Psychosocial Stress Experience and DNA Methylation in Humans – Implications for Stress-Adaptation and -Resilience

Inaugural Dissertation

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by

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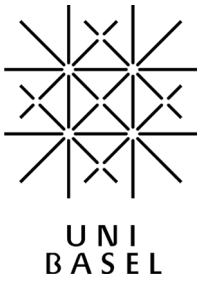

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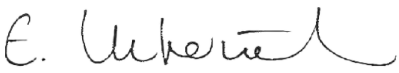
Declaration of Authorship

I hereby declare that I have written the submitted doctoral thesis „Psychosocial Stress Experience and DNA Methylation – Implications for Stress-Adaptation and Resilience“ without any assistance from third parties not indicated. Furthermore, I confirm that no other sources have been used in the preparation and writing of this thesis other than those indicated.

Place and date: Basel, 4.2.2014

Eva Unternaehrer: 

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Abbreviations

AMY	amygdala
AUCg	area under the curve with respect to ground
AUCi	area under the curve with respect to increase
BDNF	brain-derived neurotrophic factor (human gene nomenclature: *BDNF*)
CAR	cortisol awakening response
CpG	cytosine-guanine di-nucleotide
DAY	diurnal cortisol profile
DNA	deoxyribonucleic acid
EPDS	Edinburgh Postnatal Depression Scale
GR	glucocorticoid receptor (human gene nomenclature: *NR3C1*)
HC	hippocampus
HPA axis	hypothalamic-pituitary-adrenal-axis
HPG axis	hypothalamic-pituitary-gonadal-axis
ILE	Inventory of Life Events
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
mRNA	messenger ribonucleic acid
NGFI-A	nerve growth factor-induced protein A
OXTR	oxytocin receptor (human gene nomenclature: *OXTR*)
$OXTR_1$	oxytocin receptor gene target sequence 1
$OXTR_2$	oxytocin receptor gene target sequence 2
PBC	peripheral blood cells
PBI	Parental Bonding Instrument
PCR	polymerase chain reaction
PFC	prefrontal cortex
TICS-K	Trier Inventory of Chronic Stress – Short Form
TSST	Trier Social Stress Test

Abstract in English

Background: Psychosocial stress, especially early in life, is a risk factor for mental disorders. Recent evidence suggests that stress-related changes in epigenetic patterns, including DNA methylation, could mediate this association.

Aim: to examine a potential association between psychosocial stress exposure and DNA methylation of two stress-related genes: the oxytocin receptor (*OXTR*) and the brain-derived neurotrophic factor (*BDNF*).

Methods: We investigated DNA methylation in three target sequences: $OXTR_1$, $OXTR_2$ and *BDNF*. The psychosocial stressors included: (1) maternal stress during pregnancy (prenatal stress, N=39); (2) low versus high maternal care during childhood (maternal care, N=85) and (3) acute psychosocial stress (N=83). In the prenatal stress study, DNA methylation of $OXTR_1$ was quantified in cord-blood cells. In the maternal care and acute psychosocial stress study, DNA methylation of $OXTR_1$, $OXTR_2$ and *BDNF* was quantified in peripheral blood cells of adults.

Results: (1) Several indicators of increased prenatal stress predicted higher DNA methylation of $OXTR_1$. (2) Adults reporting low maternal care showed increased $OXTR_2$ DNA methylation compared to those reporting high maternal care. (3) Exposure to acute psychosocial stress was associated with dynamic changes in DNA methylation of *OXTR* – DNA methylation increased from pre- to post-stress in $OXTR_1$ and decreased from post-stress to follow up in $OXTR_1$ and $OXTR_2$. Some of these changes might have been due to variations in blood cell count.

Discussion: Exposure to psychosocial stress was associated with target sequence-specific changes in *OXTR* DNA methylation. These results could contribute to our understanding of epigenetic processes involved in stress-adaptation.

Abstract in German

Hintergrund: Psychosozialer Stress, insbesondere während der frühen Entwicklung, ist ein Risikofaktor für psychische Erkrankungen. Dieser Zusammenhang könnte durch stress-assoziierte epigenetische Veränderungen, z.B. in der DNA Methylierung, mediiert werden.

Ziel: ein potentieller Zusammenhang zwischen verschiedenen psychosozialen Stressoren und der DNA Methylierung zweier stress-assoziierter Gene zu untersuchen: dem Oxytozin Rezeptor (*OXTR*) und dem Brain-Derived Neurotrophic Factor (*BDNF*).

Methode: DNA Methylierung wurde in drei DNA Zielsequenzen gemessen: $OXTR_1$, $OXTR_2$ und *BDNF*. Die untersuchten psychosozialen Stressoren waren: (1) mütterlicher Stress während der Schwangerschaft (pränataler Stress, N=39); (2) mütterliche Zuwendung in der Kindheit (N=85) und (3) akuter psychosozialer Stress im Erwachsenenalter (N=83). In der Studie zu pränatalem Stress wurde DNA Methylierung von $OXTR_1$ im Nabelschnurblut gemessen; in den Studien zu mütterlicher Zuwendung und akutem psychosoziale Stress wurde DNA Methylierung von $OXTR_1$, $OXTR_2$ und *BDNF* in peripherem Blut gemessen.

Resultate: (1) Mehrere Indikatoren von pränatalem Stress sagten eine stärkere $OXTR_1$ DNA Methylierung vorher. (2) Erwachsene, welche von wenig mütterlicher Zuwendung berichteten, hatten eine stärkere Methylierung in $OXTR_2$ im Vergleich zu denjenigen mit mehr Zuwendung. (3) Akuter psychosozialer Stress war mit dynamischen Veränderungen in *OXTR* DNA Methylierung assoziiert: eine Erhöhung von Prä-Stress zu Post-Stress in $OXTR_1$ und eine Erniedrigung von Post-Stress zu Follow-Up in $OXTR_1$ und $OXTR_2$, wobei einige dieser Veränderungen allenfalls durch Variationen in der Blutzell-Verteilung zustande kamen.

Diskussion: Psychosozialer Stress war assoziiert mit Veränderungen in der DNA Methylierung des *OXTR*. Die Resultate könnten zu einem besseren Verständnis von epigenetischen Stress-Adaptionsmechanismen beitragen.

"Adaptability is probably the most distinctive characteristic of life."

