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Maternal adversities during pregnancy and cord blood oxytocin receptor (*OXTR*) DNA methylation FREE

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Abstract

The aim of this study was to investigate whether maternal adversities and cortisol levels during pregnancy predict cord blood DNA methylation of the oxytocin receptor (*OXTR*). We collected cord blood of 39 babies born to mothers participating in a cross-sectional study ($N = 100$) conducted in Basel, Switzerland (2007–10). Mothers completed the Inventory of Life Events (second trimester: T2), the Edinburgh Postnatal Depression Scale (EPDS, third trimester: T3), the Trier Inventory of Chronic Stress (TICS-K, 1–3 weeks postpartum) and provided saliva samples (T2, T3) for maternal cortisol profiles, as computed by the area under the curve with respect to ground (AUCg) or increase (AUCi) for the cortisol awakening response (CAR) and for diurnal cortisol profiles (DAY). *OXTR* DNA methylation was quantified using Sequenom EpiTYPER. The number of stressful life events ($P = 0.032$), EPDS score ($P = 0.007$) and cortisol AUCgs at T2 (CAR: $P = 0.020$; DAY: $P = 0.024$) were negatively associated with *OXTR* DNA methylation. Our findings suggest that distinct prenatal adversities predict decreased DNA methylation in a gene that is relevant for childbirth, maternal behavior and wellbeing of mother and offspring. If a reduced *OXTR* methylation increases *OXTR* expression, our findings could suggest an epigenetic adaptation to an adverse early environment.

Keywords: early life stress, epigenetics, intrauterine exposures delayed effect, intrauterine programming, psychosocial stress during pregnancy

Issue Section: Original Articles

Introduction

Maternal adverse psychosocial experiences during pregnancy are risk factors for poor birth outcome and physical and mental disorders in the offspring later in life (Kinsella and Monk, 2009; Tegethoff *et al.*, 2009, 2010, 2011a,b,, 2012; Meinlschmidt *et al.*, 2010; Meinlschmidt and Tegethoff, 2015). A recent line of evidence suggests that the association between the intrauterine environment and offspring's health could be epigenetically mediated (Monk *et al.*, 2012), for instance by changes in offspring DNA methylation (for a review see Mehler, 2008; Lutz and Turecki, 2014). To date, several intrauterine predictors of cord blood DNA methylation in human newborns have been identified, including maternal diet (Li *et al.*, 2014), maternal substance consumption (Breton *et al.*, 2009; Joubert *et al.*, 2012) and maternal mood and stress experience (Lutz and Turecki, 2014). Of note, the latter predictor was associated with offspring DNA methylation of several genes involved in stress-adaptation. For instance, maternal depressed mood during pregnancy was related to decreased cord blood DNA methylation in the serotonin transporter gene (*SLC6A4*) (Devlin *et al.*, 2010). Furthermore, maternal experience of pregnancy-related anxiety and augmented maternal cortisol levels, particularly in the second trimester, predicted increased cord blood DNA methylation at several CpG sites of the human glucocorticoid receptor gene (*NR3C1*) promoter (Hompes *et al.*, 2013). Similarly, maternal exposure to war stress, material deprivation and daily hassles during pregnancy were associated with increased cord blood DNA methylation in the *NR3C1* promoter region (Mulligan *et al.*, 2012). Studies in peripheral tissue of adolescent offspring of mothers who were exposed to adversities during pregnancy also suggest long-term epigenetic adaptations in the offspring after intrauterine exposure to maternal psychosocial adversities: In detail, experience of intimate partner violence during pregnancy was positively associated with *NR3C1* DNA methylation in adolescent offspring (Radtke *et al.*, 2011). Moreover, offspring of mothers who experienced a natural disaster during pregnancy showed objective hardship related changes in DNA methylation profiles across 957 genes mainly implicated in immune function (Cao-Lei *et al.*, 2014).

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In sum, DNA methylation of stress-related genes seems to be responsive to maternal mood and stress experience during pregnancy. However, further candidate genes should be investigated to improve our understanding of how the maternal psychosocial environment could shape the offspring's epigenome. One candidate gene is the oxytocin receptor gene (*OXTR*), which is expressed in several brain regions implicated in social behavior and bonding, as well as in various peripheral tissues (Kimura *et al.*, 2003). The oxytocinergic system is implicated in reproductive behavior, such as induction of labor and lactation, and has important function in mother–child bonding and maternal

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psychological wellbeing, including depression and anxiety (Lonstein *et al.*, 2014). Fluctuations in maternal oxytocin levels from early to mid pregnancy and the early postpartum show great variety between individuals, ranging from stable to increased or even decreased oxytocin levels during the course of pregnancy (Levine *et al.*, 2007). Intriguingly, mothers showing an increase in plasma oxytocin from the first to early third trimester reported augmented prenatal bonding compared to mothers with stable or decreasing oxytocin levels (Levine *et al.*, 2007). Moreover, higher oxytocin levels at early postpartum, as well as lower cortisol levels, predicted a higher proportion of positive maternal behaviors during a mother infant interaction (Feldman *et al.*, 2007) and might be protective against postpartum depression (Skrundz *et al.*, 2011). Oxytocin is also found in cord blood at delivery, but is likely to be secreted by the fetus and not transmitted from mother to child through the placenta (Kumaresan *et al.*, 1975). This barrier between maternal and fetal oxytocin stands in contrast to maternal corticosteroids, which might be increasingly transmitted to the child, following impairment of placental 11beta-hydroxysteroid dehydrogenase type 2 activity, induced by chronic stress (Welberg *et al.*, 2005).

Interestingly, prenatal stress is associated with changes in the oxytocin system. For instance, prenatally stressed adult male rats raised by their stress-exposed mothers not only showed impairment in social interactions but also a reduced number of oxytocin-positive neurons in the paraventricular nucleus as compared to adult male offspring born and raised by non-stressed mothers (de Souza *et al.*, 2013). Additionally, elevated activity of the oxytocinergic system is also implicated in a dampening of the stress response by interacting with the hypothalamic–pituitary–adrenal (HPA) axis and sympathetic nervous system (Light *et al.*, 2000; Neumann, 2002; Lee *et al.*, 2009; Grewen and Light, 2011).

