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In utero cortisol and testosterone exposure and fear reactivity in infancy

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Abstract

Fetal programming is emerging as a major conceptual model for understanding developmental origins of health and disease, including behavioral outcomes. As part of a larger study of prenatal stress and child development, we examined the association between prenatal hormone exposure and fear reactivity, a temperament dimension that is a predictor of long-term behavioral adjustment. Amniotic fluid was collected from a sample of women undergoing clinically indicated amniocentesis for later analysis of cortisol and testosterone. Children with normal birth outcomes were recalled for follow-up assessment at 17 months, at which time we administered an observational assessment of temperament (lab-TAB; n=108). Information on pregnancy and obstetric outcome was included as covariates. Results indicated that there was a significant association between prenatal testosterone and observed fear reactivity in boys (r(53)=0.34, p=0.01); no significant effect was found in girls (r (54)=-.07, ns); the effect remained when obstetric, psychosocial, and parental anxiety were controlled for. There was not a significant association between fetal cortisol exposure and fear reactivity. The prediction from in utero testosterone exposure to fear reactivity in boys extends prior research on prenatal testosterone, and may represent an association with a general predisposition to greater arousal and reactivity.

Keywords

fear; reactivity; prenatal exposure; amniotic fluid; testosterone

There is considerable evidence, dating back many years, that prenatal steroid hormone exposure can have a lasting impact on health and development. One line of research, based on the organizational hypothesis (Phoenix et al., 1959), focuses particularly on the link between prenatal exposure to testosterone and sexual development and reproductive behavior. A

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somewhat separate line of research, based on a developmental programming model (Gluckman et al., 2008), examines how prenatal glucocorticoid exposure predicts offspring stress physiology and other outcomes. To a considerable extent, these findings rely on experimental animal work and assess a limited set of behaviors. In the current study we extend existing research to human behavioral development, focusing on a key early marker of behavioral adjustment, fear reactivity, in a normative sample of infants.

A dominant hypothesis in research into the effects of prenatal androgen exposure proposed that prenatal androgens “organized” neural mechanisms serving reproductive and mating behavior (Phoenix et al., 1959). That is, the prenatal period constituted a particularly sensitive time in which exposure had organizing and lasting influence on neural mechanisms (even if it is not yet clear what those neural mechanisms are). Substantial evidence now indicates that prenatal testosterone shapes sexual development and reproductive behavior in animal models (Thornton et al., 2009); the applications to human development are not yet certain. There are, for example, several sets of findings linking prenatal testosterone to sex-typical play in children, with some but not others finding the effect limited to girls (Auyeung et al., 2009a; Ehrhardt et al., 1981; Hines et al., 2002). Another set of studies, derived in part from the hypothesis that prenatal testosterone may be associated with autistic-like traits (Auyeung et al., 2009b), shows that prenatal testosterone is associated with impaired social behavior (Knickmeyer et al., 2005) and poorer cognitive and language abilities (Jacklin et al., 1988; Knickmeyer et al., 2006). The extent to which prenatal testosterone may have effects that extend beyond these behavioral outcomes is not known.

A largely separate line of research that also proposes long-term effects of prenatal exposure on health and disease susceptibility is the developmental or fetal programming model. According to this model, the organism adapts to prenatal input and that this “set point” (e.g., in relation to metabolic functioning) is carried forward into adult life (Barker, 1992; 1998; Gluckman, 2008; Gluckman & Hanson, 2005). Few studies consider developmental programming hypotheses for behavioral or psychological phenotypes (O'Donnell et al., 2009; Rutter & O'Connor, 2004). That may be changing, however, as human research begins to translate the experimental animal findings showing that prenatal stress is a central paradigm for demonstrating long-term effects of prenatal exposures on behavioral and biological development (Coe et al., 2003; Maccari et al., 2003; Weinstock, 2005). Abundant animal work using the prenatal stress paradigm points to glucocorticoid exposure as a leading candidate mechanism (Maccari et al., 2003; Matthews, 2000; Roughton et al., 1998; Seckl & Holmes, 2007; Weinstock, 2005). The specific mechanisms of action underlying the programming effect are unconfirmed, but may include increased activation of the offspring HPA axis, alteration of glucocorticoid receptor sensitivity, and alteration of genetic expression in the stress response system. These findings underscore the need for further human work on glucocorticoid exposure on child outcomes. Particularly needed is research to examine whether key behavioral outcomes linked with prenatal stress and anxiety, such fear and emotionality (Bergman et al., 2007; Davis et al., 2007; O'Connor et al., 2002; van den Bergh & Marcoen, 2004; see Talge et al., 2007 for a review), may be explained by glucocorticoid exposure.