Hans Selye (1956)

Introduction

Mental health problems are accounting for a high proportion of the social and financial burden worldwide (Kessler & Ustun, 2011; Schuler & Burla, 2012; Wittchen et al., 2011). In the latest Swiss Health Observatory survey, 17% of the general Swiss population reported to suffer from at least moderate psychological strain, and the overall financial costs caused by psychological problems added up to around 11 billion Swiss Francs (Schuler & Burla, 2012). A major factor contributing to the diathesis of mental disorders is exposure to chronic or severe stress, especially during early development (Carr, Martins, Stingel, Lemgruber, & Juruena, 2013; Lupien, McEwen, Gunnar, & Heim, 2009; Schlotz & Phillips, 2009; Scott, 2012). This association is illustrated by the National Comorbidity Survey Replication, in which early adverse experiences accounted for 44.6% of childhood-onset disorders and for around 30% of later-onset mental disorders (Green et al., 2010).

The effects of psychosocial stress experiences can be observed as early as during fetal development: intrauterine exposure to psychosocial stress experiences of the mother during pregnancy affects birth outcome and offspring behavior and health later in life (Entringer, Buss, & Wadhwa, 2010; Schlotz & Phillips, 2009). During childhood, adverse psychosocial experiences – such as interpersonal loss, family difficulties, abuse or neglect, and physical or economic adversity and disaster – might exceed the child's coping resources and induce a continued state of stress (Green et al., 2010). The relationship between early psychosocial stress and mental health is most likely mediated by developmental (mal-) adaptations in i) psychobiological systems, particularly those involved in the stress response (Andrews, Ali, & Pruessner, 2013; Lupien et al., 2009; Phillips & Jones, 2006; Tarullo & Gunnar, 2006) as well as ii) in brain structures related to cognitive functioning and emotional regulation (Pechtel & Pizzagalli, 2011). Therefore, scrutinizing the molecular basis underlying stress-adaptation is highly relevant for understanding the diathesis of mental disorders, as well as the processes

underlying resilience (Hellhammer & Hellhammer, 2008). Moreover, consolidated knowledge of these processes could have implications for the prevention and treatment of stress-related mental disorders.

A recent line of evidence suggests that DNA methylation – an epigenetic process regulating gene activity – could provide one of the molecular mechanisms mediating these psychobiological consequences of early life stress. This behavioral epigenetic research has suggested that early psychosocial experiences could shape epigenetic patterns of genes involved in stress-adaptation, such as the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituitary-gonadal (HPG) axis, neurotrophins, as wells as other neurotransmitter and hormonal systems (e.g. Champagne & Curley, 2009; Lutz & Turecki, 2013). Furthermore, findings indicate that certain epigenetic patterns might contribute to the pathogenesis of stress-related mental disorders (e.g. Docherty & Mill, 2008; Dudley, Li, Kobor, Kippin, & Bredy, 2011). Today, behavioral epigenetic research questions are in the focus of basic and clinical research worldwide (Lester et al., 2011). However, since only a limited number of studies have been published, further research scrutinizing potential changes in epigenetic patterns related to psychosocial stress is necessary, especially in humans. Additionally, a highly neglected research question concerns the dynamics of epigenetic changes after psychosocial stress experiences.

Taken together, epigenetic research – especially on candidate genes interacting with the stress-response, as well as on the dynamics of epigenetic changes after acute psychosocial stress – could improve our understanding of how psychosocial stress affects molecular processes of stress-adaptation with potential consequences for mental health and disease.

Scope of the Thesis

Objectives.

This thesis and the presented scientific papers aim to examine changes in DNA methylation associated with exposure to psychosocial stress, and to discuss the relevance of the findings with regard to stress-adaptation and their potential implications for resilience. The research presented here is based on three studies investigating DNA methylation of two stress-related candidate genes – the oxytocin receptor (*OXTR*) and the brain-derived neurotrophic factor (*BDNF*) – after exposure to different psychosocial stressors, including (1) intrauterine exposure to maternal adversities (prenatal stress), (2) low maternal care during childhood and adolescence (low maternal care) and finally (3), acute psychosocial stress in adulthood (acute psychosocial stress). Up to date, both genes were not in the direct focus of epigenetic studies on psychosocial stress in humans, but might play a role in stress-adaptation and resilience (Cicchetti & Rogosch, 2012; Duclot & Kabbaj, 2013; Karatsoreos & McEwen, 2013; Ozbay et al., 2007; Taliaz et al., 2011). Scrutinizing epigenetic patterns after psychosocial stress might improve our understanding of how epigenetic processes could contribute to the development of stress-related mental disorders in the future.

Research Questions

Based on previous animal and human research, the papers presented here address the following specific and overall research questions:

- *Prenatal stress:* Do different indicators of maternal psychosocial stress during pregnancy predict DNA methylation in a target sequence in the *OXTR* in cord blood at birth?

- *Low maternal care:* Is maternal care during childhood and adolescence associated with DNA methylation of *OXTR* and *BDNF* in peripheral blood collected in adults?
- *Acute psychosocial stress:* Are there any dynamic changes in DNA methylation of *OXTR* and *BDNF* in peripheral blood after exposure to psychosocial stress in adults?
- *Overall research question 1:* Can we observe stress-associated changes in DNA methylation of two genes involved in stress-adaptation (*OXTR* and *BDNF*) in human blood cells?
- *Overall research question 2:* Are potential changes in DNA methylation specific to different stressors?

Since epigenetic research is only at the beginning and exposure to psychosocial stress has been associated with bi-directional changes in DNA methylation in previous studies (depending on stressor, gene and tissue investigated), we did not state specific directions of stress-related changes in DNA methylation in any of the three studies.

Approach.

To answer our research questions, we assessed DNA methylation in two *OXTR* (*$OXTR_1$*, *$OXTR_2$*) and one *BDNF* target sequence in whole blood samples after different kinds of psychosocial stress exposure:

- *Prenatal stress:* The indicators of maternal stress during pregnancy included i) exposure to life-changing events during the two years prior to the second trimester; ii) cortisol profiles during the second trimester; iii) depressive symptoms during the third trimester; and finally iv) chronic stress during the course of pregnancy. DNA methylation of *$OXTR_1$* was measured in cord blood collected at birth.
- *Early life stress:* we compared peripheral blood DNA methylation of *$OXTR_1$*, *$OXTR_2$*, *BDNF* between adults reporting high and low maternal care during the first 16 years of life.

- *Acute psychosocial stress:* dynamic changes in DNA methylation of $OXTR_1$, $OXTR_2$, *BDNF* was assessed in peripheral whole blood collected one minute before (pre-stress), 10 minutes after (post-stress) and 90 minutes after (follow-up) an acute psychosocial stressor.

The insight from the specific and overall research questions could have implications for i) the epiphenotype of psychosocial stress; ii) the potential use of peripheral blood DNA to investigate stress-related epigenetic patterns; iii) the understanding of stress-related dynamics in DNA methylation; and iv) the understanding of stress specificity of the investigated target sequences.