Although the neuropeptide oxytocin has been investigated more extensively than its receptor, a handful of studies have investigated DNA methylation of the *OXTR* gene. Kusui *et al.* (2001) reported that higher DNA methylation of the *OXTR* CpG island spanning across exon I to III is associated with decreased expression of this gene. Increased DNA methylation across this CpG island has been linked to autism spectrum disorder (Gregory *et al.*, 2009), psychopathy (Dadds *et al.*, 2014) and increased brain activity during a social task (Jack *et al.*, 2012). Furthermore, we found that exposure to acute psychosocial stress was associated with a short-term increase in *OXTR* methylation, followed by a decrease during the stress recovery period (Unternaehrer *et al.*, 2012). Finally, adults reporting low maternal care during childhood had elevated levels of DNA methylation in one of two examined *OXTR* target sequences (Unternaehrer *et al.*, 2015).

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To conclude, stress-related epigenetic adaptations in the oxytocinergic system might result in changes in socio-emotional and socio-cognitive development, stress responsivity and vulnerability for mental disorders. Despite the relevance of the oxytocin system, and particularly the OXTR, for early neuronal and social development, social cognition and behavior, as well as mental health (Meyer-Lindenberg *et al.*, 2011; Brune, 2012; Kumsta *et al.*, 2013; Wade *et al.*, 2015) and potential involvement of the *OXTR* in resilient functioning (Cicchetti and Rogosch, 2012), DNA methylation of the *OXTR* has—to the best of our knowledge—not been examined in cord blood of newborns at birth.

The aim of this study was to investigate different maternal psychosocial adversities and cortisol levels during pregnancy as predictors of cord blood DNA methylation of the *OXTR*. To cover a spectrum of maternal adverse experiences during pregnancy (Nast *et al.*, 2013), we included several measures of maternal adversities: (i) life changing events during the two years prior to the second trimester and current strain experienced by those events; (ii) chronic stress experience during the course of pregnancy and (iii) maternal depressive symptoms during the third trimester. Additionally, we assessed (iv) salivary cortisol levels [cortisol awakening response (CAR) and diurnal cortisol profiles (DAY)] as an indicator of HPA axis activity. Given the potentially opposing effects of prenatal cortisol exposure dependent on the timing of exposure (Davis and Sandman, 2010), cortisol profiles were assessed during the second as well as the third trimester.

Because maternal adverse experiences during pregnancy were associated with an increase or decrease in DNA methylation depending on the investigated gene in the above-mentioned previous studies, we did not state explicit hypotheses regarding the direction of the association. Kusui *et al.* (2001) have shown that higher methylation of the *OXTR* CpG island decreased gene expression, which might be linked to increased OXTR levels. If so, a stress-related increase in *OXTR* methylation might contribute to an offspring risk phenotype, whereas a decrease would support the notion of phenotypic adaptation, as stated by the predictive adaptive response model (Gluckman *et al.*, 2005).

Materials and Methods

Participants

A sample of 100 pregnant women was enrolled in this cross-sectional study with multiple measuring time-points, which was conducted in Basel, Switzerland from 2007 to 2010. This initial sample was recruited using different strategies, including promotion at local hospitals and advertisements in local newspapers and television stations. We

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recruited pregnant women between their 21st and 32nd week of gestation. Inclusion criteria for study participation were assessed during a telephone screening, and at a personal appointment at the facilities of the University of Basel, where we conducted a structural clinical interview and applied a biomedical questionnaire. Inclusion criteria were (i) no current mental disorder; (ii) no severe medical complications; (iii) no acute or chronic physical disease, such as metabolic disease or thyroid dysfunction; (iv) no signs of fetal malformation, (v) pre-pregnancy body mass index (BMI) below 32 kg/m^2; (vi) no cigarette, alcohol or drug consumption beyond the 10th week of gestation; (vii) good knowledge of the German language. A total of four women were excluded after study enrollment due to giving birth to twins, preterm delivery or not meeting inclusion criteria, which was detected only after the clinical interview. One woman terminated study participation before delivery. Cord blood samples could not be analyzed for 56 of the remaining 95 women, due to sample unavailability or insufficient quality or quantity of the cord blood sample. A subsample of 39 women provided enough cord blood tissue for DNA methylation analysis. A flowchart of study participants is depicted in Figure 1. Participants and pregnancy characteristics ($N = 39$), birth parameters and descriptive values of predictors are shown in Table 1.

Fig. 1.

