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Antenatal Maternal Anxiety is Related to HPA-Axis Dysregulation and Self-Reported Depressive Symptoms in Adolescence: A Prospective Study on the Fetal Origins of Depressed Mood

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Depressive symptomatology can proceed from altered hypothalamic-pituitary-adrenocortex (HPA)-axis function. Some authors stress the role that early life stress (ELS) may play in the pathophysiology of depressive symptoms. However, the involvement of the HPA-axis in linking prenatal ELS with depressive symptoms has not been tested in a prospective-longitudinal study extending until after puberty in humans. Therefore, we examined whether antenatal maternal anxiety is associated with disturbances in HPA-axis regulation and whether the HPA-axis dysregulation mediates the association between antenatal maternal anxiety and depressive symptoms in post-pubertal adolescents. As part of a prospective-longitudinal study, we investigated maternal anxiety at 12–22, 23–32, and 32–40 weeks of pregnancy (wp) with the State Trait Anxiety Inventory (STAI). In the 14–15-year-old offspring ($n = 58$) HPA-axis function was measured through establishing a saliva cortisol day-time profile. Depressive symptoms were measured with the Children's Depression symptoms Inventory (CDI). Results of regression analyses showed that antenatal exposure to maternal anxiety at 12–22 wp was in both sexes associated with a high, flattened cortisol day-time profile ($P = 0.0463$) which, in female adolescents only, was associated with depressive symptoms ($P = 0.0077$). All effects remained after controlling for maternal smoking, birth weight, obstetrical optimality, maternal postnatal anxiety and puberty phase. Our prospective study demonstrates, for the first time, the involvement of the HPA-axis in the link between antenatal maternal anxiety/prenatal ELS and depressive symptoms for post-pubertal female adolescents.
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INTRODUCTION

Depressive disorders are among the most common psychiatric disorders, with prevalence estimates for major depressive disorder (MDD) ranging from 5% to a maximum of 20%. After puberty, a maturational and potentially stressful period, the risk of developing depression increases and the prevalence among females becomes one and a half to three times that of males (Angold *et al*, 1998; Ge *et al*, 2006; Hankin and Abramson, 2001; Hankin, 2006; Kessler, 2003).

Early-life exposure to adverse environmental cues during critical windows of time in the prenatal and/or early postnatal life period (eg, nutrient restriction or maternal anxiety) could predispose the individual for somatic and mental diseases. This especially holds for stress-related disorders such as depression in which HPA-axis dysregulation plays a pathophysiological role. The above is in line with the 'fetal (or developmental) programming-hypothesis' which has been tested in numerous preclinical experimental studies (eg, Coe *et al*, 2003; Kreider *et al*, 2006; Ladd *et al*, 2000; Maccari *et al*, 2003; Macri *et al*, 2007; Owen *et al*, 2005; Weaver *et al*, 2001; Welberg and Seckl, 2001). Early-life stress (ELS) at moments when critical developmental processes are taking place in parts of the nervous system or neuronal circuits involved in (later) HPA-axis functioning, may induce epigenetic changes that program the HPA-axis. This possibly results in some individuals in distinct and stable patterns of dysregulations that are associated with altered emotional processing and heightened responsiveness to stress (Heim *et al*, 2004; Meaney and Szyf, 2005;

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Weaver *et al*, 2001, 2005). In humans, the effects of ELS were tested in epidemiological studies (see Kajantie, 2006; Phillips, 2004; Thompson *et al*, 2001) and in retrospective (see Heim *et al*, 2000, 2004; Gunnar and Quevedo, 2007; Kaufman and Charney, 2001; Tarullo and Gunnar 2006) and prospective (see Davis *et al*, 2005; Egliston *et al*, 2007; Huizink *et al*, 2004; Talge *et al*, 2007; Van den Bergh *et al*, 2005a) clinical and quasi-experimental studies.

Some prospective studies established the nature and timing of ELS exposure in relation to HPA-dysregulation in 5 $\frac{1}{2}$ year olds (Gütteling *et al*, 2005), in prepubertal (Young *et al*, 2006), 10-year-old (O'Connor *et al*, 2005), and 13-year-old children (Halligan *et al*, 2004). To the best of our knowledge, only one prospective study has established, via mediation analysis, the potential involvement of the HPA-axis in the link between ELS (namely maternal postnatal depression) and depressive symptomatology in 16-year-old postpubertal adolescents (Halligan *et al*, 2006). The present study builds on this work in extending the early-life indices to the prenatal period with the use of antenatal maternal anxiety measures.

Studies of the past 35 years have revealed that MDD is characterized by a hyperactive HPA-axis induced by alteration at different levels of the HPA-axis. Corticotropin-releasing hormone (CRH)-hyperdrive (at hypothalamic and extrahypothalamic levels), probably potentiated by the action of arginin vasopressin, as well as a reduced negative feedback have been proposed as the primary factor in the pathogenesis of MDD that contribute to the observed elevation of cortisol in MDD. However, the exact pathophysiology of MDD, which also involves changes in monoamine neurotransmitters and the immune system, remains to be unraveled further (see Claes, 2004; Claes and Nemeroff, 2005; Chrousos, 1998; Heim *et al*, 2004; Holsboer, 2001; Pruessner *et al*, 2003; Raison and Miller, 2003; Swaab *et al*, 2006; van Praag *et al*, 2004; Van Den Eede *et al*, 2005; Van West *et al*, 2006).