Outline of the Thesis

This thesis is structured as follows: The "*Introduction*" described the general background and relevance of the research presented in this thesis. The "*Theoretical Framework*" will cover the theoretical background and current state of research in the field of psychosocial stress, behavioral epigenetics and the selected candidate genes. The "*Methods*" section will give an overview on the research designs, samples, applied instruments and biological and statistical analyses. The section "*Results and Main Conclusions*" contains a summary of the results and the main conclusions for the stated research questions. Finally, the "*General Discussion*" will consider the general psychobiological implications and the implications for stress-adaptation and resilience. Additionally, strengths, limitations and outlook for future research will be presented. A detailed description for the theoretical background, methods, results and discussion of the three presented scientific papers can be found in Appendix A to D.

Theoretical Framework

Psychosocial Stress

Stress is a reaction to a psychological or physiological demand drawing on an organism's resources (Margraf, Lieb, & Pschyrembel, 2012). Thereby, psychosocial and other stressors[1] (e.g. physical or physiological) can destabilize an organism's psychological, endocrine and physiological balance. After stress exposure, the balance of the organism has to be reestablished by processes of adaptation in order to maintain health (Chrousos & Gold, 1992; Conrad, 2011; Selye, 1950). The interplay between the psychological, the endocrine and the physiological systems orchestrates the stress response: a stressful event is perceived and centrally processed to evoke an emotional reaction, which initiates the activation of the physiological and endocrine stress system (Andrews et al., 2013). Activation of these three systems induces psychobiological processes supporting a fight or flight response, which promotes the organism's survival. However, an inability to adapt to the stress – for instance due to the severity or chronicity of the stressor, or due to a dysfunctional stress response system – threatens physical and mental health (e.g. Hellhammer & Hellhammer, 2008). Therefore, the identification of psychobiological processes underlying stress-adaptation might improve our understanding of stress-related mental disorders.

Psychobiological Concepts of the Stress Response and Stress-Adaptation.

Hans Selye's "*General Adaptation Syndrome"* (Selye, 1950) was one of the first modern concepts of the stress response and stress-adaptation. The model postulates three stages: i) Phase of alarm: a stressor triggers an alarm reaction with rapid activation of the sympathetic nervous system and release of stress hormones, which mobilizes energy resources to improve performance. ii) Phase of resistance: the organism needs to adapt to the stress, in order to

[1] the term stressor refers to the condition causing a stress response, whereas the term stress refers to the organism's psychophysiological reaction to the stressor (Margraf et al., 2012). Whether a certain condition is perceived as a stressor depends on the individual's evaluation (Lazarus, 1993).

reestablish normality and homeostasis. iii) Phase of exhaustion: since adaptation energy is restricted, exposure to prolonged stress will eventually result in exhaustion and impaired health. Subsequent psychobiological concepts of stress-adaptation aimed to describe how different psychobiological stress-systems interact, how individual patterns of stress-reactivity in these systems are established and how these individual patterns could contribute to maintaining mental health. For example, the "*Biopsychosocial Stress Model"* (Dienstbier, 1989) categorizes psychophysiological stress responses according to the interaction between cognitive processes (demands versus individual resources) and the associated release of catecholamine and cortisol. The "*Biological Sensitivity to Context Model"* (Boyce & Ellis, 2005) takes individual differences of the stress systems into account and states that stress reactivity – e.g. autonomic, adrenocortical and immune system reactivity – moderates the association between environmental adversities and the risk to develop a disorder. Finally, the "*Adaptive Calibration Model*" (Del Giudice, Ellis, & Shirtcliff, 2011) suggests that environmental factors (e.g. parental investment) and population competence (e.g. age or status) shape different interacting stress response systems, which results in an individual pattern of sympathetic nervous system and HPA axis reactivity, with relevance for mental health. Thereby, it seems that particularly early psychosocial experiences determine an individual's stress-response pattern and potential for stress-adaptation, which might contribute to the vulnerability to develop a stress-related disorder later in life (Hankin, 2005; Heim, Meinlschmidt, & Nemeroff, 2003).

Early Psychosocial Stress and Mental Health.

The effects of chronic or severe psychosocial stress during the early stages of development have extensively been investigated (Carr et al., 2013; Lupien et al., 2009). For example, intrauterine exposure to maternal psychosocial stress, including adverse life events, depressed mood and increased cortisol levels during pregnancy, was associated with adverse birth

outcomes, as well as behavioral and stress-related problems later in life (Baibazarova et al., 2013; Bolten et al., 2011; Brand, Engel, Canfield, & Yehuda, 2006; Coussons-Read et al., 2012; Dancause et al., 2011; Duthie & Reynolds, 2013; Engel, Berkowitz, Wolff, & Yehuda, 2005; King & Laplante, 2005; Li et al., 2012; Martini, Knappe, Beesdo-Baum, Lieb, & Wittchen, 2010; Meinlschmidt, Martin, Neumann, & Heinrichs, 2010; Tegethoff, Greene, Olsen, Meyer, & Meinlschmidt, 2010; Tegethoff, Greene, Olsen, Schaffner, & Meinlschmidt, 2011, 2012; Tegethoff, Pryce, & Meinlschmidt, 2009; Yehuda et al., 2005). During the stages of childhood and adolescence, exposure to childhood abuse, neglect or restrictions in parental care, increased the risk for physical and mental disorders across life (Carr et al., 2013; Green et al., 2010; McLaughlin et al., 2010a, 2010b). Research on the biological mechanisms mediating the association between early psychosocial stress experience and physical and mental health suggests that psychosocial early life experiences shape psychoneuroendocrine systems affecting an individual's stress response patterns and neuronal development (Engert et al., 2010; Heim et al., 2003; Heim et al., 2000; Lovallo, Farag, Sorocco, Cohoon, & Vincent, 2012; Pechtel & Pizzagalli, 2011; Tarullo & Gunnar, 2006). Thereby, dysfunctional adaptations in stress-related psychobiological systems might contribute to an individual's vulnerability to stress later in life (Gutman & Nemeroff, 2003; Heim, et al., 2009; Heim & Nemeroff, 1999; Heim, Newport, Bonsall, Miller, & Nemeroff, 2001; Lovallo, 2013). Recent studies have indicated that these psychobiological systems could be shaped by changes in epigenetic patterns of stress-related genes (e.g. Heim & Binder, 2012; Kofink, Boks, Timmers, & Kas, 2013; Lutz & Turecki, 2013).

Taken together, stressful psychosocial experiences induce a stress response involving the activation of different stress-related psychobiological systems. These psychobiological stress-systems could be shaped by psychosocial stress experiences, particularly during early

development, with consequences for stress reactivity and health. Evidence suggests that these processes might be epigenetically mediated.