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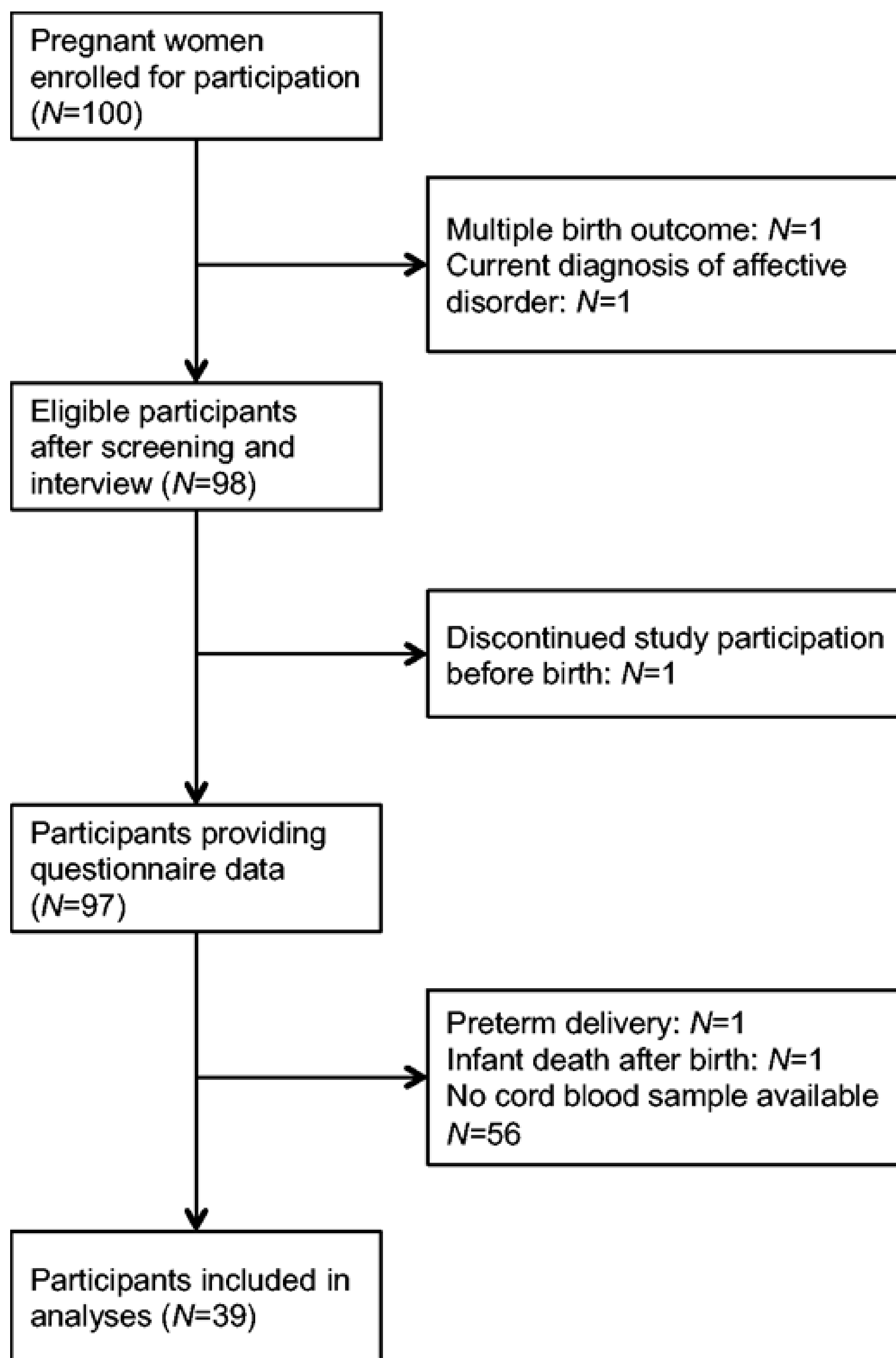

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Flowchart of study participants.

Table 1.

Characteristics of participants (N = 39), pregnancy and birth outcomes and descriptive statistics of predictors

	Mean	*SD*	Range
Maternal age (years)	31.9	3.9	24 to 40

	Mean	*SD*	Range
Education (years)	15.2	4.0	8 to 24
Pre-pregnancy BMI (kg/m^2)	22.5	3.8	16.2 to 31.2
Length of gestation (days)	277	8.2	256 to 291
Birth weight (g)	3313	430	2270 to 4440
Life changing events (ILE score)			
Number of life events	7.08	3.67	1 to 15
Total strain	14.95	8.87	2 to 35
Average strain per life event	2.05	0.47	1 to 2.8
Depressivity (EPDS score)	4.68	4.42	0 to 17
Chronic stress (TICS-K score)	34.08	11.00	16 to 60
Salivary cortisol[a]			
Second trimester CAR AUCg	15.68	4.87	8.96 to 26.79
Second trimester CAR AUCi	4.14	3.91	−2.31 to 12.41
Second trimester DAY AUCg	111.80	34.71	58.83 to 208.65
Second trimester DAY AUCi	−80.28	35.03	−161.75 to − 26.58
Third trimester CAR AUCg	18.00	5.28	9.81 to 31.17
Third trimester CAR AUCi	6.87	4.21	1.01 to 18.54
Third trimester DAY AUCg	143.16	45.63	68.62 to 315.82
Third trimester DAY AUCi	−56.78	55.60	−133.59 to 99.23
	N	%	
Household income rating[b]			
‘Income is not enough’	0	0	

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	Mean	*SD*	Range
'Income is just enough'	6	17	
'Income is good to live with'	29	83	
Parity[c]			
First	25	66	
Second	11	29	
Third (or more)	2	5	
Delivery mode			
Vaginal	24	63	
Caesarean section	14	37	

AUCg, area under the curve with respect to ground; AUCi, area under the curve with respect to increase; CAR, cortisol awakening response (nmol/l*hours); DAY, diurnal cortisol profile (nmol/l*hours); EPDS, Edinburgh Postnatal Depression Scale; ILE, Inventory of Life Events; SD, standard deviation; TICS-K, Trier Inventory of Chronic Stress - Short Version.

[a] measurement units for CAR (nmol/l*hours) and for DAY (nmol/l*hours).

[b] Missing data ($n = 4$).

[c] Missing data ($n = 1$).

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Procedure

At gestational weeks 21–32, participants completed a telephone screening to assess inclusion criteria and underwent the standardized DIA-X Munich-Composite International Diagnostic Interview (M-CIDI) (Wittchen and Pfister, 1997; Reed *et al.*, 1998; Wittchen *et al.*, 1998). Women who fulfilled inclusion criteria completed questionnaires on stressful life events during the past 2 years (Inventory of Life Events, ILE) (Siegrist and Geyer, 2003) at gestational weeks 21–24 or at the earliest time point possible. Additionally, they provided saliva samples to assess CAR and DAY during the second trimester (T2). During gestational weeks 32–34, they completed a questionnaire on depressive symptoms during the past 7 days (Edinburgh Postnatal Depression Scale, EPDS) (Cox *et al.*, 1987; Bergant *et al.*, 1998) and provided saliva samples to assess CAR

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and DAY during the third trimester (T3). At birth, cord blood samples were collected for DNA methylation analysis. One to three weeks postpartum, the mothers were asked to complete a questionnaire on chronic stress experience during the course of their pregnancy (Trier Inventory of Chronic Stress—Short Version, TICS-K) (Schlotz and Schulz, 2005). The local ethics committee (Ethikkomission beider Basel) approved the study protocol, which was carried out in accordance with the latest version of the Declaration of Helsinki. All participants gave written informed consent after explanation of all study procedures.