In summary, the current study sought to add to, and connect, two lines of research emphasizing prenatal hormone exposure on offspring development: one focusing on prenatal testosterone and a second on glucocorticoids, which we index by cortisol. We include both measures study for two reasons. First, as noted, both testosterone and cortisol have been implicated in studies of human behavioral development, although the emphasis derived from different developmental models. A second reason is that, unlike in the adult, cortisol and testosterone are positively correlated in the fetus (Gitau et al., 2005; Sarkar et al., 2007b). Accordingly, before drawing conclusions about the effect of either on behavioral development, it is necessary to consider a possible joint activation and confound between the two.

We focus on fear reactivity because it is an index of a behavioral phenotype with a long history of developmental research (Rothbart et al., 2000) and because psychobiological work suggests it may be relevant to both cortisol and testosterone (Charney, 2004). By capitalizing on the leverage provided from amniotic fluid, the current study provides the most direct test in humans of a link between prenatal exposure to testosterone and cortisol and fear-related behavior in infancy.

Materials and Methods

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 in a large urban maternity hospital between December, 2001 and January, 2005; women were referred to the clinic for karyotyping. Written informed consent was obtained in accordance with local research ethics committee requirement. All English-speaking mothers with full-term (≥37 weeks), healthy and singleton infants, whose birth outcomes were known, were invited to return to the pediatric clinic in the hospital when the child was between 14 and 19 months old. Initially, 365 women were recruited at amniocentesis, of whom 109 were excluded because of known abnormalities, incomplete data on birth outcome, or because the procedure was for non-routine amniocentesis. Of the 256 remaining mothers, 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 from London), resulting in 125 children who were eligible and agreed to participate. For 17 children it was not possible to complete the temperament assessment, primarily because of fatigue or time constraints in the lab visit, resulting in a sample of 108, for whom there were 108 cases with valid prenatal cortisol and 107 cases with valid prenatal testosterone. (There were two children living in non-native English speaking homes; excluding these children did not alter the findings and so they were included given the non-language nature of the outcome.) Sample sizes in multivariate analyses differ slightly because of missing data on some covariates.

Amniotic fluid sampling and cortisol and testosterone analysis

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. Mean gestational age at the time of sampling was 17.2 weeks (median was 16 weeks; the range was 15-32, with 91% between 15 and 20 weeks). 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 (Sarkar et al., 2007). The intra and inter assay coefficients of variation for the amniotic fluid cortisol assay were 4.4% and 6.5% respectively.

Total testosterone in amniotic fluid was measured by radioimmunoassay Coat-a-Count (DPC, Los Angeles, CA) after prior extraction by diethylether to minimize cross-reactivity. The intra- and inter-assay coefficients of variation of our testosterone assay procedures were 7.5 % and 8.9 % respectively. Some questions have been raised about the reliability of the above method for analyzing amniotic fluid testosterone. Therefore, as a check on the reliability of that method, a random subset of the samples was analyzed by liquid chromatography/mass spectrometer (LCMS). Steroid hormone concentrations determined by an Agilent 1100 LCMS equipped with an electrospray ionization source and Chemstation software version A 10.02 were undertaken in the Assay Services Laboratories of the Wisconsin National Primate Research Center. All steroid hormones used as reference preparations were obtained from Steraloids (Newport, RI). The LCMS methods were validated using Federal Drug Administration protocols (May, 2001) and adapted from those previously described (Abbott et al., in press). Briefly, using positive ion identification for testosterone (m/z 289), the LCMS standard curves

for testosterone used 0-4 ng/ml. Deuterated testosterone was added as internal standard to monitor recovery. The lower limit of quantitation was 0.02 ng/ml. The within-day coefficients of variation (for samples determined on the same day) were 2.7% and the between-day coefficients of was 5.1%. The correlation between LCMS testosterone data and radioimmunoassay testosterone data was $r(40) = .82$, $p < .001$).