The secretion of cortisol, the hormonal endproduct of HPA-axis activation, is kept within an optimal time-integrated narrow range, which is quite stable in an individual subject (Chrousos *et al*, 2004). Normally, basal cortisol increases markedly upon awakening to peak at 30–40 min post-awakening, followed by a declining pattern until the evening and a quiescent period (a dip) with very low cortisol around 2000–0200 hours (van Praag *et al*, 2004), and finally a rise into the next awakening surge. Changes in or dysregulation of the diurnal rhythm of cortisol are increasingly being studied in depression and other stress-related disorders in which the HPA-axis is involved (Chrousos and Gold, 1998; den Hartog *et al*, 2003; Deuschle *et al*, 1997; Gunnar and Vazquez, 2001; Heim *et al*, 2000; Holsboer *et al*, 1984; Miller *et al*, 2007; Rosmond *et al*, 1998; Sachar *et al*, 1970). A high, flattened diurnal profile has been shown to be indicative of a chronically stressed, hyperactive HPA-axis (Deuschle *et al*, 1997; McEwen, 2002: McBruce and Wingfield, 2003; Rosmond *et al*, 1998) and has been observed in depressed adult (den Hartog *et al*, 2003; Sachar *et al*, 1970; Young *et al*, 2006) and adolescent (Kaufman and Charney, 2001; Forbes *et al*, 2006) outpatients. Such a profile has moderately lower awakening output with less decline in the afternoon/evening than would be expected, thus leading to a higher daily output. Other dysregulations imply low cortisol levels at awakening which remain constant throughout the day. Such a low, flattened diurnal profile (ie, lacking of expected diurnal rhythm) reflects reduced cortisol output (hypocortisolism) and is seen in adult patients with post-traumatic stress disorders, atypical depression, or chronic fatigue syndrome (Gunnar and Quevedo, 2007; Heim *et al*, 2000, 2004; Miller *et al*, 2007; Van Praag *et al*, 2004) and in neglected children (eg, orphanage-reared children; see Gunnar and Vazquez, 2001; Gunnar and Quevedo, 2007). Total cortisol secretion measures or single measures at one moment during the day do not take account of circadian or diurnal variability and may therefore be less sensitive markers of vulnerability than measures of diurnal or circadian cortisol rhythm (Chrousos and Gold, 1998). Therefore, this paper will focus on measures of cortisol rhythm.

In a recent meta-analysis, Miller *et al* (2007) concluded that even when a person does not develop a full-blown psychiatric condition, the extent of distress is positively associated with HPA-activation. To the extend that people reported higher levels of distress, they show greater daily cortisol output and afternoon/evening cortisol, although morning levels are somewhat lower. However, the major limitation of the work on which that conclusion is based is that is has been largely cross-sectional in nature. Miller *et al* (2007) accentuate that future work in this area needs to be prospective. Such work could identify psychopathological or 'predisease' pathways at the medium-low part of the continuum of depressive symptomatology, with the use of biological markers—*vulnerability markers*. This may lead to a significant progress in the early identification of vulnerable persons and to the elaboration of early intervention programs to delay or prevent diseases later in life (McEwen, 2002). Moreover, post-pubertal adolescents have been understudied with respect to diurnal cortisol rhythms. It is suggested that the full maturation of the HPA-axis—including the full diurnal cortisol rhythm—which is reached with the attainment of Tanner stage three, may have implications for the heightened risk of psychopathology noted among post-pubertal adolescents (Gunnar and Quevedo, 2007).

In the present study, we aimed to investigate the possible fetal origins of disturbance in diurnal cortisol rhythms and depressive symptomatology in post-pubertal adolescents. To do so, we tested the following two hypotheses in a sample of an ongoing prospective-longitudinal study started at 12–22 weeks of pregnancy (Van den Bergh, 1992, 2005b, 2006; Van den Bergh and Marcoen, 2004): (1) antenatal exposure to maternal anxiety is, in post-pubertal adolescents, associated with disturbance in the diurnal secretion pattern of cortisol; (2) disturbance in the diurnal secretion pattern of cortisol mediates the association between exposure to antenatal maternal anxiety and self-reported depressive symptoms in post-pubertal adolescents. Given the evidence outlined above, a disturbance in the diurnal cortisol profile could be reflected in a flattened profile. We also tested the effect of gender and of timing of anxiety and controlled for potential confounders. Significant results may be indicative that ELS during one or more critical gestation periods plays a role in the pathophysiology of depressive symptomatology and may provide preliminary evidence that 'programming' of the HPA-axis by exposure to antenatal maternal anxiety/stress may predispose individuals to depressive symptomatology even much later in life.

MATERIALS AND METHODS

Participants

The study started with 86 healthy Dutch-speaking mothers (18–30-year-old) and their firstborn. Most of them were well educated and married and none of them had a psychiatric disorder. All babies were delivered between 36 and 41 weeks of pregnancy (wp) and had 5-min Apgar scores of 9 or 10 (2 babies with score 8). This sample has been assessed at 36–38 wp, at 1, 10, and 28 weeks after birth (Van den Bergh, 1992) and at ages 8–9 (Van den Bergh and Marcoen, 2004). At 14–15 years of age, 68 (79.1%) adolescents and their parents participated (Van den Bergh *et al*, 2005b, 2006); they all gave their informed consent. The ethical committee for experiments on human beings of our university approved the study.

