well as maternal plasma cortisol. Amniotic fluid cortisol was significantly associated with concurrently measured prenatal maternal plasma cortisol ($r(92) = .24$, $p < .05$), but was not with maternal prenatal anxiety or stressful life events (all r's $< .10$).

Amniotic fluid cortisol predicts infant cortisol response to an acute stress

A repeated measures multivariate analysis of variance was conducted with pre-/post-Strange Situation cortisol as the dependent variable; amniotic fluid cortisol was the between-subjects predictor; between-subjects covariates were gestational age and time of amniotic fluid collection, maternal age and education, smoking and alcohol use in pregnancy, birthweight adjusted for gestational age, child sex and time of infant cortisol collection (Table 2). We found a significant effect of pre/post separation-reunion X amniotic fluid cortisol ($F(1,91)=5.70$, $p<.05$), indicating that the association between amniotic fluid cortisol and infant cortisol differed for the pre- and post-stress assessment. For follow-up analyses, displayed in Figure 1, we examined the pre-/post-Strange Situation X amniotic fluid cortisol interaction using a median split for amniotic fluid cortisol for illustrative purposes (standardized pre- and post-stress cortisol values are shown after residualizing the covariates for each assessment; raw cortisol values were, for the infants in the high amniotic fluid cortisol group, 5.45 (5.33) and 4.68 (4.43), for pre- and post-stress, respectively; for low amniotic fluid cortisol, infant cortisol value pre- and post-stress were 4.13 (5.61) and 4.38 (3.95), respectively). The Figure shows that children exposed to low levels of amniotic fluid cortisol showed an increase over time in cortisol in response to the Strange Situation stress; however, children exposed to elevated levels of amniotic fluid cortisol exhibited a higher pre-stress cortisol level and a slight decrease over time. One other significant effect was detected: a pre/post X child sex interaction ($F(1,91)=4.95$, $p<.05$) indicated that girls showed a higher pre-Strange Situation cortisol level than boys (1.38 [.76] compared with 1.07 [.73], respectively), but comparable levels at post-stress (1.23 [.70] and 1.19 [.79], respectively). There was, in addition, a marginal pre/post X time of day effect ($F(1,91)=3.75$, $p=.056$) suggested, as shown above, that children assessed in the morning showed an increase in cortisol whereas those assessed in the afternoon showed a decrease in cortisol from pre- to post-Strange Situation. No other within- or between-subjects effects were detected.

We then repeated the analysis, including multiple measures of maternal postnatal mood: state anxiety from the STAI, postnatal life events, and the EPDS. Results (not shown) indicated that none of the postnatal mood and stress measures were significantly associated with infant cortisol; neither did they substantively change the magnitude of the amniotic fluid cortisol prediction in the regression model above, which remained significant at $p < .05$.

Amniotic fluid cortisol, infant cortisol, and infant-parent attachment

A repeated measures analysis of variance was conducted with cortisol levels pre- and post-Strange Situation as the within-subjects factor and time of assessment and attachment security as between-subjects factors; other covariates were included as above. We then tested the hypothesis that the association between amniotic fluid cortisol exposure and infant cortisol was moderated by attachment classification. There was neither a main effect nor a significant interaction between amniotic fluid cortisol and infant pre- and post-Strange Situation cortisol according to attachment classification (whether attachment was classified as Secure/Insecure, Disorganized/Organized, or 4-way classification; the pre/post X attachment classification was $F(3,104)=1.76$, $p=.16$; the pre/post X attachment classification was also non-significant using the Secure/Insecure and Organized/Disorganized categorizations).

Supplementary analyses

We conducted a series of supplementary analyses to examine the robustness of the amniotic fluid finding and to examine exploratory hypotheses. The first set of supplementary analyses considered further any possible bias introduced with the variation in gestational age at amniocentesis or time of day of infant cortisol collection. As noted above, we re-analyzed the data eliminating the small subset of mothers whose amniotic fluid data were collected

outside of the 15-20 week gestation window and found substantively identical effects to those reported above. In addition, analyses were re-run eliminating the small subset of infants whose cortisol data were collected in the afternoon (post 2pm); again, we obtained substantively identical effects to those reported above. In addition, we re-ran the final model and also included maternal prenatal plasma cortisol as a between-subjects variable. Repeated measures analyses indicated that maternal prenatal plasma cortisol was not significantly associated with infant cortisol, nor did it alter the amniotic fluid prediction of infant cortisol (which remained significant at $p<.05$). In short, it was only amniotic fluid cortisol that predicted infant cortisol; no other index of prenatal stress was a significant predictor of infant cortisol, and neither did it alter the moderately strong prediction from amniotic fluid. In addition, we tested a series of interactions with amniotic fluid to examine if its effect on infant cortisol was moderated by child sex, time of infant cortisol sampling, gestational age of the amniotic fluid sampling; none of these interactions was significant at $p<.05$.