Questionnaires

The ILE (Siegrist and Geyer, 2003) assessed number of life-changing events during the last 2 years, such as being a victim or witness of assault, severe illness or death of a loved one. A total of 32 life events were listed, including two open questions. Additionally, the ILE measured current strain caused by the respective life event on a 4-point Likert scale ranging from 1 to 4, whereby a high score indicated high levels of strain. The questionnaire provided information on three scales: (i) number of life events (ILE life events); (ii) total strain caused by all experienced life events (ILE total strain) and (iii) average strain per experienced life event (ILE average strain per event). Reliability and criterion validity of the ILE are considered sufficient (Siegrist and Geyer, 2003).

The EPDS (Cox *et al.*, 1987; Bergant *et al.*, 1998) is a 10-item scale, which assessed maternal depressive symptoms. Respondents had to indicate their mood during the last 7 days, such as experience of joy, anxiety or sadness. Answers were given on a 4-point Likert scale ranging from 0 to 3, with higher scores on the EPDS indicating higher levels of depressive symptoms. The EPDS was identified as gold standard to assess maternal depressive symptoms during pregnancy, due to its high reliability and validity coefficients and the exclusion of somatic symptoms overlapping with pregnancy-related somatic changes (Bergant *et al.*, 1998).

The short version of the TICS (Schlotz and Schulz, 2005) consists of 30 items assessing chronic stress in different areas of everyday life. Women rated the amount of stress they experienced during the course of their pregnancy on a 5-point Likert scale ranging from 0 (‘I never made this experience’) to 4 (‘I made this experience very often’). The subscales of the TICS-K included work and social overload, excessive work demands, lack of social recognition, work discontent, social tensions and isolation, pressure to perform and in communication, and chronic worrying. The TICS scales possess good to very good reliability and sufficient validity (Schlotz and Schulz, 1999, 2005). For the statistical analyses, we calculated an overall sum score.

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We collected sociodemographic information, including maternal age (in years), education (in years) and family income rating (categories: 'income is not enough for living', 'income is just enough for living', 'income is good to live with') using a sociodemographics interview. Information on pre-pregnancy BMI, parity and birth outcomes (length of gestation, delivery mode, birth weight) was collected from medical records and a biomedical questionnaire.

Salivary cortisol

Women were requested to collect saliva six times a day during two consecutive workdays during the second and third trimester using salivettes (Sarstedt, Nuembrecht, Germany). Samples were collected immediately after awakening, 30, 45 and 60 min after awakening, at 1500 h and at 2000 h. We asked mothers to report sampling time and excluded samples from the analyses if they were collected outside the following time frames: (i) ±15 min for the first three samples (T2: two samples excluded; T3: four samples excluded); (ii) ±30 min for the fourth sample (T2: four samples excluded; T3: two samples excluded) and (iii) ±60 min for the fifth and sixth sample (no samples excluded). We applied more stringent exclusion criteria for the earlier samples, because cortisol levels rapidly increase after awakening and then slowly decline during the day (e.g. Edwards *et al.*, 2001). In the following, valid samples refer to samples provided and collected within the defined time frame. After collection, saliva samples were kept refrigerated until delivery, upon which they were stored at −20 °C until further analysis. Saliva samples were centrifuged at 2000 × *g* for 6 min before free salivary cortisol levels were measured using a time-resolved fluorescence immunoassay with fluormetric detection (DELFIA). All intra- and inter-assay coefficients of variation were below 10%.

To analyse the CAR within the first hour of awakening (samples one to four) and DAY (samples one to six), we calculated the area under the curve with respect to ground (AUCg) and increase (AUCi) to obtain information on total hormonal output and HPA axis sensitivity (Pruessner *et al.*, 2003). AUC values were calculated for the two consecutive days separately and were then averaged to obtain indicators (AUCg and AUCi) for CAR (nmol/l*hours) and DAY (nmol/l*hours) for T2 as well as for T3. In the case of participants providing a complete set of valid samples (CAR: first four samples; DAY: all samples) for one of the two collection days only, the AUC calculated for the day with the complete set of valid samples was used for further statistical analyses. Nine and four participants did not provide any saliva sample on any of the 2 days at T2 or T3, respectively. Among those mothers providing at least one valid sample at T2, 25 mothers provided a complete set of valid samples to calculate the CAR AUCs on both days, three mothers on 1 day, and two mothers did not provide a complete set of valid samples on any of the 2 days. For DAY AUCs at T2, 24 mothers provided a complete set of valid

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samples on both days, two mothers on 1 day, and four mothers did not provide a complete set of valid samples on any of the 2 days. For CAR AUCs at T3, 31 mothers provided a complete set of valid samples on both days, three mothers on 1 day, and one mother did not provide a complete set of valid samples on any of the 2 days. For DAY AUCs at T3, 28 mothers provided a complete set of valid samples on both days, five mothers on 1 day, and two mothers did not provide a complete set of valid samples on any of the 2 days. Cortisol concentrations for the valid samples ranged from 0.85 to 54.27 nmol/l. Samples available for analysis, descriptive statistics of cortisol concentrations, and average sampling time per time-point for the valid samples are depicted in Supplementary Table 1 (S1).