Scales Measuring Maternal Anxiety and Adolescent Depressive Symptomatology

We used Dutch, psychometrically validated versions of standardized English symptom severity rating scales. The State Trait Anxiety Inventory (STAI) consists of a state and a trait subscale, each containing 20 items scored from 1 to 4, and with reliability coefficients, Cronbach's alphas, of 0.95 and 0.93, respectively (Van der Ploeg *et al*, 1980). State anxiety is conceptualized as a transient emotional condition, whereas trait anxiety reflects a dispositional anxiety proneness. The mothers completed the STAI at all assessments; all data were used in the present study. The Children's Depression Inventory (CDI; Kovacs, 1992; Timbremont and Braet, 2002), which was completed by the adolescents, entails 27 items measuring the severity of depressive symptoms, scored from 0 to 2. Subjects are asked to refer to their feelings, cognitions, and behavior during the past 2 weeks. According to the authors of the CDI, a score of 19 best distinguishes between nondepressed and clinically depressed persons rated with DSM-IV criteria, whereas levels between 13 and 18 are regarded as subclinical or minor depressive episode. In a Dutch-speaking reference population of 13–15-year-olds (Timbremont and Braet, 2002) the Cronbach's α is 0.84 and the test-retest reliability coefficient (Pearson product moment correlation) is 0.86.

HPA-Axis Dysregulation: Day-Time Cortisol Profile (Shortened Version)

Saliva sampling, in the morning as well as during the day, is best done with strict reference to the timing of awakening rather than on exact, predefined hours; the latter underlies large inter-and intraindividual variation (Edwards *et al*, 2001; Pruessner *et al*, 1997, 2003). We used a short version of the day-time cortisol profile and samples were collected at awakening, at approximately 4 (noon) and 12 h (evening) after awakening, at home, on a typical weekend day. For reasons of feasibility, we planned the saliva collection on a weekend day (eg, for an adolescent on a normal school day it is difficult to collect the noon sample 4 h after the awakening sample). It is shown that there is not a significant impact of genetic factors on this day-time cortisol profile (Wüst *et al*, 2000). Considering our interest in prenatal environmental factors, a short day-time cortisol profile should thus be an appropriate measure.

Samples were collected by spitting in a small plastic tube (Sarstedt, Germany), without using aids to salivation or swabs. Detailed instructions were outlined on a sheet provided with the tubes. The tubes were kept refrigerated and brought along to the laboratory visit, together with the questionnaires they had completed at home. All samples were stored at -60°C upon arrival. Cortisol in saliva was measured with a revised version of the protocol provided by the manufacturer of the Coat-a-Count Radio-Immuno-Assay Kit (Euro DPC, Llanberis, Wales). Two hundred microliters of saliva and diluted standards was used, with incubation for 3 h at room temperature, modified by an additional period overnight (15–18 h) at $+4^{\circ}$C (cf., O'Connor *et al*, 2005).

Statistical Analyses

The first hypothesis, on the relation between ELS and HPA-axis dysregulation, is tested using longitudinal repeated measurements regression. The outcomes were the three cortisol measurements that were encoded as 0 (awakening), 4 (noon), and 12 (evening). The associations among these measurements were best modeled using a 'heterogeneous first-order autoregressive' covariance structure. The independent ELS variables were the six prenatal maternal anxiety measures. Using six anxiety measures should bring about caution in the interpretation of the results, as we have six opportunities to find a relationship between prenatal anxiety and HPA-axis dysregulation. However, previous research has shown that fetal programming may occur during various gestational periods (for a review, see Talge *et al*, 2007; Van den Bergh *et al*, 2005a). It is supposed that the final programming effects are related to the kind of critical developmental processes that were taking place in organs or biological systems involved, at the time of ELS exposure. Hence, it is important to investigate the effect of exposure to maternal anxiety at different pregnancy periods. Repeated measurements allow the investigation of the whole cortisol profile and can thus be used to investigate the potential importance of a flattened profile. As an additional analysis, we also investigated the effect of prenatal anxiety on often-used summary measures of cortisol such as area under the curve (AUC). Note that these measures summarize the cortisol profile and as such loose a good deal of information.

The second hypothesis, on the mediational role of the HPA-axis between ELS and adolescent depression, was tested by a mediation analysis consisting of three steps, as required by the classical approach (Baron and Kenny, 1986). Using ordinary least-squares (OLS) regression, we assessed the influence of: (1) antenatal maternal anxiety on HPA-axis function (ie, cortisol day-time profile); (2) antenatal maternal anxiety on adolescent depressed mood; and (3) HPA-axis function on adolescent depressed mood when statistically controlling for antenatal anxiety. If the effect of antenatal anxiety on adolescent depressed mood (second regression) is less strong when HPA-axis function is also added to the model (third regression) and antenatal anxiety is associated with HPA-axis function (first regression), altered HPA-axis function can be seen as a mediator of the

effect of antenatal anxiety on depressed mood. Interaction effects with gender were always added to the regressions to investigate the moderating effects of gender.

As an alternative to the classical approach to detect mediation, the recently suggested and highly powerful bias-corrected bootstrap method (MacKinnon *et al*, 2004) was also used. The mediation effect is quantified by multiplying the parameter estimate of the antenatal maternal anxiety effect in the first regression with the parameter estimate of the HPA-axis function effect in the third regression. A 95% confidence interval of this mediation effect estimate was computed using the bias-corrected bootstrap method (MacKinnon *et al*, 2004).