Behavioral Epigenetics

Background.

The genome contains the blueprint that defines all organisms from bacteria to humans. Thereby, every cell of an individual organism comprises exactly the same genetic information. During embryonic development, tissue-specific gene activity has to be established and propagated. Hereby, epigenetic programming determines cell fate and consequently defines tissue characteristics. Certain aberrations in epigenetic patterns are associated with pathological cellular processes and cancer (for a review see Sharma, Kelly, & Jones, 2010). Several lines of evidence suggest a limited potential for dynamic changes within the epigenome (Bergman & Cedar, 2013; Caldji, Hellstrom, Zhang, Diorio, & Meaney, 2011; Szulwach & Jin, 2014). These dynamic processes might provide the organism with a certain degree of phenotypic plasticity and the ability to adapt to specific environmental conditions through regulation in gene expression (e.g. Gluckman, Hanson, & Low, 2011; Weaver, 2009). A prime epigenetic mechanism is DNA methylation, which affects the chromatin architecture and regulates gene transcription (for further reading on epigenetic mechanisms see Allis, 2007).

DNA methylation is a type of DNA modification defined as the addition of a methyl-group to typically a cytosine-guanine dinucleotide (CpG) in the DNA strand (for a review see Auclair & Weber, 2012). DNA sequences containing high CpG density are called CpG islands and are mainly located within regulatory promoter regions of a gene. A high degree of DNA methylation within a promoter sequence or an exon is usually associated with a closed chromatin state and a reduced accessibility for transcription factors, which in turn is associated with a silencing of the respective gene (Allis, 2007). DNA methylation can be

influenced by genetic determinants (Lienert et al., 2011; Xie et al., 2012), other epigenetic mechanisms (e.g. modifications of the histone code) and through the biological translation of environmental cues, including psychosocial experiences (e.g. McGowan et al., 2011; Weaver et al., 2004).

Animal Models of Behavioral Epigenetics.

The most cited behavioral epigenetic studies investigated the "*epigenetic programming*" of the stress-response. These studies compared epigenetic patterns of the glucocorticoid receptor gene (*GR*) – a gene involved in the negative feedback system of the HPA axis in the hippocampus – between the offspring of rat mothers showing a high or low amount of pup licking/grooming and arched-back nursing (Francis, Diorio, Liu, & Meaney, 1999; Weaver et al., 2004). In more detail, these findings suggest that offspring of low caring mothers showed i) increased hippocampal DNA methylation of an important regulatory CpG site located at an NGFI-A (nerve growth-factor inducible protein A; a transcription factor) consensus region of the *GR* exon 1_7 promoter (Weaver et al., 2004); ii) This DNA hypermethylation and associated histone modifications resulted in a decreased NGFI-A binding to its consensus region (Weaver et al., 2004); iii) this reduced *GR* expression in offspring raised by low caring mothers, iv) causing decreased HPA axis feedback sensitivity, v) a less moderate stress response and vi) augmented fear behavior (Caldji et al., 2011; Francis et al., 1999; Liu et al., 1997). These results indicated that epigenetic modifications in the *GR* could mediate the association of low maternal care with augmented stress reactivity and the behavioral consequences thereof. Of note, the effects of received maternal care were confirmed in cross-fostering experiments (Cameron et al., 2008; Francis et al., 1999; Weaver et al., 2004), which rules out an exclusive role of epigenetic inheritance. Moreover, the effects induced by low maternal care were reversed by pharmacological treatment targeting the closed chromatin structure, which resulted in epigenetic patterns, hippocampal *GR* expression, GR protein

levels and stress responses comparable to offspring raised by high caring mothers (Weaver et al., 2004). Subsequent animal studies demonstrated stress-related changes in DNA methylation patterns in additional candidate gene, as well as on a global level. Thereby, the investigated stressors included i) prenatal stress (Boersma et al., 2013; Jensen Pena, Monk, & Champagne, 2012; Matrisciano et al., 2013; Mueller & Bale, 2008; Mychasiuk, Harker, Ilnytskyy, & Gibb, 2013; Mychasiuk, Ilnytskyy, Kovalchuk, Kolb, & Gibb, 2011; Mychasiuk, et al., 2011; Onishchenko, Karpova, Sabri, Castren, & Ceccatelli, 2008; Paternain et al., 2012; Petropoulos, Matthews, & Szyf, 2014); ii) early life stress (Anier et al., 2013; Blaze, Scheuing, & Roth, 2013; Champagne & Meaney, 2006; Chen et al., 2012; Edelmann & Auger, 2011; Franklin et al., 2010; Kember et al., 2012; McGowan et al., 2011; Murgatroyd et al., 2009; Qin et al., 2011; Roth, Lubin, Funk, & Sweatt, 2009; Wang, Meyer, & Korz, 2013; Zhang et al., 2010) and iii) exposure to psychosocial stress in adulthood (Elliott, Ezra-Nevo, Regev, Neufeld-Cohen, & Chen, 2010; Roth, Zoladz, Sweatt, & Diamond, 2011; Sterrenburg et al., 2011).

Human Studies on Behavioral Epigenetics.

Several studies reported an association of early life stress with DNA methylation in humans. One of the first behavioral epigenetic human studies was conducted on post-mortem brain tissue, which revealed increased DNA methylation in the human glucocorticoid receptor (*NR3C1)* promoter 1_F[2] in the hippocampus of suicide victims who suffered from childhood abuse compared to suicide victims and non-suicide controls without experiences of abuse (McGowan et al., 2009). Subsequent findings from human post-mortem studies have extended our knowledge on stress-related alterations in DNA methylation after psychosocial stress in neuronal tissue (Keller et al., 2010; Labonté et al., 2012; McGowan et al., 2008; Suderman et al., 2012). Notably, recent studies suggest that an association between psychosocial stress

[2] homologue to rat exon 1_7

exposure and mental disorders with changes in DNA methylation can also be observed in DNA derived from peripheral blood and buccal cells (for a comprehensive review see Klengel, Pape, Binder, & Mehta, 2014).

In sum, behavioral epigenetic studies in animal models and humans suggest that psychosocial experiences, especially those occurring early in development, could shape the stress-response system by alterations in epigenetic patterns of stress-related genes. These epigenetic processes might contribute to an individual's vulnerability for stress-related physical and mental disorders (Zannas & West, 2013). However, behavioral epigenetic research is only at the beginning and the number of studies is limited, especially in humans and with regard to candidate genes participating in stress-adaptation beyond the HPA axis.