Cord blood sample preparation and DNA methylation analysis

Cord blood was collected by medical staff immediately after birth using 2.7 ml S-Monovette (Sarstedt, Nuembrecht, Germany). Samples were centrifuged at room temperature at 1650 × *g* for 10 min. DNA was extracted from whole blood using the standard protocol of the Gentra Puregene Cell Kit (QIAGEN, Hilden, Germany) and stored at −20 °C until further analyses. Five hundred forty nanograms of genomic DNA was bisulfite converted using EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA), according to manufacturer's protocol. Using the Sequenom EpiDesigner software, the *OXTR* target sequence (chromosome 3, p25, nt 8 809 275–8 809 534; Figure 2) was designed to be located in the protein-coding region of *OXTR* exon III, which is part of a CpG island spanning across exons I to III and which was previously described to be associated with transcriptional regulation (Kusui *et al.*, 2001). Bisulfite PCR amplification of the target sequence was conducted using Hot Star *Taq* DNA polymerase (QIAGEN, Hilden, Germany). We quantified DNA methylation (%5meC) using EpiTYPER 1.0 (Sequenom Inc., San Diego, CA). For each run, we included a fully methylated positive control (New England BioLabs Inc., Ipswich, MA) and a blank control (distilled water). Resolution of Sequenom EpiTYPER yielded 10 individual CpG units including a total of 22 CpG sites (Figure 2). Two CpG units (CpG unit 3 and CpG unit 6) could not be analysed due to high mass detection limit. CpG unit 4 (CpG site 9) was excluded because data were available for <10% of the participants.

Fig. 2.

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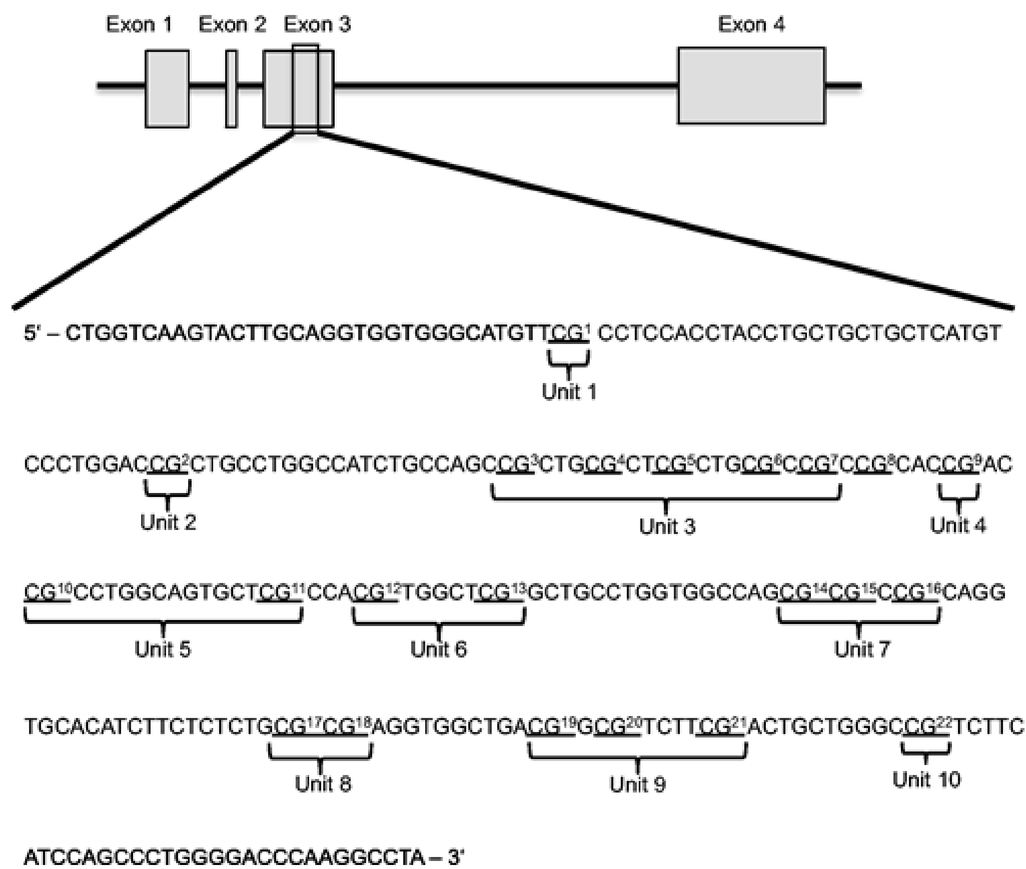

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Schematic view of the oxytocin receptor gene (*OXTR*). CpG sites within the target sequence are underlined and numbered consecutively. Unit 1 to 10 indicate the CpG units based on resolution yielded by the Sequenom EpiTYPER.

Statistical analyses

Data preparation included inspection for assumption of normality distribution (qq-plots, histograms, boxplots, Kolmogorov–Smirnov test) of all variables. The sum score on the EPDS and DAY AUCg at T3 were the only variables with indications of non-normal distribution and were therefore transformed using natural logarithm.

For all statistical analyses on DNA methylation we used linear mixed models, with CpG unit as within subject factor. We specified an unstructured variance-covariance matrix for the within-subject factor as this pattern led to the best model fit, based on the Akaike information criterion (AIC). Each model contained batch number as covariate to statistically adjust for measurement bias due to batch effects. Then, each maternal predictor of adversity (ILE, EPDS, TICS-K, salivary CAR and DAY AUCg and AUCi at T2 and T3) was analysed in a separate model to avoid model over-fitting. In order to obtain information about possible confounders or mediators in the association between maternal predictors and DNA methylation, we examined sociodemographic data, pregnancy characteristics and birth outcome (see Questionnaires section) (Schlinzig *et al.*, 2009; Hoyo *et al.*, 2012; Turan *et al.*, 2012). To assess the effect of a specific predictor

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we used the Log-likelihood-Ratio (L-Ratio) test, comparing the mixed model including the respective predictor with the corresponding model excluding it. In addition to the TICS-K overall sum score we also analysed its 10 subscales, thereby adjusting for multiple testing using the Bonferroni–Holm method (Holm, 1979).

To test whether the subsample of women who provided cord blood for DNA methylation analysis *vs* those who did not provide these samples differed with regard to sociodemographic data, pregnancy characteristics or birth outcomes, we used *t*-tests, Whitney U-test or χ^2-test according to data characteristics. The descriptive statistics and subsample comparisons were conducted using IBM SPSS 20. All mixed model analyses were performed using R version 3.0.1 (16 May 2013) (R Core Team, 2015) using the gls function of the nlme package (Pinheiro *et al.*, 2015). A P value <0.05 was considered as statistically significant.