Apart from gender, five covariates were considered: (a) smoking during pregnancy (binary); (b) birth weight controlled for gestational length (continuous); (c) obstetric optimality (continuous; a list of 52 optimal obstetrical conditions); (d) postnatal maternal trait anxiety (continuous: *z*-score resulting from a principal component analysis on trait anxiety measures at 1, 10, 28 weeks, 8/9 and 14/15 years after birth); and (e) morphological pubertal stage (ordinal: schematic drawings of secondary gender characteristics; according to Tanner, 1962, stage $\geqslant$III indicates post-puberty).

Due to the small sample size and the complexity of the statistical analyses, the influence of different prenatal anxiety measures in the repeated measurements analysis and of the covariates in the mediation analyses were checked using model selection procedures. As a measure of effect size in OLS regression, the omega squared (ω^2) was computed; it represents the proportion of the variance of the outcome variable that is accounted for by a predictor variable. Based on the studentized residuals (absolute value >2.00), we defined cases as suspect (possible outliers). Removal of those cases did not change the conclusions, therefore they were retained in the analyses.

RESULTS

Preliminary Statistics

Of the 68 mother-child pairs, 58 (85.3%) had complete data for all cortisol measures (29 males, 29 females; mean age $\pm$ SD = 15 years 0 month $\pm$ 3 months; range = 14 years 6 months to 15 years 6 months). For the 58 complete cases, descriptive statistics are given in Table 1. State and Trait anxiety scores in the highest quartile correspond with deciles 6–9 (for anxiety at 23–31 and at 32–40 wp) and 6–10 (for anxiety at 12–22 wp) of a nonclinical female community sample (Van der Ploeg *et al*, 1980), indicating that for 25% of the pregnant women anxiety levels were at least higher than average.

Table 1 Descriptive Statistics for Key Variables ($n=58$)

Variable	Mean (SD)	Median	Q1–Q3	Min–Max
Maternal anxiety 12–22 wp				
Trait anxiety 12–22 wp	36.0 (8.44)	35.5	29–39.5	21–59
State anxiety 12–22 wp	39.4 (8.83)	37.0	33–44	24–62
TAI12–22	0.0 (6.51)	0.2	−4.0–4.1	−16.0–14.7
Trait anxiety 23–31 wp	35.0 (8.25)	34.5	28.0–41.0	21.0–52.4
State anxiety 23–31 wp	35.0 (8.83)	34.0	29.0–39.4	20.0–62.0
Trait anxiety 32–40 wp	35.1 (7.87)	36.3	30.0–40.0	20.0–53.0
State anxiety 32–40 wp	35.8 (7.21)	34.7	30.0–40.0	20.0–58.0
Cortisol (nmol/l)				
Awakening cortisol level	12.3 (4.99)	11.8	9–15.4	1.5–25.4
Noon cortisol level	6.1 (4.24)	5.1	3.3–7.3	0.4–22.2
Evening cortisol level	2.2 (3.26)	1.3	0–3.3	0.0–17.8
DIF (awakening-evening cortisol)	10.1 (5.21)	9.6	7.6–14	−1.9–22.6
DIF (females)	9.4 (6.54)	8.7	5.6–14.1	−1.9–22.6
DIF (males)	10.8 (3.38)	9.9	9.0–13.3	3.9–16.7
Adolescent depressive symptoms				
Children's depression inventory	8.9 (5.44)	7.5	5–12	1–25
Gender and covariates				
Adjusted birth weight	−0.011 (0.964)	−0.045	−0.76–0.90	−1.85–1.88
Obstetric optimality	45.5 (3.34)	46.0	44–48	30–51
Postnatal maternal anxiety	0.013 (1.032)	−0.055	−0.72–0.81	−2.12–2.34
Pubertal development	4.13 (0.053)	4.00	4–4.5	3–5
Gender (29 males, 50%)	/	/	/	/
Prenatal smoking ($n=14$, 24%)	/	/	/	/

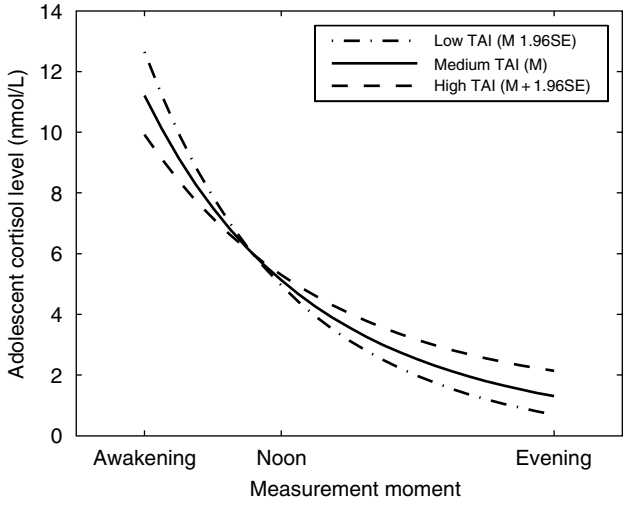

Figure 1 Day-time cortisol profiles as predicted by prenatal anxiety (TAI12–22) in a repeated measurements regression analysis.

As expected, the intraindividual variation in cortisol levels is large (see Table 1). The mean absolute levels of cortisol secretion at awakening, noon, and evening and the day-time profile in our sample (see also Figure 1) are in accordance with community samples with depressive outpatients and controls (eg, Peeters *et al*, 2004; Young *et al*, 2006).