Stress-Related Candidate Genes

Two genes presumably involved in stress-adaptation and mental health are the oxytocin receptor (*OXTR*) and the brain-derived neurotrophic factor (*BDNF*).

Oxytocin Receptor (OXTR).

The oxytocin receptor is expressed in a wide range of central and peripheral tissues and binds to oxytocin, a neurohypophyseal hormone (Gimpl & Fahrenholz, 2001). The primary functions of oxytocin signaling include induction of childbirth, lactation and maternal as well as sexual behavior (Grewen, Davenport, & Light, 2010; Lee, Macbeth, Pagani, & Young, 2009; Pena, Neugut, & Champagne, 2013). Notably, the oxytocin system might also be relevant in social bonding and stress-adaptation (Carter, 2003; Gimpl & Fahrenholz, 2001): First, oxytocin signaling was shown to be involved in the regulation of the autonomic nervous system, having especially parasympathic action, and to interfere with the HPA axis to dampen the stress response (Grewen & Light, 2011; Holst, Uvnas-Moberg, & Petersson, 2002; Liberzon & Young, 1997; Neumann, 2002). Second, oxytocin signaling might mediate the

effects of social support and interpersonal relationships on the stress system (Ditzen et al., 2013; Ditzen et al., 2009; Grewen, Girdler, Amico, & Light, 2005; Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003; Heinrichs, Ditzen, Kirschbaum, & Ehlert, 2003; Holt-Lunstad, Birmingham, & Light, 2008; Light, Grewen, & Amico, 2005; Olff et al., 2013; Pedersen & Boccia, 2002). Third, adverse early experiences were associated with decreased oxytocin concentration in plasma, urine and cerebrospinal fluid (Fries, Ziegler, Kurian, Jacoris, & Pollak, 2005; Heim, et al., 2009; Opacka-Juffry & Mohiyeddini, 2012), decreased oxytocin sensitivity (Meinlschmidt & Heim, 2007), and reduced OXTR levels in specific rodent brain regions (Francis, Champagne, & Meaney, 2000; Francis, Young, Meaney, & Insel, 2002). Last, disturbances in the oxytocin system are associated with a variety of mental disorders (Olff et al., 2013), including schizophrenia (Goldman, Marlow-O'Connor, Torres, & Carter, 2008; Keri, Kiss, & Kelemen, 2009) and depression (Cyranowski et al., 2008; Skrundz, Bolten, Nast, Hellhammer, & Meinlschmidt, 2011).

To date, only a few studies investigated *OXTR* DNA methylation, although the *OXTR* seems to be a promising epigenetic candidate gene (Kumsta, Hummel, Chen, & Heinrichs, 2013; Kusui et al., 2001). Recent studies proposed that *OXTR* DNA methylation could be involved in the pathology of autism spectrum disorders (Gregory et al., 2009), and be associated with callous-unemotional traits in teenage boys diagnosed with conduct disorder (Dadds et al., 2013). Finally, a recent fMRI study demonstrated that *OXTR* DNA methylation was associated with brain activity in a social animacy task (Jack, Connelly, & Morris, 2012).

Brain Derived Neurotrophic Factor (BDNF).

BDNF is a neurotrophin associated with neuronal development, -differentiation, -function and -plasticity, as well as long-term potentiation (Binder & Scharfman, 2004; Cirulli & Alleva, 2009; Huang & Reichardt, 2001; Thoenen, 1995). Although BDNF plays a key role in the central nervous system, it crosses the blood-brain barrier and is also synthesized in blood cells

(Braun et al., 1999; Gielen, Khademi, Muhallab, Olsson, & Piehl, 2003; Kerschensteiner et al., 1999; Pan, Banks, Fasold, Bluth, & Kastin, 1998). Animal models suggest that stress exposure bi-directionally affects BDNF expression, depending on brain structure (Bath, Schilit, & Lee, 2013). Furthermore, BDNF signaling could mediate the association between early psychosocial stress experience and neuronal development with consequences for mental health (e.g. see Cirulli et al., 2009). For instance, reduced peripheral BDNF protein levels were associated with depression (Bocchio-Chiavetto et al., 2010; Karege et al., 2002; Pandey et al., 2010), suicide (Kim et al., 2007; Sher, 2011), schizophrenia (Palomino et al., 2006; Toyooka et al., 2002), symptoms of insomnia (Giese et al., 2013) and posttraumatic stress disorder (Angelucci et al., 2014; Dell'Osso et al., 2009).

Animal models showed that exposure to various environmental factors was associated with changes in *BDNF* DNA methylation in several exon and promoter regions (table 1) (Blaze et al., 2013; Boersma et al., 2013; Gomez-Pinilla, Zhuang, Feng, Ying, & Fan, 2011; Lubin, Roth, & Sweatt, 2008; Mizuno, Dempster, Mill, & Giese, 2012; Onishchenko et al., 2008; Roth et al., 2009; Roth et al., 2011; Sui, Wang, Ju, & Chen, 2012). Additionally, human studies reported that early psychosocial stress, psychopathology and prenatal toxin exposure were associated with differences in *BDNF* DNA methylation as compared to subjects not exposed to early life stress or toxins, or healthy controls, respectively (table 1) (Fuchikami et al., 2011; Keller et al., 2010; Kordi-Tamandani, Sahranavard, & Torkamanzehi, 2012; Mill et al., 2008; Perroud et al., 2013; Toledo-Rodriguez et al., 2010).

Table 1

Animal and Human Studies investigating DNA Methylation of BDNF

Authors	Variable	Species and Tissue	Change in DNA Methylation
Blaze et al. (2013)	early life stress	rat PFC	↑↓[a]
Boersma et al. (2013)	prenatal stress	rat HC, AMY	↑
D'Addario et al. (2012)	bipolar disorder II	human PBC	↑
Fuchikami et al. (2011)	major depression	human PBC	↑↓[a]
Gomez-Pinilla et al. (2011)	exercise	rat HC	↓
Ikegame et al. (2013)	schizophrenia	human PBC	↑
Keller et al. (2011)	suicide	human WERN	↑
Kordi-Tamandani et al. (2012)	schizophrenia	human PBC	↓
Lubin et al. (2008)	fear conditioning	rat HC	↑↓[a]
Mizuno et al. (2012)	fear conditioning	mouse HC	↓
Onishchenko et al. (2008)	prenatal toxin exposure	mouse HC	↑
Perroud et al. (2013)	early life stress	human PBC	↑
Perroud et al. (2013)	BPD	human PBC	↑
Roth et al. (2009)	early life stress	rat PFC	↑
Roth et al. (2011)	traumatic stress	rat HC	↑↓[b]
Sui et al. (2012)	long-term potentiation	rat PFC	↓
Toledo-Rodriguez et al. (2010)	prenatal maternal smoking	human PBC	↑