Results

The ILE score for number of life events (L-Ratio = 4.606; P = 0.032), EPDS sum score (L-Ratio = 7.183; P = 0.007), and CAR AUCg at T2 (L-Ratio = 5.454; P = 0.020) and DAY AUCg at T2 (L-Ratio = 5.125; P = 0.024) were all negatively associated with cord blood DNA methylation, whereas the ILE scores for total strain (L-Ratio = 2.514; P = 0.113), strain per life event (L-Ratio = 1.267; P = 0.260), sum score on the TICS-K (L-Ratio = 0.135; P = 0.713), T2 CAR AUCi (L-Ratio = 3.637; P = 0.057), T2 DAY AUCi (L-Ratio = 0.314; P = 0.575) and all T3 AUCs (CAR AUCg: L-Ratio = 0.082; P = 0.775; CAR AUCi: L-Ratio = 1.330; P = 0.249; DAY AUCg: L-Ratio = 2.224; P = 0.136; DAY AUCi: L-Ratio = 0.613; P = 0.434) were not (Table 2). Of the 10 subscales of the TICS-K, only the subscale assessing social isolation was negatively associated with cord blood DNA methylation (L-Ratio = 4.181; P = 0.041). However, this result was not significant after correction for multiple testing for the 10 TICS-K subscales.

Table 2.

Results from mixed model analysis (N = 39)

	Model parameters				Parameter estimates	
Predictors	***AIC***	***df***	**L-Ratio**	***P***	**Estimate**	***SE***
Model without predictors	1189	36				

	Model parameters				Parameter estimates	
Predictors	***AIC***	***df***	**L-Ratio**	***P***	**Estimate**	***SE***
Life changing events (ILE score)						
Number of life events	1134	37	4.606	0.032	−1.48E-01	6.03E-02
Total strain	1136	37	2.514	0.113	−4.69E-02	2.64E-02
Average strain per life event	1138	37	1.267	0.260	6.51E-01	5.05E-01
Depressivity (EPDS score)[a]	1112	37	7.183	0.007	−7.09E-01	2.37E-01
Chronic stress (TICS-K score)	1095	37	0.135	0.713	−7.62E-03	2.02E-02
Salivary cortisol						
Second trimester CAR AUCg	863	37	5.454	0.020	−1.08E-01	4.02E-02
Second trimester CAR AUCi	865	37	3.637	0.057	−1.18E-01	5.31E-02
Second trimester DAY AUCg	799	37	5.125	0.024	−1.56E-02	5.99E-03
Second trimester DAY AUCi	803	37	0.314	0.575	4.22E-03	5.46E-01
Third trimester CAR AUCg	1030	37	0.082	0.775	−1.36E-02	4.39E-02
Third trimester CAR AUCi	1029	37	1.330	0.249	7.23E-02	5.29E-02
Third trimester DAY AUCg[a]	1003	37	2.224	0.136	−1.31E+00	7.87E-01
Third trimester DAY AUCi	1004	37	0.613	0.434	−3.50E-03	4.21E-03

Note: Model parameters and estimates from mixed model analysis ($N = 39$) calculated for each predictor of *OXTR* DNA methylation. Shown is goodness of model fit (AIC), degrees of freedom of the model (*df*), L-Ratio between model with and without the predictor of interest and *P* value of model comparison. Parameter estimates indicate the direction and estimated value with standard error (*SE*) of the predictor. AIC, Akaike Information Criterion; AUCg, area under the curve with respect to ground; AUCi, area under the curve with respect to increase; CAR, cortisol awakening response (nmol/l*hours); DAY, diurnal cortisol levels (nmol/l*hours); df, degrees of freedom; EPDS, Edinburgh Postnatal Depression Scale; ILE, Inventory of Life Events; L-Ratio, Loglikelihood Ratio; SE, standard error of the estimate; TICS-K, Trier Inventory of Chronic Stress-Short Version.

[a] Transformed using natural logarithm; quantity without dimensions.

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None of the potential confounding (e.g. sociodemographic factors) or mediating factors (e.g. birth outcomes) listed in Table 1 were associated with *OXTR* DNA methylation. Although prepregnancy BMI (Sharp *et al.*, 2015), offspring birth weight (Sharp *et al.*, 2015) and caesarean section (Schlinzig *et al.*, 2009; Almgren *et al.*, 2014; Franz *et al.*, 2014) have been linked to DNA methylation previously, we could not confirm an association with *OXTR* methylation (pre-pregnancy BMI: L-Ratio = 2.922; $P = 0.087$; birth weight: L-Ratio = 0.686; $P = 0.407$; caesarean section: L-Ratio = 0.001; $P = 0.972$). Therefore, we did not investigate these factors further and did not include any of them in the primary analyses.

Coefficients of inter-correlation between the predictors are shown in Supplementary Table 2 (S2). The numbers of participants indicating a specific life event on the ILE are illustrated in Supplementary Figure 1 (S3). Graphical illustration of mean scores and standard deviations for the TICS-K subscales are shown in Supplementary Figure 2 (S4).

Differences between subsets of participants providing *vs* those not providing cord blood for DNA methylation analysis were not statistically significant with regard to birth weight ($t(88)=-0.553$, $P = 0.582$), sociodemographic variables (maternal age: $t(95)=-1.860$, $P = 0.066$; maternal education: $U=-0.163$, $P = 0.870$; household income rating: $U=-1.095$, $P = 0.274$), length of gestation: $t(89)=-0.462$, $P = 0.645$), pre-pregnancy BMI ($t(87)=-0.415$, $P = 0.679$), gender of the child ($\chi(1)=0.710$, $P = 0.836$), scores on the maternal adversity questionnaires (ILE number of events: $t(94)=0.937$, $P = 0.051$; ILE total strain: $t(92)=1.374$, $P = 0.173$; ILE average strain per event: $t(92)=-0.100$, $P = 0.921$, EPDS: $t(87)=1.672$, $P = 0.098$); TICS-K sum: $t(84)=1.470$, $P = 0.145$) and cortisol profiles at T2 (CAR AUCg: $t(72)=0.459$, $P = 0.648$; CAR AUCi: $t(72)=0.464$, $P = 0.644$; DAY AUCg: $t(70)=0.308$, $P = 0.759$; DAY AUCi: $t(70)=0.115$, $P = 0.909$). However, we found that those providing cord blood samples generally showed lower cortisol levels at T3 (CAR AUCg: $t(78)=-2.618$, $P = 0.011$; CAR AUCi: $t(78)=-2.901$, $P = 0.005$; DAY AUCg: $t(78)=-2.585$, $P = 0.012$; DAY AUCi: $t(78)=-0.920$, $P = 0.360$) as compared to those not providing cord blood samples.