Child temperament

The Laboratory Temperament Assessment Battery (Lab-TAB) – Locomotor Version (Goldsmith and Rothbart, 1999) was used to assess infant temperament. The Lab-TAB is a leading observational measure of childhood temperament, with considerable support for its validity, and clinical and predictive value (Rothbart, Derryberry, and Hershey, 2000). It consists of 20 paradigms that are designed to elicit fear, anger/frustration, joy/pleasure, interest/persistence and activity level. We used the unpredictable mechanical toy paradigm from the fear reactivity subscale and the paradigm for joy/pleasure.

During the unpredictable mechanical toy paradigm, the child sat at a table facing a puppet theater and a robotic dog was presented on the table when the child was calm and alert. Each trial lasted about 20 seconds and would consist of the dog barking walking towards the child as its eyes, mouth and head moved. Three trials were presented to each infant unless the child was too distressed by an earlier trial to complete a subsequent trial. We report findings from the first trial (findings for the composite across trials – which carried forward scores for those unable to complete latter trials – were substantively identical to those from the first trial only). The episode was videotaped from behind the puppet theater and observational measures were later rated from videotape by a researcher blind to maternal data using standard scoring procedures. A composite score was determined based upon intensity of facial fear (0-3), bodily fear (0-3), escape behavior (0-3) and distress vocalizations (0-5). Inter-rater reliability was calculated on 22 randomly selected tapes with a rater who was blind to child and parent data. Intraclass correlations were 0.80 for facial expression, 0.70 for body posture, 0.92 for vocalizations, and 0.89 for escape behavior (all p's <0.001); a composite score composed of the four indicators had an intraclass correlation of 0.93.

In the paradigm to assess joy/pleasure, the experimenter presented a scripted puppet show lasting about one minute. A composite score was determined based on intensity of smiling (0-3) and presence or absence of laughter, positive vocalizations and positive motor activity (0-1) in all five trials. Inter-rater reliability on the previously noted random sample of tapes was .93 for laugh, .82 for positive movements, .95 for smile, and .80 for vocalizations, and .93 for the composite.

Psychosocial and obstetric covariates

Information on maternal age, gestational age at amniocentesis, time of collection of amniotic fluid, parity, ethnicity (categorized as Caucasian; Asian – Indian/Subcontinent; Black or Black-British; Middle-Eastern; Asian – Far-Eastern; or Unknown), smoking (cigarettes per day; given the limited smoking reported we collapsed this into yes/no), and alcohol (units per week) use were collected at recruitment. Few women reported taking any prescription medication during pregnancy (11% had taken any prescription drug categories, with the most common being anti-asthmatic, n=7) and we had very limited verifiable information on dose and timing, and not enough to exclude any individual on a priori grounds. Given that prescription medication during pregnancy was unassociated with study variables of interest (see below; perhaps because of the limited available data), they are not excluded from analyses. Information regarding birth outcomes was collected from the child's hospital notes after delivery, including birth weight, gestational age at birth, method of delivery and child sex. Standard deviation score (SDS) of birth weight adjusted for gestational age and sex was calculated using commercially available

software based upon 1990 British Growth Reference data. At the follow-up appointment we collected information on child age at testing.

Maternal stress and mental state

Mothers completed a 26-item Stressful Life Events Questionnaire (SLEQ; adapted from Barnett, Hanna, & Parker, 1983), at the postnatal visit, and reported if the event occurred and whether the event "affected me a little" or "affected me a lot." Example items include: "you had a major financial problem"; "your partner lost his job"; "your partner was emotionally cruel to you." Mothers reported if the event occurred antenatally or postnatally (birth to follow-up) or both. The SLEQ is similar to measures of stressful life events used in studies of non-pregnant adults. The number of events experienced is used as the index of stress. In addition, the Spielberger State-Trait Anxiety Inventory (STAI) (Spielberger, Gorsuch, and Lushene, 1983) was used to quantify levels of current (state) and habitual (trait) anxiety and to control for the heritability of anxiety-like traits in the child. The STAI is a widely used index of anxiety symptoms and has considerable validity, reliability, and clinical utility. The state and trait measures were administered at the postnatal visit; only state anxiety was included in the prenatal/amniocentesis visit.