Finally, we re-analyzed the data using the area under the curve approach (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003) within a multiple regression format. We constructed a measure of area under the curve with respect to initial value; higher/positive values index greater response to the separation-reunion stress whereas lower/negative values index less response from pre- to post-stress. Using the same covariates as in the repeated measures analysis above (time of day and gestational age of amniocentesis, maternal age and education, smoking and alcohol use in pregnancy, birthweight adjusted for gestational age, child sex and time of infant cortisol), we obtained essentially identical results to those obtained from the repeated measures analyses: amniotic fluid cortisol was associated with significantly less area under the curve (B -2.33 SE .97, $t=-2.39$, $p<.05$; significant effects were also obtained for child sex (B 2.34 SE 1.07, $t = 2.22$, $p<.05$) and time of collection (B -.64 SE .33, $t=-1.94$, $p<.06$). Again, there was no evidence that parent-child attachment moderated the effect of amniotic fluid cortisol on infant cortisol. Neither was there any evidence that the amniotic fluid effect was altered with the inclusion of maternal plasma cortisol or maternal prenatal anxiety or life stress in pregnancy.

Discussion

Decades of animal studies and several years of human research underscore the role of the HPA axis in normal and abnormal development. Accordingly, there is a considerable need for research to identify factors associated with its development. Findings from the current study, based on typically developing children, indicate that prenatal exposure to cortisol is one factor associated with HPA axis function in the child. These results help translate a considerable database from experimental animal studies and expand a small set of human investigations. Specifically, we found that, across two distinct strategies for analyzing infant cortisol, amniotic fluid cortisol, a direct index of prenatal exposure, predicted infant cortisol at approximately 17 months of age: elevated prenatal exposure predicted higher initial levels and a dampened response to the acute separation-reunion stress. This effect held after controlling for measures of obstetric risk, socioeconomic risk, and postnatal maternal mood disturbance. None of the other indicators of prenatal stress or anxiety, including maternal plasma cortisol or self-report ratings, was significantly associated with infant cortisol. Finally, we found no evidence that child-parent attachment predicted infant cortisol response or moderated the link between prenatal exposure and infant cortisol reactivity.

A link between prenatal exposure to cortisol and infant HPA axis function was strongly predicted from the animal data (Maccari & Morley-Fletcher, 2007). However, translating the animal findings to human development is beset with a variety of difficulties, including the differential distribution of glucocorticoid receptors in the brain (Sanchez, Ladd, & Plotsky, 2001) and the fact that rats, which are the target of much of the research on prenatal stress,

are born much at an earlier stage in ontogeny than are humans, confounding conclusions about prenatal versus postnatal effects. Furthermore, the tendency in experimental animal work to assess specific, precisely timed stressors in pregnancy has limited application to humans where stresses are multiplicative and typically chronic.

The use of amniotic fluid cortisol offers particular leverage for drawing a (more) direct link between prenatal exposure and infant HPA axis than maternal questionnaire or salivary data which have been used in prior research. Additionally, the current study adds to the field by demonstrating that prenatal exposure to cortisol was associated with altered baseline and HPA response to an acute stress in infancy, even after accounting for obstetric risk, indices of socio-economic status, postnatal maternal mood and stress, and caregiving quality. These findingsextend animal and prior human work in providing further evidence that the child's HPA axis may be programmed by prenatal exposures.

A human model for understanding the effect of prenatal anxiety or stress on the child is being constructed from animal data. Several components of this model have received support. For example, there is some suggestion that anxious pregnant women have elevated cortisol levels, especially in the evening (Evans, Myers, & Monk, 2008; Kivlighan, DiPietro, Costigan, & Laudenslager, 2008; Obel et al., 2005). Second, maternal prenatal cortisol is directly associated with fetal exposure (Sarkar, Bergman, Fisk, O'Connor, & Glover, 2007b), notwithstanding the role of the barrier enzyme 11 beta-hydroxysteroid dehydrogenase 2 [11 β-HSD-2] in metabolizing maternal cortisol. More recent data suggest that prenatal anxiety may also down-regulate this enzyme in the placenta, which would also lead – indirectly – to greater fetal exposure to maternal prenatal cortisol in prenatally anxious women (Glover, Bergman, Sarkar, & O'Connor, 2009; O'Donnell et al., 2011). Findings from the current study and somewhat similar studies offer evidence for a third component of the model: prenatal exposure to cortisol alters the developing HPA axis of the child.