Note. The variable column gives the investigated exposure, model or mental disorder. Change in DNA methylation is indicated as increase (↑) or decrease (↓) as compared to unexposed/healthy subjects. Only results on *BDNF* DNA methylation of a respective study are given. PFC=prefrontal cortex; HC=hippocampus; AMY=amygdala; PBC=peripheral blood cells; WERN=Wernicke Area; BPD=borderline personality disorder. [a]Depending on genomic target region (e.g. exon number). [b]Depending on target tissue (e.g. brain region)

In sum, there is evidence that oxytocin and BDNF signaling might mediate the association of early psychosocial experiences with the stress response, neuronal plasticity and mental health. Moreover, DNA methylation of the genes encoding BDNF and OXTR seems to be sensitive to psychosocial experiences. Therefore, DNA methylation of *OXTR* and *BDNF* is a promising

molecular candidate mechanism underlying stress-vulnerability and -resilience (Cicchetti & Rogosch, 2012; Duclot & Kabbaj, 2013; Karatsoreos & McEwen, 2013; Ozbay et al., 2007; Taliaz et al., 2011).

The overall aim of the research presented here is to explore stress-related differences in DNA methylation of *OXTR* and *BDNF*. The investigated stressors included prenatal stress, low maternal care during childhood and acute psychosocial stress in adulthood.

Methods

This section intends to give an overview on study design, sample and methods applied in the three studies. A detailed description for each study is given in the respective paper, which can be found in Appendices A to C. All studies applied a cross-sectional study design. A more detailed description of DNA methylation analysis is given in Appendix D.

Prenatal Stress

The sample of the prenatal stress study (*N*=39) consisted of pregnant mothers participating in the "Individual Project C: Psychobiological Programming of the Stress Response", which was conducted between the years 2007 to 2010 in Basel, Switzerland, as a part project of the National Center of Competence in Research, Swiss Etiological Study of Adjustment and Mental Health (NCCR sesam). We assessed indicators of maternal psychosocial stress experience during pregnancy and DNA methylation of an *OXTR* target sequence in cord blood at birth. The indicators of maternal stress included: i) number of stressful life events up to two years prior to the second pregnancy trimester and current strain caused by the reported events; ii) depressive symptoms during the past seven days assessed in the third trimester; iii) chronic stress during the course of pregnancy and iv) cortisol awakening response (CAR) and diurnal cortisol profiles (DAY) in the second trimester.

Table 2

Main Predictor Measures for the Prenatal Stress Study

Applied instrument	Construct	Reference
Prenatal stress		
Inventory of Life Events (ILE)	number and strain caused by stressful life events	(Siegrist & Geyer, 2003)
Edinburgh Postnatal Depression Scale (EPDS)	depressive symptoms	(Bergant, Nguyen, Heim, Ulmer, & Dapunt, 1998)
Trier Inventory of Chronic Stress (TICS-K)	chronic stress	(Wolff Schlotz & Schulz, 2005)
Cortisol awakening response (CAR, AUCg)	anticipation of upcoming demands	(E. Fries, Dettenborn, & Kirschbaum, 2009)
Diurnal cortisol profile (DAY, AUCi)	HPA axis sensitivity	(Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003)

Note. AUCg=area under the curve with respect to ground; AUCi=area under the curve with respect to increase; HPA=hypothalamic-pituitary-adrenal

Low Maternal Care

Within the scope of the NCCR sesam "Pre-Study II: Epigenetic Information and Metabolic Profiling", which was conducted from 2007 to 2008 in Basel, Switzerland, we screened an ad-hoc sample of university students (N=709) for perceived parental care and parental protection during the first 16 years of their life using the Parental Bonding Instrument (Parker, Tupling, & Brown, 1979). We then recruited participants scoring within the two extreme groups of the maternal care subscale (N=85) and compared peripheral whole blood DNA methylation of the two *OXTR* and the *BDNF* target sequence between subjects reporting to have experienced high (n=40) or low maternal care (n=45). Additionally, we assessed blood cell count as a potential mediator in the association between maternal care and DNA methylation.

Acute Psychosocial Stress

We recruited a cohort sample of elderly participants (*N*=83, age 60-67) from the research project "Sequelea of a Persisting HPA Axis Hyperactivity", which was funded by the German Research Foundation and conducted at the University of Trier, Germany from 2006 to 2008. All participants underwent the Trier Social Stress Test, which is an established procedure to induce an acute psychosocial stress response (TSST, see Foley & Kirschbaum, 2010). Blood was drawn one minute before start of the TSST (pre-stress), one minute after completion of the TSST (post-stress) and 90 minutes after stress (follow-up). DNA methylation of the three blood samples of both *OXTR* and the *BDNF* target sequences was assessed. Additionally, we measured blood cell count as a time-varying covariate.

Analysis of DNA Methylation

While sample collection and DNA extraction differed between studies (see Appendices A to C), DNA methylation analysis was performed identically for all three studies reported here. Blood samples were collected in cord blood at birth (prenatal stress) or from brachial vein in adults (maternal care, acute psychosocial stress). DNA was bisulfite-converted (EZ-96 DNA methylation kit, ZYMO research) and target sequences in *OXTR* and *BDNF* were amplified using bisulfite polymerase chain reaction (PCR). The $OXTR_1$ and $OXTR_2$ target sequences are located within *OXTR* exon 3, which is part of a CpG island. The *BDNF* target sequence is located within exon 6, which is expressed in the periphery (Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007). DNA methylation was quantified using the standard protocol for Sequenom EpiTYPER, which applies base-specific cleavage and matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS). The resolution of EpiTYPER does not always yield single CpG sites, but combines CpGs into CpG units. CpG units with more than 20% missing data, for example due to high or low detection limits, were

excluded from the analysis (see Appendix D for detailed information on analysis of DNA methylation).

Statistical Analysis.

To analyze the associations between psychosocial stress and DNA methylation, we applied mixed model analyses. The statistical mixed models performed in the prenatal stress and early life stress study allowed unstructured covariance matrices between CpG units, because these models yielded highest model fit compared to more restricted models. The applied mixed models could provide a statistical solution for future studies using a similar approach to investigate DNA methylation across a target sequence consisting of multiple CpG units.

Results and Main Conclusions

This section provides a brief summary of the results and the main conclusions drawn in the three research papers. For more detailed information on results, see Appendices A to C.