Statistical analyses

A P-P plot was used to check the normality of distribution for all numeric data. Non-normally distributed variables (amniotic fluid testosterone and cortisol) were log transformed. As we had *a priori* expectations for differences in effects, particularly for testosterone, separate-sex analyses were conducted. Multiple regression analysis was used to test the robustness of the study hypothesis by controlling for covariates. Based on *a priori* considerations, maternal age, and smoking and alcohol use in pregnancy were included as covariates, but other covariates are included in the final regression model only if they were significantly associated with either fear reactivity or prenatal hormone levels. In the final regression model we also included as covariates concurrent maternal anxiety symptoms to provide a strong test of the hypothesis that the effects of amniotic fluid testosterone on child outcomes are not explained by current maternal mental state. Hypotheses concerning amniotic fluid testosterone are carried out using the RIA-derived measures, but we also report findings using the smaller sample of LCMS-derived measures of amniotic fluid testosterone to confirm reliability of the result.

Results

Sample characteristics are provided in Table 1. Preliminary analyses indicated one expected sex difference: amniotic fluid testosterone was higher in males than females (untransformed means were, for males, n=53; .80 (SD=.40); for females, (n=54; .24 (SD=.19); $F(1,105) = 86.55$, $p<.001$), although there was overlap in the range of levels between the sexes. There were no difference in amniotic fluid cortisol according to child sex ($F(1,106)=1.06$,$p=.31$); neither was the fear reactivity or the joy/please temperament measure associated with child sex (p's > .3). For those children on whom we had temperament data, amniotic fluid testosterone and cortisol were positively correlated ($r(107)=.36$, $p<.01$); in females, ($r(54)=.46$, $p<.01$) and in males ($r(53)=.30$, $p<.05$). In the range of gestational age in this sample, gestational age was weakly positively associated with amniotic fluid cortisol ($r(108)=.17$, $p=.08$) but not with amniotic fluid testosterone ($r(107)= -.07$). Additional bivariate analyses indicated that gestational age at amniocentesis, time of collection of amniotic fluid, parity, ethnicity, method of delivery, birth weight, and prescription drug use during pregnancy were not significantly related to fear reactivity; neither were these variables was associated with amniotic fluid testosterone.

Amniotic fluid cortisol and testosterone and infant fear reactivity

Amniotic fluid cortisol was not significantly associated with fear reactivity, either in the whole sample (r(108)=.10) or in boys and girls assessed separately. In contrast, amniotic fluid testosterone was significantly associated with fear reactivity in males r(53)=0.34, p=0.01 (Figure 1), but not females (r(54)=-0.07, ns (Figure 2). The association was not significant when boys and girls were combined (r(107)=0.07, ns). The significant association in males and the greater association in males than females were confirmed in the subsample for whom prenatal testosterone was assayed by HPLC/mass spectrometry: the correlation between amniotic fluid testosterone and fear reactivity in boys was r(24)=0.62 p=0.001, and in girls r (16)=0.26, ns.

The final regression model (using radioimmunoassay data) is presented in Table 2, which includes a formal test of the child sex × amniotic fluid testosterone interaction. Results show that the link between prenatal testosterone and fearfulness in males remained significant when multiple covariates were included in the model (the significant main effect for sex that indicated higher scores for females was only evident only when the interaction was included in the model).

Supplementary analyses (not shown) indicated that the findings were unchanged when prenatal cortisol was included. Additional supplementary analyses (not shown) indicated that the prediction of fear reactivity from amniotic fluid testosterone in boys did not differ according to gestational age; neither did eliminating the few cases in which amniotic fluid was obtained after 30 weeks gestation alter the findings. We also considered the possibility that potential outliers may have had undue influence on the findings. Results indicate that this was not the case: dropping the case with the highest prenatal testosterone and fear reactivity values diminished the effect slightly but it remained small/moderate in magnitude; using the arguably more sensitive liquid chromatography/mass spectrometer approach, the effect is diminished slightly but remained moderate/large in magnitude. That is, the effect sizes are comparable with and without this case according to both assays. Dropping the highest scoring female on prenatal testosterone had minimal effect on the association in females.

Observed Joy/Pleasure was not significantly associated with amniotic fluid cortisol or testosterone.