Discussion

The aim of this study was to investigate maternal adversities as predictors of *OXTR* cord blood DNA methylation. Total number of stressful life events up to 2 years prior to the second pregnancy trimester, but not the strain caused by these events at the time of assessment predicted cord blood *OXTR* DNA methylation at birth. Increased total

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hormonal output (AUCg) related to the CAR and the diurnal profile during the second trimester and maternal depressive symptoms were associated with decreased cord blood *OXTR* DNA methylation. At the same time, there was no statistically significant association between chronic stress during the course of pregnancy and changes in cortisol profiles (AUCi) during the second trimester or any of the cortisol AUCs during the third trimester with cord blood *OXTR* DNA methylation. The result that some, but not all indicators of adversities predicted *OXTR* methylation are intriguing, and could indicate that specific adversities might be more relevant for adaptations in DNA methylation of *OXTR* in cord blood as compared with others. Some of these adversity-specific effects will be discussed in more detail in the respective section below.

The observed epigenetic changes we report here are in line with the predictive adaptive response model (Gluckman *et al.*, 2005): If reduced levels in *OXTR* methylation relate to increased expression levels (Kusui *et al.*, 2001) and elevated OXTR abundance, we speculate that this epigenetic adaptation might compensate some of the effects of maternal adversities during pregnancy and postpartum. While several studies indicated that oxytocin receptor expression and OXTR binding is elevated in several brain areas during parturition and breastfeeding and linked to reduced anxiety and depression in the mother (Lonstein *et al.*, 2014), oxytocin signaling in offspring is less well studied. Intriguingly, rodent studies suggest that exposure to prenatal and postnatal maternal stress decreased the number of oxytocin positive cells in adult offspring as compared with offspring from non-stress exposed mothers (de Souza *et al.*, 2013). Furthermore, prairie vole neonates injected with oxytocin display higher parental behavior, facilitated partner bonding and decreased corticosterone after a swim test later in life, as compared to animals injected with an oxytocin antagonist (Carter, 2003). Finally, female rat offspring receiving high levels of maternal licking and grooming showed elevated oxytocin receptor binding in the amygdala and stria terminalis (Francis *et al.*, 2002).

Taken together, based on our findings and results from the animal and human literature as well as the predictive adaptive response model, we hypothesize that maternal adversities predict not only an adverse future environment, but also reduced levels of maternal caring behavior. These cues are transmitted to the developing fetus, e.g. through corticosteroids through the placenta or dietary compounds, inducing processes of adaptation of the developing child. Our results suggest that these adaptations include reduced offspring *OXTR* methylation, potentially relating to higher OXTR availability and oxytocin sensitivity. This in turn might facilitate social bonding and reduced stress reactivity. However, these hypotheses remain rather speculative. Furthermore, since our sample consisted of healthy mothers with mostly none to mild levels of stress and symptoms of depression, the results have to be interpreted carefully and cannot be translated to traumatic stress exposure or a clinically depressed population. Finally, it is

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unclear how DNA methylation in cord blood relates to methylation in neuronal or peripheral tissues in the newborn.

Maternal stress and *OXTR* methylation in cord blood

The absolute number of maternal critical life events before the second trimester—rather than event-related strain that women experienced or chronic stress during pregnancy—predicted *OXTR* DNA methylation (Table 2). Previous studies imply that maternal exposure to stressful life events even prior to conception increase the risk for infant mortality and low birth weight (Class *et al.*, 2013; Witt *et al.*, 2014). The finding that life changing events prior to the second trimester, but not stress experience during pregnancy *per se* predicted cord blood *OXTR* DNA methylation are in line with evidence that stressful life events during pregnancy, such as war-related stressors or exposure to natural disaster, might be a stronger predictor of DNA methylation status in the offspring as compared to more chronic stressors or subjectively perceived stress (Mulligan *et al.*, 2012; Cao-Lei *et al.*, 2014). Therefore, our findings extend these results by indicating a stronger relevance of life events as compared with chronic stress for *OXTR* DNA methylation in cord blood. However, in contrast to the two studies examining war stress and exposure to a natural disaster, the stressors reported in our study are not *per se* traumatic. For instance, the three most frequent life events included change in close co-workers, change in living situation or disappointment/insult by a close person (Supplementary Figure 1). Thus, the reported life events might better be described as 'challenges' as compared with life-threatening situations. Nevertheless, on the one hand we see changes in DNA methylation associated with these non-traumatic events. On the other hand, the reduced severity could be a reason why we observed a stress-related reduction in *OXTR* methylation as compared with increased DNA methylation often observed after more severe forms of stress exposure. Future studies in pregnant women exposed to more severe events could answer whether severe stressful life events might have similar or opposite effects, as compared to our findings.

Maternal depressive symptoms and *OXTR* methylation in cord blood

More symptoms of maternal depression were associated with lower *OXTR* DNA methylation in cord blood (Table 2). Therefore, DNA methylation of *OXTR* seems to be sensitive to maternal depressed mood. This result extends previous studies showing that maternal depressive symptoms predicted decreased *SLC6A4* and increased *NR3C1* cord blood DNA methylation (Oberlander *et al.*, 2008; Devlin *et al.*, 2010). With regard to these bidirectional changes in DNA methylation—increase in *NR3C1* and decrease in *SLC6A4* and *OXTR* DNA methylation after depressive symptoms during pregnancy—we speculate

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that offspring DNA methylation might be sensitive to maternal mood during pregnancy in a gene-specific manner.