The fetal programming hypothesis predicts that the developing fetus adapts to *in utero* exposures in an adaptive manner, in preparation for subsequent environmental demands. Documenting a *bona fide* "programming" effect in humans, especially with respect to the HPA axis, presents several challenges. One is that few studies have assessed prenatal maternal stress and offspring HPA axis function past infancy (O'Connor, et al., 2005; Van den Bergh, Van Calster, Smits, Van Huffel, & Lagae, 2008); it may be premature to label an effect observed in infancy as "programming" given the ongoing nature of HPA axis development and considerable changes observed (Gunnar, Brodersen, Krueger, & Rigatuso, 1996). Human studies, which are mostly limited to infants and young children and based on observational designs, are not able to differentiate key methodological and conceptual questions concerning the programming of the HPA axis, such as the persistence of effects and the role of prenatal timing. Second, there is the question of what pattern of cortisol response would be most typical of a programmed response, that is, how a programming effect would be operationalized. We found that amniotic fluid cortisol was associated with elevated initial level of cortisol rather than increased reactivity following a stressor; the blunted response to the separation-reunion stress is presumably a reflection of an initially high value. That finding differs from Davis et al(Davis, et al., 2010) who did not find baseline differences but rather only post-stress differences. These discrepancies may be to do with the age period of study (neonates in the Davis et al. report), the salience of the stressor, or any of several other factors that might predict the cortisol response. In fact, as indicated in the literature review above, findings are inconsistent with respect to whether or not prenatal anxiety or prenatal cortisol exposure predicts infant baseline or reaction to stress measures. Of course, there is at least a moderate negative link between baseline or initial value and response to stress: individuals showing higher initial values show less increase in

cortisol in response to stress. Therefore, it may be artificial to draw too clear a distinction between these two measures of cortisol. Interestingly, only one study found evidence for a down-regulation of the HPA axis in children whose mothers reported stress (Yehuda et al., 2005); that was in the special case of women who were pregnant during the 9/11 attacks in New York city. It may be that prenatal maternal trauma and PTSD have a different impact on the fetus' HPA axis than the worry, fear, and stress that composed anxiety in other studies.

A third consideration is whether or not the fetal adaptation to increased levels of amniotic fluid cortisol is "adaptive" in the evolutionary sense implied in the fetal programming or "predictive adaptive response" model. Too little is known about the contexts of human development in which an elevated baseline level or accentuated stress response would be adaptive. Work of that kind is needed to substantiate that the programming effect is adaptive rather than, for example, more simply an index of risk for compromised behavioral, endocrinological, or immune development.

Two non-significant findings are worth mentioning. One is that infant cortisol was not predicted from maternal self-reports in pregnancy or from maternal plasma cortisol obtained at the amniotic fluid visit. Failure to find a link between maternal plasma or self-reports contrasts with some prior studies (Davis, et al., 2010; Tollenaar, et al., 2011). These discrepancies may be accounted for by variation in methods, particularly concerning maternal cortisol assessment. These inconsistencies underscore the incomplete picture of the mechanisms by which stress and anxiety in pregnancy may influence child development. Additionally, it was noteworthy that we found no evidence that caregiving quality moderated the link between prenatal exposure to cortisol and infant cortisol response. There are obviously difficulties in interpreting a non-finding, and so it is notable that the measure of caregiving quality was the leading index available, and that the sample size was comparatively large for studies of this kind. Previous analyses in this sample indicated that caregiving quality did moderate the effects of amniotic fluid cortisol on cognitive ability (Bergman, et al., 2010). Accordingly, failure to find a moderating link of child-parent attachment on infant HPA axis function may imply that this effect does not exist or is much weaker.

Limitations

Several limitations of the study deserve mention. One is the single assessment of amniotic fluid in pregnancy. We sought to control for biases associated with the single assessment by controlling for gestational age at collection and time of day; their inclusion in analyses controlled for any biases that might have been introduced. There are obvious trade-offs between the proximity or directness of exposure and intensity of measurement. We opted in this study to obtain a direct index of exposure in amniotic fluid, which meant that we would not be able to conduct repeated assessments. A second feature of the design is the inclusion of a comparatively normative risk sample in terms of both mothers and babies. A minority of mothers in the current sample reported elevated symptoms of mood and stress, and so it may be that the effects observed here are not limited to clinical extremes. Third, sampling women undergoing amniocentesis could introduce bias. We controlled for the most obvious biases by excluding those participants for whom there was a clinical finding and including multiple possible confounding factors as covariates, including maternal age and obstetric risk. Fourth, it is not possible to unambiguously determine the source of amniotic fluid cortisol; it is possible that we were detecting infant-derived cortisol in amniotic fluid that then predicted later infant cortisol basal values and response to stress.