Analyses to test the mediational model linking prenatal stress and fear reactivity

We previously reported that stressful life events in pregnancy predicted observed fear reactivity (Bergman et al., 2007). The availability of amniotic fluid data in the current study allowed us to test the mediational model that prenatal hormone exposure mediated the link between prenatal stress and observed fear reactivity in the child. There was no evidence for this mediation hypothesis for cortisol given the lack of association between prenatal cortisol exposure and fear reactivity noted above. On the other hand, it was possible that prenatal testosterone mediated the significant association between prenatal life event stress and fear reactivity in boys. Several analyses, however, indicated that this was not the case. For instance, there was not a significant correlation between amniotic fluid testosterone and prenatal stress (r(124)=-.05, ns; in males only, r(53)=-.03). Neither was prenatal testosterone significantly associated with prenatally assessed maternal state anxiety (r(124)= -.01). In addition, for boys, a regression model indicated that both prenatal testosterone and prenatal life event stress were independently associated with fear reactivity (for prenatal testosterone, B= 5.34, SE 1.83; beta=.38, p<.01; for prenatal stress, B= .66, SE .24; beta=.36, p<.01); the magnitude of one was little affected by the inclusion of the other in the model. Further supplementary analyses (not shown) indicated that the findings for prenatal testosterone and prenatal stress were

unchanged after including prenatal cortisol, prenatal anxiety, postnatal stress and the interaction between prenatal stress and prenatal testosterone.

Discussion

The idea that early exposures may have significance for child or adult health is not new (e.g., Mackenzie, 1906), but it is only recently that the broad conceptual and health implications of this notion have been coupled with a methodological sophistication for researching biological mechanisms. The current study provided an extension to existing human research by testing the hypothesis that direct prenatal exposure to cortisol and/or testosterone predicted a) a key behavioral outcome, fear reactivity and b) mediated the effect of maternal prenatal stress. The novel finding of this study is that amniotic fluid testosterone level predicted fear reactivity in boys; that adds to the existing work on prenatal hormone exposure and behavioral development. We did not find that prenatal testosterone mediated the link between prenatal stress and fear reactivity. Additionally, there was no support for the hypothesis that amniotic fluid cortisol predicted fear reactivity.

Before discussing the conceptual significance of the finding, we first discuss the specific phenotype indexed by the observational assessment. The fear reactivity phenotype, as observed here, is not a “pure” measure of anxiety or behavioral inhibition. Indeed, the unpredictable toy also elicited general emotional arousal and activation. It is quite possible that greater exposure to testosterone during fetal development might also predispose the male child to a greater propensity for arousal and reactivity. In that regard, it is possible that what we detected is along the lines of the link between testosterone and increased tension (van Honk et al., 1999). Perhaps relatedly, van Wingen and colleagues (2008) reported that endogenous testosterone levels were positively correlated with amygdala reactivity, an association that was further supported with results from an experimental administration of testosterone. Further follow-up of these infants may be necessary to clarify the particular behavioral phenotype associated with prenatal testosterone.

The current findings build on and extend prior work on prenatal testosterone exposure in several ways. First, it extends prior findings on sex-typical play and autistic-like behavior and language problems. It is possible that observed fear reactivity is indexing part of a broader phenotype of sex-linked behaviors. However, the general lack of mean differences by sex in fear reactivity and related behaviors in very young children makes that somewhat unlikely, and others report associations between prenatal testosterone and additional psychiatric and behavioral phenotypes, including eating disorders (Culbert et al., 2008; Procopio & Marriott, 2007). The implication is that prenatal testosterone exposure may have a role in the development of a wider range of behavioral/psychiatric phenotypes, beyond autism and language problems, especially in boys.

Second, prior work linking testosterone to behavior tended to emphasize its impact on increased aggression and decreased anxiety. Robust associations of that kind have been reported in adults (Archer et al., 1998; Hermans et al., 2006); somewhat weaker and qualified findings have been reported in younger samples (Constantino et al., 1993; Granger et al., 2003; Sanchez-Martin et al., 2000; Schaal et al., 1996; van Bokhoven et al., 2006). Whether or not the current findings are contrary to this general pattern is not clear: we did not have a direct index of aggression; it may be that the arousal/distress observed is as good a marker of (later) aggressive as anxious behavioral development. In any event, a potential limitation of many studies is a reliance on concurrent associations, little attention to co-morbid patterns of behavior that may confound associations (e.g., such as co-occurring anxiety and aggression in some children), and lack of inclusion of young children and infants. As regards the latter concern, Marcus et al. (1985), in one of the few studies of infants, found no association between testosterone from umbilical

cord blood and several mood ratings from parental diary. It may be that an association between testosterone and aggressive behavior emerges only later in development (e.g., post puberty) or, as the current findings imply, a prenatal effect has different and distinguishable long-term effects than subsequent postnatal hormone exposure in middle childhood or adolescence. Further follow-up of the current sample might resolve this question. On the other hand, recent fMRI findings in males suggest that testosterone levels are positively associated with amygdala activation to fearful faces (Derntl et al., 2009), which may be more compatible with the results reported here.