The whole range of depressive symptomatology measured by the CDI is covered by our sample. If we use the cut-off scores defined by the authors of the CDI, 22 % ($n = 13$; eight girls, five boys) of our sample can be seen as reporting at least subclinical depression symptoms (38% of which had a CDI score higher than 18 ($n = 5$; four girls, one boy). An even higher percentage may suffer from depressed mood. This incidence seems comparable with the incidence in nonclinical adolescent samples, in which the likelihood of depressed mood ranges between 25–40% (girls) and 20–35% (boys) (Hankin and Abramson, 2001).

To correct for non-normality of the depressive symptoms scores and the cortisol values, a square-root, respectively log transformation was used for these variables.

Repeated Measurements Analysis

The effects considered were all six prenatal anxiety main effects, the linear, and quadratic time effects of cortisol, and the interactions of all six prenatal anxiety measures with the linear time effect (14 effects). After model selection, the model contained the linear and quadratic time effects of cortisol and all main and interaction effects related to anxiety at 12–22 wp (six effects). There was a linear and a quadratic time effect ($P < 0.0001$, respectively $P = 0.0363$), suggesting that the cortisol level strongly decreased towards the evening and that this decrease was very strong at first, but less strong in the end, when approaching the quiescent period. Trait anxiety at 12–22 wp emerged as the only prenatal anxiety predictor of the offspring day-time cortisol profile (interaction with the linear time effect: $b \pm \mathrm{SE} = 0.0027 \pm .0014$, $t_{(112)} = 2.00$, $P = 0.0476$). Although surprising, the prenatal trait anxiety measures did not appear stable. The 12–22 wp period seemed especially influential for the mothers. Although traits are considered stable entities over short time spans, the trait anxiety in the 12–22 wp period tended to differ nonetheless from the two subsequent prenatal measures (Wilcoxon signed rank test: $S = 216.5$, $P = 0.09$). This lack of stability may explain why the three trait measures do produce different results in our analysis.

The interaction between trait anxiety 12–22 wp and the linear time effect suggested that adolescents whose mothers were anxious during 12–22 wp have a flattened day-time cortisol profile. It was also observed that state anxiety acted as a suppressor of trait anxiety. (Suppression is the counterintuitive situation where, in its classical form, a suppressor variable X2 is unrelated to outcome Y even though its inclusion in the model improves the prediction by making variable X1 an improved predictor of Y. In such a situation, X2 is correlated with a part of X1 that is uncorrelated with Y such that the addition of X2 to the model removes or suppresses this noise or unwanted variance in X1, thus enhancing the predictive ability of X1. Readers are referred to the seminal textbook by Cohen and Cohen (1983) and to the recent paper by Friedman and Wall (2005)). Therefore, we decided to control for state anxiety at 12–22 wp in further analyses concerning trait anxiety at 12–22 wp. A parsimonious way to do so is to take the residuals of trait anxiety when it is regressed on state anxiety; this allows us to avoid the suppressing effect of state anxiety without having to include both variables in analyses. This variable is named 'trait anxiety index 12–22 wp' (TAI12–22). The correlation between trait anxiety at 12–22 wp and TAI12–22 is 0.77.

Higher TAI12–22 predicted a flattened day-time cortisol profile (ie, a smaller decrease from awakening cortisol to evening cortisol; see Figure 1). Also, the weak main effect of TAI12–22 indicated that it appears to be the absolute decrease in cortisol that is most important. These results suggested that the difference between the awakening and evening cortisol level—a measure named 'DIF'—was a valid proxy for the short day-time cortisol profile. DIF is a parsimonious measure to indicate the flatness of the cortisol profile; it has been used in previous research (Wüst *et al*, 2000). As the third regression in the mediation analysis uses the HPA-axis function as a predictor variable, DIF is interesting because it allows us to summarize the cortisol profile with one number reflecting that part of the profile, which is related to maternal anxiety. A profile in which awakening output is only somewhat lower, but in which there is less decline in the afternoon/evening than would be expected, results in a high, flattened profile, whereas a profile with low cortisol levels at awakening which remain constant throughout the day, results in a low, flat profile. Only in the latter case DIF will be very small, almost reaching zero.

We did additional analyses to check whether our results are related to a high flat profile or to a low flat profile. First, we redid all analyses, whereas excluding the four cases with the lowest awakening cortisol (which all had a low, flat profile and a low DIF). All results were similar and the conclusions were the same as with analyses including these four cases. Second, we compared adolescents whose mothers were not very anxious (25% mothers with lowest TAI; <Q1) and those that were highly anxious (25%

mothers with highest TAI; >Q3). We observed that the relation between TAI and DIF was not due to cases with low awakening cortisol; these cases typically had mothers with an average TAI (ie between Q1 and Q3). Adolescents of highly anxious *vs* low anxious mothers had the following median cortisol levels: 13.3 at awakening (mean = 12.7), 5.9 at noon, 1.5 in the evening *vs* 13.2 at awakening (mean = 14.4), 5.0 at noon, 0.4 at evening.

Using OLS regression analysis, no strong effects of antenatal maternal anxiety on the cortisol awakening or AUC measures were found (all $P > 0.21$).

Mediation Analyses

The first regression conceptually replicated the results of the repeated measurements analyses. The TAI12–22 anxiety index is used to predict the diurnal cortisol change (DIF). No effects involving gender or the covariates were important. TAI12–22 was associated with lower DIF, thus with less decreasing (ie, flattened) cortisol profiles ($b \pm SE = -0.210 \pm 0.103$, $t_{56} = -2.03$, $P = 0.0467$, $\omega^2 = 0.05$). Obviously, this result is nearly identical to the interaction effect described in the previous section.