Prenatal Stress

The number of life events up to two years prior to the second trimester (ILE), an augmented cortisol awakening response (CAR) during the second trimester and increased symptoms of depression (EPDS) during the third trimester were associated with decreased DNA methylation of $OXTR_1$. Neither the strain caused by the number of stressful life events at the time of assessment, nor diurnal cortisol profile (DAY) during the second trimester or chronic stress (TICS-K) during the course of pregnancy predicted DNA methylation of $OXTR_1$. We concluded that intrauterine exposure to several indicators of maternal psychosocial stress predicted decreased *OXTR* DNA methylation in cord blood at birth. This suggests that maternal psychosocial stress experience during pregnancy could be linked with alterations in accessibility of the *OXTR* for transcription. This conclusion would support the notion that the developing embryo might get prepared for a potentially stressful future environment (Gluckman, Hanson, & Spencer, 2005).

Low Maternal Care

In the second study, we found that subjects reporting low maternal care during childhood and adolescence had increased DNA methylation in $OXTR_2$. There was no indication that this association was mediated by blood cell count. We therefore concluded that low maternal care during childhood is associated with decreased DNA methylation of the *OXTR* in peripheral blood in adults. This could indicate a psychobiological mechanism of how low maternal care might becomes a risk factor for later mental disorders.

Acute Psychosocial Stress

Exposure to acute psychosocial stress induced by the TSST was associated with dynamic changes in *OXTR* DNA methylation: DNA methylation in the $OXTR_1$ sequence increased from pre-stress to post-stress and decreased from post-stress to follow-up, even below baseline levels. The decrease from post-stress to follow-up remained significant after controlling for time-related variation in blood cell count. In $OXTR_2$ we found a decrease from post-stress to follow-up, which lost significance after controlling for blood cell count. We concluded that DNA methylation of the *OXTR* seems to be dynamically regulated after stress experiences, which could provide a potential mechanism by which an organism adapts to a short-term psychosocial stressors. To the best of our knowledge, this was the first study reporting dynamic changes in DNA methylation after acute psychosocial stress.

Overall Research Question 1

The first overall research question was whether or not stress-associated changes in DNA methylation could be observed in genes involved in stress-adaptation in human blood cells. This question could be affirmed for *OXTR*. In the three studies presented here, we observed stress-related changes in the *OXTR* target sequences, while DNA methylation of the *BDNF* target sequence remained stable. Whether blood cell DNA methylation of this particular *BDNF* target sequence is generally not sensitive to psychosocial stress or to other environmental factors, remains to be investigated in future studies.

Overall Research Question 2

The second overall research question was whether stress-related changes in DNA methylation are stressor specific. Indeed, the results from the three studies indicated that this might be the case, even across target sequences within the same gene. In conclusion, DNA methylation of

OXTR1 and *OXTR2* could be sensitive to specific psychosocial stress experiences, while DNA methylation of *BDNF* remained stable. The results are summarized in table 3

Table 3

Summary of the Results

Psychosocial Stressor	*OXTR1*	*OXTR2*	*BDNF*
Prenatal stress	↓	NA	NA
Low maternal care	=	↑	=
Acute psychosocial stress[a]	(↑) ↓	= (↓)	= =

Notes. Observed changes in DNA methylation: ↑=increase in DNA methylation; ↓=decrease in DNA methylation. Arrows in brackets indicate that the change in DNA methylation was not significant after controlling for blood cell count. *OXTR*=oxytocin receptor; *BDNF*= brain-derived neurotrophic factor; NA=not analyzed; equals sign=no changes.

[a] left arrow: change from pre-stress to post-stress; right arrow: change from post-stress to follow-up

General Discussion

The aim of the three research papers presented here was to investigate different psychosocial stressors with regard to changes in DNA methylation of three target sequences within two candidate genes (*OXTR$_1$*, *OXTR$_2$* and *BDNF*). First, intrauterine exposure to several indicators of maternal psychosocial stress during pregnancy predicted decreased *OXTR$_1$* DNA methylation. Second, experience of low maternal care during childhood and adolescence was associated with increased *OXTR$_2$* DNA methylation. Third, DNA methylation of *OXTR$_1$* was increased immediately after exposure to acute psychosocial stress and fell even below baseline levels 90 minutes after stress exposure. This DNA methylation decrease could also be observed in *OXTR$_2$*. While the decrease from post-stress to follow-up remained significant after accounting for time-related variations in blood cell count, all other dynamic DNA methylation changes did not.

General Psychobiological Implications

The three studies reported here have identified stress-related changes in DNA methylation of the *OXTR*. I speculate that these changes might be relevant for gene activity (Gregory et al., 2009). If so, the findings might provide insight into how exposure to psychosocial stress could epigenetically shape the oxytocin system. These results not only expand the existing literature on stress-related epigenetic changes to an additional candidate gene, but also contribute to the increasing literature on stress-related changes in the oxytocin system (Kumsta et al., 2013).

Results on the *BDNF* target sequence suppose that DNA methylation of this sequence was neither associated with acute psychosocial stress nor low maternal care. Whether *BDNF* is epigenetically regulated by other stressors or environmental factors, or whether different *BDNF* target sequences (e.g. comprising other exons) possess different stress-sensitivity, should be investigated in future studies.

Overall, the reported results have some general psychobiological implications:

- *Stress-related changes in peripheral blood:* Up to date, only few studies investigated epigenetic patterns in living humans and most results on early life stress and DNA methylation are derived from animal models. The fact that stress-related changes in DNA methylation could be observed in DNA samples extracted from peripheral blood supports the potential gain of investigating epigenetic patterns in this highly accessible tissue (Davies et al., 2012), although several methodological questions remain to be answered (see limitations and future directions).
- *Stress-related dynamic changes:* Stress-related epigenetic processes are highly dynamic. Stress-related changes in *OXTR* DNA methylation were not only associated with prolonged psychosocial stress experiences in early development, such as intrauterine adversities and maternal care during childhood and adolescence, but also after an acute psychosocial stressor. Whether stress-related epigenetic adaptations after early life stress are associated with the observed dynamic changes in *OXTR* DNA methylation in adulthood could be investigated in future studies.
- *Stress-specificity:* stress-related changes in DNA methylation of the assessed target sequences are – at least to some degree – specific to the psychosocial stressor. Results suggest that DNA methylation of $OXTR_1$ might be sensitive to prenatal stress and to acute psychosocial stress in adulthood while DNA methylation of $OXTR_2$ seems sensitive to maternal care. This is especially noteworthy with regard to the fact that the *OXTR* target sequences were in close proximity, indicating that changes in *OXTR* DNA methylation might not only be stressor-, but also target sequence-specific.
- *Direction of stress-related changes in DNA methylation:* The stress-related changes in DNA methylation were bi-directional and seemed specific to the psychosocial stressor (table 3). However, it might also be possible that it is not the psychosocial stressor *per*

se that defines direction of the observed changes but rather the timing of stress exposure, which could be investigated in future studies.