The findings also add to developmental programming models of prenatal stress and anxiety. What has been largely missing from human work is direct evidence of a mediating mechanism accounting for the effect of prenatal stress and anxiety on child outcomes. The current findings add to and complicate the general model. On the one hand, the link between prenatal testosterone and fear reactivity, an outcome commonly linked to prenatal stress, provides valuable new evidence for prenatal hormonal influence. This finding may be particularly useful for understanding developmental origins of outcomes that are more often found in males, such as symptoms of ADHD and mixed handedness (Glover et al., 2004; Obel et al., 2003). On the other hand, prenatal testosterone did not account for the prediction from prenatal stress to fear reactivity. Furthermore, and contrary to our predictions, amniotic fluid cortisol did not predict child fear reactivity (neither was the amniotic fluid cortisol associated with either prenatal anxiety or prenatal stress). The absence of a link between prenatal glucocorticoid exposure and fear reactivity was surprising given the robust associations between prenatal stress and fear- and distress-related temperament outcomes in humans, and the strong support in the animal literature for the glucocorticoid mediating hypothesis. Human studies showing an association between prenatal cortisol – whether derived from amniotic fluid or from maternal saliva – and offspring outcome are limited. For example, one study of 17 mother-infant pairs found that 4 of 15 behaviors of young infants during everyday routines were associated with maternal saliva cortisol in pregnancy (de Weerth et al., 2003). It is possible that the lack of association with amniotic fluid cortisol is a false negative finding (see limitations below), but it is also possible that an HPA-mediated link is weaker or more complicated than has been assumed. In fact, other human studies that do not support a simple mediated link between prenatal cortisol and child outcomes. For example, Davis et al (2007) found that maternal prenatal salivary cortisol predicted maternal reported infant temperament independently of prenatal stress. Although Davis et al. did not assess amniotic fluid cortisol, and maternal salivary cortisol may be too weak an index of fetal exposure to detect an effect, their results are nevertheless consistent with the current findings and raise important conceptual and methodological questions about the mechanisms accounting for the prenatal programming of child behavior.

The current findings also leave open other questions about mediating mechanisms. Our postulated mechanism of fetal adrenal activation would have predicted an association with both cortisol and testosterone, not testosterone alone. Nevertheless, the differential effects of testosterone in boys and girls is consistent with the research cited above, as well as the prior work of Geschwind and others (Geschwind & Galaburda, 1987). It may suggest an association between increased activity of the testes rather than the adrenal as a predictor of fear reactivity. It is also possible that there is a linked mechanism of control of both *in utero* testosterone level and later child fear reactivity, but that the raised testosterone does not play a direct fetal programming role. And, given that the source(s) of amniotic fluid cortisol and testosterone cannot be deciphered with precision (possibilities include fetal urine, maternal circulation), questions remain about the mechanisms within the fetal-placental-maternal unit.

The study had several limitations, including a modest over-representation of higher income and older age. It is unlikely that these biases had much influence on the findings, however, because these variables are not known to predict fear reactivity and maternal age, an important

variable associated with amniocentesis sampling, was included as a covariate. The most important limitation of the study is that prenatal exposure to cortisol and testosterone were assessed on a single occasion, and at an early mean gestational age (17 weeks) That is an insurmountable limitation because repeat assessments of amniotic fluid for research purposes are neither possible nor ethical; furthermore, those mothers who undergo more than one amniocentesis will be at very high risk for developmental problems. It is possible that raised cortisol exposure at a later (or earlier) gestational age than is common for amniocentesis is associated with increased fear reactivity and were therefore missed from our assessment. That may also account for the failure to detect a mediated effect with prenatal testosterone. Also, we could not rule out alternative explanations for these observations, including genetics. Including maternal anxiety as a covariate provides some, but limited, protection against a simple genetic transmission hypothesis in which genetically anxious mothers have fearful children. Additionally, we were unable to examine the impact of postnatal hormone levels in the children. We previously reported that attachment classification moderated the association between maternal self-reported stress in pregnancy and fear reactivity (Bergman et al., 2008); however, we found no such moderation effect on amniotic fluid cortisol. Whether this constitutes a non-robust effect is unclear. Lastly, we were unable to address questions about timing, which are so far unresolved in the human literature (cf. Laplante et al., 2004; O'Connor et al., 2002; van den Bergh et al., 2005), or its specific impact in the brain, such as the amygdala. Offsetting these limitations were several strengths of the study, including observational assessments of temperament – which have substantial methodological advantages over maternal self-report, longitudinal follow-up, adequate sample size to detect modest effects, and the inclusion of an index of prenatal exposure to both cortisol and testosterone. Finally, in contrast to many studies that assess clinic populations (e.g., girls with CAH, boys with autistic-like behavior), the current study included a community sample.