In the *second regression*, the gender-TAI12–22 interaction ($b \pm SE = 0.083 \pm 0.035$, $t_{54} = 2.36$, $P = 0.0220$, $\omega^2 = 0.07$; Figure 2) revealed that higher TAI12–22 predicts higher depressed mood only in adolescent females (females: $P = 0.0027$, $\omega^2 = 0.14$; males: $P = 0.8470$, $\omega^2 = 0.00$). Including the other covariates only yielded a significant positive effect of postnatal maternal anxiety. This effect did not eliminate the gender/TAI12–22 interaction ($P = 0.0502$, $\omega^2 = 0.04$).

In the third regression, postnatal maternal anxiety was again the only covariate retained in the model. A gender–DIF interaction ($b \pm SE = -0.096 \pm 0.048$, $t_{51} = -2.02$, $P = 0.0492$, $\omega^2 = 0.04$; Figure 3) showed that smaller DIF (ie, a flattened profile) predicts higher depressed mood, again in females only (females: $P = 0.0077$, $\omega^2 = 0.08$; males: $P = 0.4216$, $\omega^2 = 0.00$). Importantly, the strength of the gender-TAI12–22 interaction was smaller than in the second regression ($P = 0.1735$, $\omega^2 = 0.01$). This indicates that, only in females, there is an effect of antenatal exposure to maternal anxiety on depressed mood in adolescence that can be explained, in part, by an effect of the flattened cortisol profile on depressed mood (this is mediation according to the classical approach) (Figure 4, panel b).

Regression analyses in which cortisol or depressed mood were regressed on measures of anxiety at 23–31 and 32–40 wp did not reveal independent effects of anxiety during these periods of pregnancy.

The alternative bias-corrected bootstrap method (MacKinnon *et al*, 2004) to investigate mediation also suggested a mediating role of DIF for girls. Because the gender-dependent effect of TAI12–22 on CDI is at least partially mediated by a general effect of TAI12–22 on DIF and a gender-dependent effect of DIF on CDI, the mediation effect could be quantified by multiplying the parameter estimates of the effect of TAI12–22 on DIF (first regression)

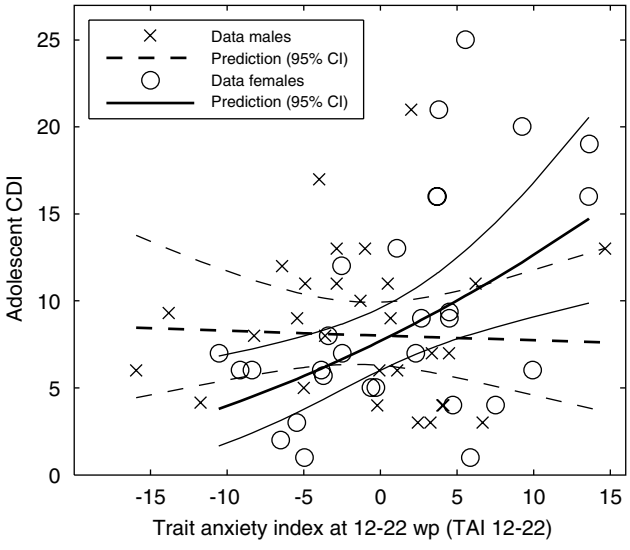

Figure 2 CDI depressive symptoms scores as predicted by prenatal maternal anxiety (TAI12–22) and gender in the second regression.

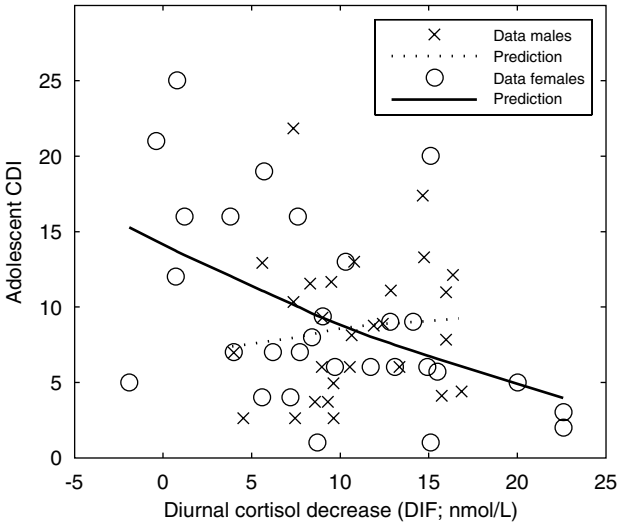

Figure 3 CDI depressive symptoms scores as predicted by diurnal cortisol decrease (DIF) and gender in the third regression. The prediction lines are smoothed to filter out the influence of other variables.

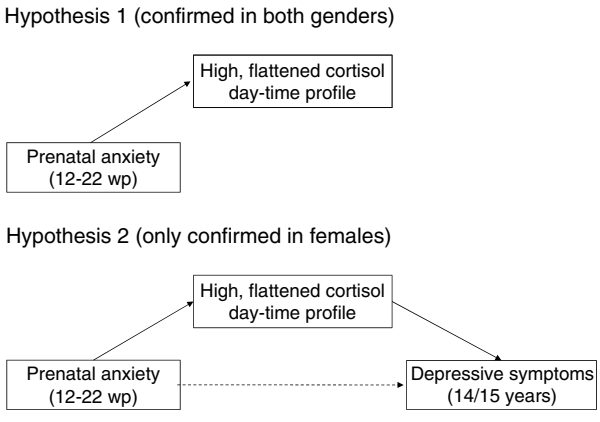

Figure 4 Graphical presentation of the results. Results in (a) represent the effect of prenatal anxiety on a flattened day-time cortisol profile. Results in (b) represent a 'mediated moderation' model in which a gender-related effect of prenatal anxiety on adolescent depressive symptoms (dashed line) is mediated by a flattened day-time cortisol profile.

and the gender-dependent effect of DIF on CDI: −0.021 *−0.096 = 0.0202. The 95% confidence interval of this mediation effect equaled (0.0025; 0.0603), suggesting the presence of mediation.