Clinical implications

It may be too early to draw strong clinical conclusions from this and related studies because of the uncertain clinical applications of elevated infant cortisol for current and later functioning. Nonetheless, these findings underscore the need for research that investigates ways of reducing prenatal maternal anxiety, stress and cortisol levels. Randomized clinical trials would offer valuable clinical information and experimental leverage not possible in observational studies to advance our understanding of if and how prenatal anxiety and cortisol may have lasting effects on the heath and development of the child.

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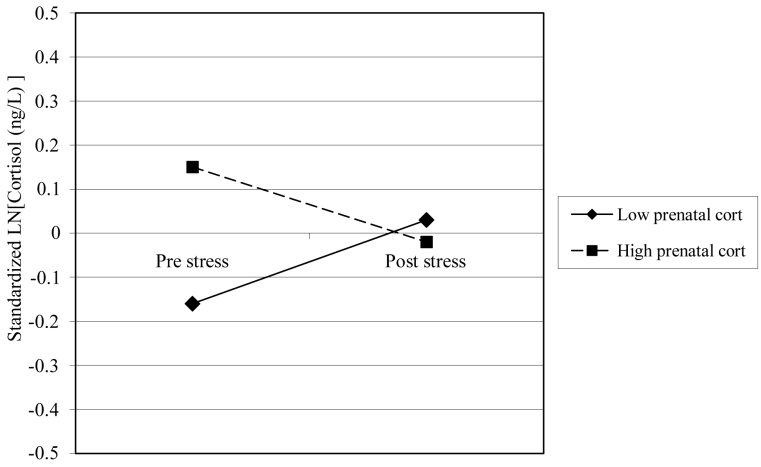

Figure.

Cortisol (ln) in infancy pre- and post-separation reunion stress according to amniotic fluid cortisol.

Infant cortisol response to acute stress is predicted from prenatal exposure

Table 1

Characteristics of the study sample

	Mean (SD)/range OR number (%)
Maternal age (years)	36.68 (4.27)/25-45
Gestational age at birth (weeks)	39.47 (1.18)/37-42
Birth weight (g)	3497.10 (465.64)/2338-6000
Child age at postnatal visit (months)	16.77 (1.39)/14.37-19.80
Female sex	59 (51%)
Ethnicity/Race	
Caucasian	93 (80%)
Asian/Indian subcontinent	7 (6%)
Black	9 (8%)
Other	7 (6%)
Maternal education	
No qualifications	4 (4%)
GCSE Level or equivalent	13 (11%)
A levels or equivalent	18 (16%)
Diploma or equivalent	22 (19%)
University degree or higher	58 (51%)
Married or cohabitating	104 (90%)
Parity	
Nulliparous	45 (39%)
1 previous child	44 (38%)
2 or more previous children	27 (23%)

Note: Data are based on the sample from whom at least some prenatal cortisol data were obtained in infancy. N's range from 109-116. GCSE is the approximate equivalent to high school diploma; A level indicates that the individual passed college entrance exams.

Table 2

Pre- and Post-Stress Cortisol Values are Predicted from Amniotic fluid Cortisol: Repeated Measures MANOVA Results.

	MS	**df**	**F**
Within-Subjects effects		(1,91)	
Time (pre-post stress)	.07		.28
Time X Amniotic fluid	1.37		5.70 *
cortisol	.17		.71
Time X Gestation age at amniotic fluid collection	.03		.12
Time X Amniotic fluid collection time	.10		.43
Time X Maternal age	.04		.17
Time X Maternal education	.15		.62
Time X Prenatal Smoke	.00		.01
Time X Prenatal alcohol	.06		.23
Time X Birthweight/ gestational age	.90		3.75+
Time X Postnatal visit time	1.19		4.95 *
Time X Male child			
	MS	df	F
		(1,91)	
Between-Subjects effects	.96		1.12
Intercept	1.19		1.39
Amniotic fluid cortisol			
Gestational age at amniotic	1.55		1.81
fluid collection	.14		.16
Amniotic fluid collection time	.42		.49
Maternal age	.72		.84
Maternal education	2.10		2.45
Prenatal smoke	.04		.04
Prenatal alcohol	.01		.01
Birthweight/gestational age	2.01		2.34
Postnatal visit time	1.54		1.79
Male child			

Note: Time = the repeated measures (i.e., pre-, post-stress effect);

* p < .05,

** p <.01,

*** p < .001.