In conclusion, the study provides the first evidence in humans of a link between *in utero* testosterone exposure and fear reactivity, but we were unable to find convincing evidence for either cortisol or testosterone as potential mediators of prenatal maternal stress or anxiety on this particular behavioral outcome of the child. Further work is needed to understand the biological mechanisms underpinning the programming effects of maternal mood on fetal and child development. Investigations using amniotic fluid, placenta, and other sources will be needed to translate the animal models and elucidate their meaning for human health.

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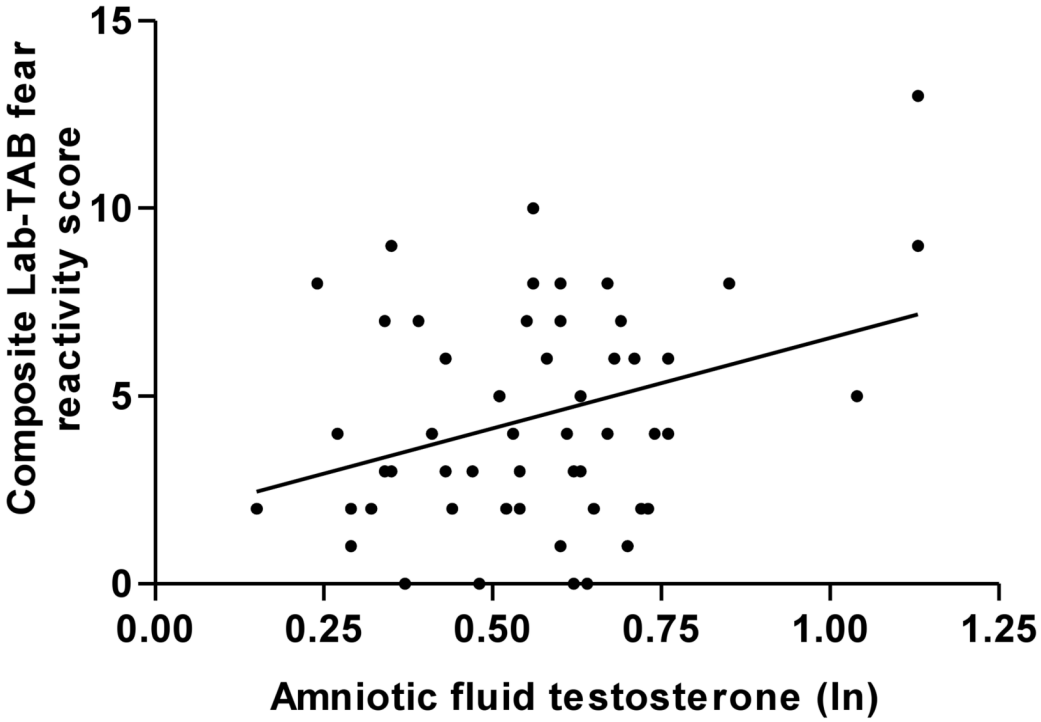

Figure 1.
Correlation between amniotic fluid testosterone(ln) and infant composite Lab-TAB fear reactivity score at 17 months in males: ($r(53)=0.34$, $p=0.01$).