DISCUSSION

This study prospectively investigated the influence of antenatal maternal anxiety on HPA-axis vulnerability and self-reported depressive symptoms in 58 14- and 15-year-olds. Our results showed that anxiety at 12–22 weeks of pregnancy was associated with a diurnal cortisol profile that was attenuated due to somewhat lower than normal cortisol output at awakening, but higher than expected secretion in the evening, in females as well as in males. Maternal anxiety at 23–31 and 32–40 weeks of pregnancy had no independent effect on diurnal cortisol profile. Furthermore, in female offspring only, the flattened profile was shown to mediate the link between maternal anxiety at 12–22 wp and depressive symptoms. These results were found after controlling for obstetric risk, postnatal maternal anxiety, and other potential confounders. These results confirm our two hypotheses and furthermore reveal an effect of gender (see Figure 4).

We have obtained several interesting findings, which of course need replication in larger samples before firm conclusions can be drawn.

First, in line with previous results of preclinical, retrospective, and prospective ELS studies, we find that exposure to adverse environmental cues during presumed critical periods of time in early life influence later vulnerability and health. We can conclude that our empirical evidence is consistent with the 'fetal programming'-hypothesis, which more recently has also been labeled 'developmental origins of health and disease (DOHaD)—hypothesis' (eg, Gluckman and Hanson, 2004).

Second, to our knowledge this is the first prospective study that tentatively illustrates the potential involvement of the HPA-axis in the link between antenatal maternal anxiety (or prenatal ELS) and depressive symptoms in post-pubertal, female adolescents. These results can be seen as an extension of the findings of Halligan *et al* (2006) up into the prenatal life period.

Although we cannot proof it, 'resetting of the HPA-axis setpoints' by antenatal exposure to maternal anxiety during critical periods leading to a hyperactive HPA-axis, seems a plausible underlying mechanism (cf., Levine, 2005; Weaver *et al*, 2001; Owen *et al*, 2005). A high, flattened diurnal profile has in other studies been empirically linked with inadequate suppression of awakening cortisol by overnight dexamethasone, for example, in chronically distressed (Rosmond *et al*, 1998) and in depressed adults (Young *et al*, 2006). According to Chrousos and Gold (1998), the findings of Rosmond *et al* (1998) suggest chronic hypersecretion of CRH and a reset of their HPA-axis. Earlier results of Deuschle *et al* (1997) (24-h blood sampling) lend support to this kind of interpretation of a high, flattened profile. If this interpretation holds also for the high flattened profile following prenatal ELS in our sample, our results may also be seen as a possible extension into the prenatal life period of the finding of Heim *et al* (2004) that CHR-overdrive is observed in adults who were maltreated as a child, and particularly, among adult survivors of maltreatment who have depression. Some studies have found that early postnatal ELS is associated with a low, flat cortisol profile (for a review, see Gunnar and Vazquez, 2001; Heim *et al*, 2000; Tarullo and Gunnar, 2006). Notwithstanding the fact that some adolescents had a low flat cortisol profile (ie, those with a very low DIF; see Table 1), this profile was not associated with prenatal ELS (ie, exposure to maternal anxiety) in our study.

Third, with no known biological markers available to diagnose mood and anxiety disorders at an early stage, researchers previously had to rely solely on the use of behavioral signs and other nonbiological risk and resiliency factors in the prediction (eg, Ong *et al*, 2006) or prevention of depression (eg, Beardslee and Gladstone, 2001). Our results seem to indicate that, at least in female offspring of highly anxious pregnant women, a high and flattened diurnal profile of cortisol secretion may be seen as a measure of 'predisease' pathway (McEwen, 2002) or as a vulnerability marker (Gottesman and Gould, 2003).

Fourth, although our conclusion concerning the gender effect may be limited by the small number of males with depressive symptoms, a plausible interpretation is that maternal anxiety induced gender-specific changes in the HPA-axis during critical gestation periods, which become clear in post-puberty, when the HPA-axis has reached its full maturation. This may render females more vulnerable: more sustained HPA-axis activation, a lower threshold for perceiving stress, increased vigilance, and sensitization of emotional and HPA-axis responses to subsequent stress may have enhanced the risk to develop depressive symptoms in females. Of course, it is also plausible that only during puberty females become more vulnerable to depressive symptoms because of crucial gender differences in gonadal hormones (Ge *et al*, 2006; Netherton *et al*, 2004; Kessler, 2003; Federman 2006; Goodyer *et al*, 2000; Kajantie and Phillips, 2005).