Oxytocin signaling has important functions for bonding between mother and child and vice versa (Nissen *et al.*, 1995; Light *et al.*, 2000; Wismer Fries *et al.*, 2005) and seems impaired in depressed or anxious mothers (Francis *et al.*, 2000; Feldman *et al.*, 2007). Thus, we hypothesize that *OXTR* DNA methylation could be considered an adaptive molecular mechanism, by which offspring with decreased DNA methylation in *OXTR* can regulate expression of this gene more flexibly. This molecular adaptation might facilitate oxytocin signaling in an environment with potentially restricted maternal care due to maternal depressive symptoms (Lovejoy *et al.*, 2000). Interestingly, a previous article by our group based on data from the same study found that decreased maternal oxytocin levels in the third trimester predicted an increased risk to develop postpartum depression (Skrundz *et al.*, 2011).

Maternal cortisol levels and *OXTR* methylation in cord blood

A higher maternal total hormonal output during the second (CAR and DAY) but not during the third trimester was associated with decreased *OXTR* DNA methylation. Thus, the results suggest two distinct phenomena: First, at T2, the AUCgs, an indicator of overall cortisol intensity (Pruessner *et al.*, 2003), predicts cord blood *OXTR* DNA methylation, rather than the AUCis, which contain information on changes over time and HPA sensitivity (Table 2). This might be related to the permeability of the placenta for corticosteroids, which might be more related to the total hormone level rather than changes during the day. Second, the described associations were limited to the second trimester, indicative of a sensitive period during the second, but not third trimester. This finding is in line with previous studies indicating that cortisol during earlier gestation had more pronounced effects on the fetus (Buss *et al.*, 2012; Sandman *et al.*, 2012). Thus, we speculate that if total cortisol levels reach a certain height during the second trimester, this might serve as an intrauterine signal for an anticipated challenging extrauterine environment (Duthie and Reynolds, 2013), in which improved accessibility of the *OXTR* gene for transcription might represent a beneficial mechanism for stress-adaptation. Interestingly, this study examines the association between maternal cortisol levels and offspring DNA methylation of a gene, which is not directly but indirectly associated with the HPA axis, specifically by interacting with the HPA axis to dampen the stress response (Neumann, 2002). Therefore, epigenetic modifications in genes of the oxytocinergic system might represent an adaptive mechanism in offspring exposed to increased maternal cortisol levels.

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In short, our findings are in line with the assumptions of the predictive–adaptive response model. This model states that a developing organism adapts to the future environment by using cues from the current environment, providing the organism with an evolutionary advantage (Gluckman *et al.*, 2005). We speculate that relevant experiences of an adverse environment of the mother before and during pregnancy are signaled to the unborn child (D'Anna-Hernandez *et al.*, 2012; Li *et al.*, 2012; Hompes *et al.*, 2013; Reynolds *et al.*, 2013). Decreased DNA methylation in the *OXTR* could result in changes in *OXTR* accessibility for transcription (Kusui *et al.*, 2001). If this resulted in facilitated oxytocin signaling and thus increased dampening of the stress response, it might be advantageous in a stressful environment. Hence, we hypothesize that the unborn child might get prepared for a potentially challenging environment by an epigenetic adaptation of the *OXTR*.

Strengths, limitations and implications for future studies

This study has several strengths. First, cord blood is an available target tissue providing information about an organism right at birth. At this stage, there was no direct postnatal exposure to environmental factors potentially affecting DNA methylation, such as offspring nutritional intake or psychosocial experiences. Second, we used statistical mixed model analysis, which was defined according to the patterns in DNA methylation across the CpG units. Using these models, we were able to account for different structures in variances and covariances between CpG units, which might give more reliable results as compared with statistical models investigating DNA methylation values averaged across a given target sequence. Last, we took a broader view on prenatal adversities by applying multiple instruments assessing different forms of maternal adversities, including maternal depressive symptoms and included CAR and diurnal cortisol levels as indicators of HPA axis activity at two different trimesters. Moreover, we did not only focus on stress experience during pregnancy, but also on periconceptional stressors.

The study has several limitations: First, the results of this study should be interpreted with caution unless replicated in a larger sample. However, our subsample did not differ from the total sample with respect to sociodemographic data, pregnancy characteristics, general birth outcome or the predictor variables, except for cortisol AUCs during the third trimester. The observed higher cortisol levels in participants not providing cord blood samples could indicate higher levels of stress at the end of pregnancy, which might have affected the mother’s decision not to provide cord blood. Second, the
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functional relevance of the assessed target sequence should be examined in future research, as we did not assess OXTR expression or protein levels. Third, due to the small amount of cord blood available, we were not able to determine blood cell count and

could therefore not statistically take into account this potential mediator (Adalsteinsson *et al.*, 2012), which should be considered in future studies. Fourth, some of the assessed parameters of maternal adversities were inter-correlated (Supplementary Table 2), suggesting that some predictors were not independent from each other. Fifth, as we recruited pregnant women not before the 21st week of gestation, we were unfortunately not able to collect data on psychosocial stress during the first trimester. Last, despite multiple measuring time-points, our study design does not allow conclusions about causality. Moreover, our results are not generalizable to pregnant women with different socioeconomic characteristics, experience of severe traumatization, suffering from physical or mental disorders, women having birth complications or multiple birth outcomes.

Conclusion

In conclusion, increased maternal stress experience, depressive symptoms and cortisol total hormonal output in the morning and across the day during the second trimester predicted decreased cord blood DNA methylation in an *OXTR* target sequence. Our data provide first evidence that an adverse maternal environment increases the accessibility of the *OXTR*. Activity of this gene potentially facilitates social bonding and stress adaptation and could therefore provide a mechanism by which the offspring adapts to a potentially challenging environment. However, not all indicators of stress yielded statistically significant relations with cord blood *OXTR* methylation, suggesting that specific maternal stress experiences or stress at specific times during pregnancy might be especially relevant for adaptations in cord blood *OXTR* methylation. If our findings are replicated in a larger sample of mother–child dyads, the results could provide information on molecular mechanisms underlying the association between maternal adversities during pregnancy and physical and mental health of the offspring.

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