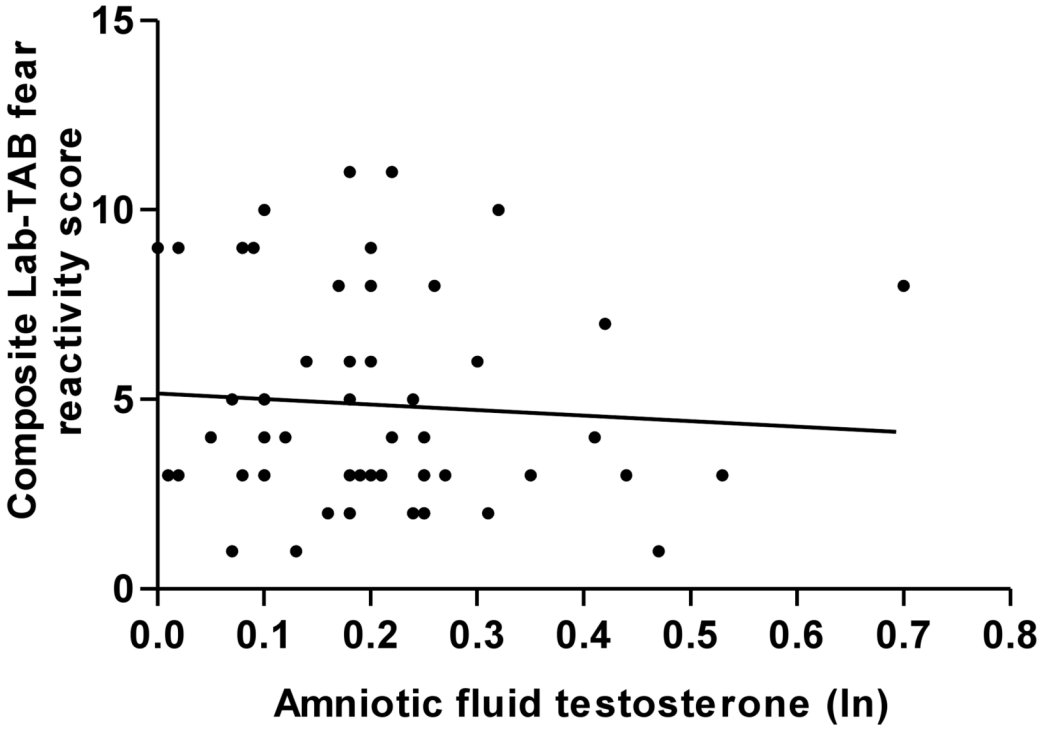

Figure 2.
Correlation between amniotic fluid testosterone(ln) and infant composite Lab-TAB fear reactivity score at 17 months in females: (r(54)=-.07, ns).

Table 1

Sample descriptive data

	Mean (SD) *Range* No. (%)		Mean (SD) *Range* No. (%)
Maternal age at amniocentesis	**36.95 (3.85)** *28 – 45*	**Gestational age at amnio (weeks)**	**17.19 (2.86)** *15– 32*
Time of collection at amnio (24 hr)	*9.05am – 13.15pm*	**Gestational age at birth (weeks)**	**39.51 (1.12)** *37 – 42*
Child sex		**Birth weight (grams)**	**3499.70 (473.94)** *2574 - 6000*
Female	54 (50.5)		
Male	53 (49.5)		
Racial background		**Parity**	
White Caucasian	87 (81.3)	Nulliparous	43 (40.2)
Asian/Subcontinent	7 (6.5)	1 previous child	41 (38.3)
Asian Far-Eastern	1 (0.9)	2 previous children	17 (15.9)
Black	10 (9.3)	3 previous children	6 (5.6)
Middle-eastern	2 (1.9)		
Smoking in pregnancy		**Alcohol use in pregnancy**	
0 per day	95 (88.8)	0 units per week	72 (67.3)
1-2/day	10 (9.4)	1-2 units per week	30 (28.0)
>2/day	2 (1.8)	>2 units per week	5 (4.7)
Child age at follow-up (months)	**16.71 (1.48)** *14.37 – 19.97*	**Method of delivery**	
		vaginal delivery	58 (54.2)
		assisted vaginal	12 (11.2)
		elective caesarean	14 (13.1)
		emergency caesarean	14 (13.1)
		Unrecorded	9 (8.4)

Note> n's range from 101-108.

Table 2
Prediction of observed fear reactivity from prenatal testosterone

	B (SE)	Beta
Maternal age	.05 (.07)	.07
Child sex (1=female,2=male)	-3.81 (1.37)	-.67**
Smoking during pregnancy	-.53 (.46)	-.11
Alcohol use during pregnancy	.16 (.17)	.10
Postnatal State anxiety	.04 (.03)	.13
Amniotic fluid testosterone (ln)	-11.43 (6.89)	-1.00
Amniotic fluid testosterone (ln) × child sex	8.19 (3.80)	1.60*

Note. N=103;

* p <.05;

** p<.01.