Fifth, in our sample, we did not have evidence of traumatic experiences such as maltreatment that may have interfered with the effects of intrauterine life. Results of this wave and of earlier waves of our longitudinal study are consistent with the idea of 'fetal programming', namely that antenatal maternal anxiety already before birth may have altered the neurodevelopmental program of the offspring. Maternal anxiety in early-mid (12–22 wp) as well as in late pregnancy (23–31 and 32–40 wp) were associated with fetal sleep and movements patterns observed at 36–38 weeks (Van den Bergh, 1992) and moreover, at 8/9 years 15% of anxiety symptoms, measured with a child-report standardized anxiety scale, were explained by prenatal maternal state anxiety at 12–22 wp (Van den Bergh and Marcoen, 2004). Enhanced anxiety in the child may reflect sensitization of the HPA-axis and may be seen as co-morbid symptoms (Halbreich, 2006) or a precursor of later depressive symptoms (Forbes *et al*, 2006).

Sixth, we observed that, in our sample, different periods exist during which the fetus is susceptible for programming. Effects of maternal anxiety on childhood disorders at 8–9 years, on cognitive functioning (Van den Bergh *et al*, 2005b, 2006) and on depressive mood at 14–15 years are confined to maternal anxiety at 12–22 wp, whereas those on fetal

sleep and movement patterns were not. This is consistent with the idea that developmental programming may operate at least during the whole prenatal period and that timing effects of ELS exposure may depend on the timing of critical developmental processes in underlying neural circuits and other biological system involved. In this regard, it is interesting that several gestational periods were reported to be critical for the development of ADHD in childhood (for a review, see O'Connor *et al*, 2003; Rodriguez and Bohlin, 2005; Van den Bergh and Marcoen, 2004); this may indicate that various brain systems, that each have different critical periods, are underlying different types of ADHD.

The limitations of our study necessitate some caution in interpreting its results and their implications. First, the sample was rather small and results might be sample specific; confirmation on larger samples is required. *Second*, our HPA-axis measures showed some shortcomings. Assessment of maternal HPA-axis during pregnancy and of HPA-dysregulation in the offspring at an earlier age would have enabled us to better test their assumed role in the chain of pathophysiological events. As it is known that saliva cortisol shows day-to-day variability, assessment of salivary cortisol on more than one day could have increased the data reliability (Pruessner *et al*, 1997; Wüst *et al*, 2000). We planned the saliva collection on a weekend day for reasons of feasibility. Adolescents sleep in on weekend days. Adolescents with depressed mood may sleep in more than adolescents without depressed mood. It can be questioned whether that may explain why adolescents with a depressed mood show a flatter cortisol profile. We do not think this is a plausible explanation for our data; whereas sleeping in on weekend day should mainly be related to lowering of the awakening cortisol, the flattening of the daytime profile was mainly a consequence of the elevated evening cortisol and not of lower awakening cortisol. Also, although preclinical studies (Maccari *et al*, 2003) and previous ELS studies (Heim *et al*, 2000; Gunnar and Quevedo, 2007) have observed that reactivity to a stressor may be a more sensitive measure of HPA-axis functioning than diurnal salivary cortisol, we were not able to include this measure in our study. Finally, we did not measure the adolescents' stress concurrently with their cortisol assessment, therefore, we cannot rule out its influence on cortisol (Goodyer *et al*, 2000).

Third, as the HPA-axis is connected with other biological systems (eg, monoamine neurotransmitters, immune system), which we did not measure, it would be premature to conclude that the HPA-axis is the only system involved in linking ELS to depressive symptoms (cf., O'Connor *et al*, 2005).

Fourth, our study has no genetic sensitive design, therefore, we cannot exclude an effect of genes or a gene–environment interaction nor can we explore the interesting hypothesis that prenatal adversity may 'amplify' eventually existing genetic differences (de Kloet *et al*, 2005).

To our defence, however, our study also has some strengths compared to earlier work in this area. First, it is based on a prospective design with a retention rate of 67 ($n=58$)−79% ($n=68$) over 15 years. Second, we have used well-validated questionnaires and, third, our sample covered the range of maternal anxiety and adolescent depressive symptoms scores seen in nonclinical populations and a substantial proportion scored in the higher range. Fourth, rater bias was almost excluded as a confounding factor, because the mother rated her anxiety and the adolescent his/her depressive symptoms. Fifth, we have used a solid mediation analysis in which also the moderating effect of gender was tested and obstetric risk, postnatal anxiety and other confounding factors were controlled.

In conclusion, our findings may suggest that 'developmental programming' of the HPA-axis by maternal anxiety in early-mid pregnancy leads to vulnerability, predisposing females to develop depressive symptoms after puberty, and probably earlier. Specifically, we have argued for a hyperactive HPA-axis as a putative neurobiological mechanism leading to an enhanced risk for depression. Although it is clear that our results cannot directly prove this programming of HPA-axis hyperactivity and surely need to be replicated before firm conclusions can be drawn, they corroborate results from numerous preclinical and several human ELS studies and as such underscore the growing need to consider not only postnatal ELS, but also early prenatal ELS in any (single or combined) model of HPA-axis; vulnerability; depressed mood and depression (Gunnar and Quevedo, 2007; Heim *et al*, 2004). Undoubtedly, ELS has complex, long-term and gender-specific influences on health and disease. To be able to optimize strategies for prevention and treatment, studies should pay more attention to the nature, duration and timing of pre- and postnatal ELS, to potential vulnerability markers and to the identification of possible genetic and nongenetic factors (including diverse therapeutic interventions) that may enhance or protect, buffer or delay the risk ELS bears for later health.

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DISCLOSURE/CONFLICT OF INTEREST

None of the authors has a potential conflict of interest. None of them has received a compensation for professional services in any of the previous three years, or anticipates receiving such compensation in the near future.

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