Implications for Stress-Adaptation and -Resilience

Oxytocin signaling was described to dampen the stress response. Therefore, I speculate that decreased *OXTR* DNA methylation and presumably augmented flexibility for regulation of *OXTR* expression could be a protective factor promoting stress-adaptation and -resilience.

Prenatal Stress.

Based on the finding that intrauterine exposure to several indicators of maternal adversities resulted in decreased DNA methylation of $OXTR_1$, I assu,e that specific prenatal adversities are associated with increased accessibility of the *OXTR* gene for transcription. Although this seems surprising, the result is in line with the assumptions of the Adaptive Calibration Model (Del Giudice et al., 2011) and the Predictive Adaptive Response Model (Gluckman et al., 2005). These models of phenotypic plasticity state that early in development, an organism produces a phenotype appropriate for an expected environment, based on cues from the immediate environment. An adverse maternal environment during pregnancy is associated with decreased positive maternal behavior (Lovejoy et al., 2000). Consequently, one could speculate that alleviated *OXTR* expression might support the infant's development by an increased dampening of its stress response and improved oxytocin signaling. This could protect the developing neuronal structures, which are highly sensitive to exposure to stress hormones and actions of the sympathetic nervous system (Li et al., 2012). Improved accessibility of *OXTR* could support stress-adaptation and resilience in a potentially stressful environment. However, one has to keep in mind, that other biological systems could still be programmed differently.

Low Maternal Care.

The quality and quantity of care provided by a primary care-person is essential for the normal development of a child (Bowlby, 1969). Based on our findings and the assumptions of the diathesis-stress model (e.g. Wittchen & Hoyer, 2011), I speculate that a reduced *OXTR* accessibility and expression could provide a molecular basis by which low maternal care contributes to the increased predisposition of an individual to develop a mental disorder, after exposure to a major stressor. Thereby, decreased *OXTR* signaling could contributes to an diminished stress-adaptation potential – for instance by a reduced ability to dampen the HPA-axis or sympathetic nervous system.

Acute Psychosocial Stress.

The response to an acute stressor consists of a fast response with increased sympathetic activity and release of stress hormones. Our finding that *OXTR* DNA methylation increased during the immediate alarm phase could indicate a molecular reaction to acute stress, in which an increased stress-response supports the organism's survival. However, due to potential contributions made by time-related changes in blood cell count, this finding has to be interpreted carefully. During the phase of resistance, DNA methylation decreased even below baseline levels. This process could support stress recovery on a molecular level, namely by an increased *OXTR* accessibility for transcription. Notably, the sample investigated grew up during World War II and it is likely that these participants were exposed to war adversities. Therefore, the results are not generalizable to other cohorts and have to be interpreted with caution. Subsequent research questions could examine whether the severity of early stress exposure associates with the quantity of stress-related changes in DNA methylation, or whether observed changes in DNA methylation are related to the stress reactivity of other psychobiological stress systems, such as HPA axis or sympathetic nervous system activity.

General Strengths and Limitations

The presented studies have some general strength and limitations.

General Strengths.

- *Stress-related candidate genes:* The studies presented here were highly innovative, because we investigated stress-related candidate genes beyond the HPA axis, which were not in the focus of epigenetic research on psychosocial stress in humans yet. Therefore, the presented studies have the potential to scrutinize the epiphenotype of psychosocial stress by providing new insights in the field of behavioral epigenetics.
- *Multiple target sequences:* In the maternal care and acute psychosocial stress study, we did not only investigate one candidate gene but two and included two target sequences within one gene. Therefore, our results could lead the presumption that psychosocial stress experiences are associated with gene- and target sequence-specific changes in DNA methylation, rather than global alterations.
- *Multiple stressors:* We investigated DNA methylation of identical target sequences in combination with three different psychosocial stressors. Since target sequences often differ between studies and are therefore not comparable, our study provides insight into the stress-specificity of our selected target sequences.
- *Assessment of blood cell count: W*e tried to account for blood-cell specific DNA methylation patterns by assessing blood cell count whenever possible.
- *Advanced statistical models:* We applied statistical mixed models that, at least in the prenatal stress and maternal care study, accounted for the independence of DNA methylation values across CpG units, which might have implications for future studies.

General Limitations.

- *Sample sizes:* The sample sizes were relatively small, especially with regard to the mixed model analyses. This resulted in difficulties with regard to the stability of the results. Moreover, we were not able to conduct subgroup analyses. For example, it would have been interesting to investigate specific exposures to life-changing events prior to the second pregnancy trimester or the influence of the severity of war exposure on the dynamic changes in DNA methylation after acute psychosocial stress.
- *Study design:* The study designs do not allow drawing conclusions about causality. Therefore, we cannot determine whether prenatal or early life stress is a cause or a simple correlate of the observed changes in *OXTR* DNA methylation. In the acute psychosocial stress study, we could have applied a randomized experimental design with a group exposed to a non-stressful control-TSST.
- *Epigenetic tissue- and cell-specificity:* DNA methylation was measured in blood samples. Due to the assumed tissue-specificity of epigenetic patterns, we cannot draw conclusions about epigenetic patterns in neuronal tissue. Additionally, we should be careful with the interpretation of DNA methylation in cord-blood samples, as we do not know to what degree these patterns relate to DNA methylation in the tissue of newborns. Moreover, even the assessment of epigenetic patterns in blood is problematic, since epigenetic patterns are not only tissue- but for some genes also blood cell type-specific (Adalsteinsson et al., 2012). However, we tried to address this issue by statistically analyzing the influence of blood cell count.
- *Assessment of epigenetic patterns:* Sequenom EpiTYPER is an established method to assess DNA methylation, but does not yield single CpG resolution. Up two seven neighboring CpG sites can be combined into a CpG unit. In our analyses, we did not weight CpG units according to the number of CpG sites included in a unit. Another

weakness is that the sole assessment of DNA methylation might not provide an overall epigenetic picture, since additional epigenetic processes affect gene expression, for instance histone modifications or microRNAs (e.g. Allis, 2007).

- *Significance of investigated target sequences:* In all studies, we did not analyze expression or protein levels of OXTR or BDNF, therefore we cannot draw conclusion about the functional relevance of the assessed target sequences. However, they were designed to cover functionally relevant regions identified in previous studies. Nevertheless, we can only speculate about the implications for stress-adaptation and resilience.
- *Generalizability:* The reported studies are not generalizable to other populations. The prenatal stress and maternal care study included low-risk samples with above-average socioeconomic backgrounds and high educational levels. The cohort of the acute psychosocial stress study grew up during World War II, which might have affected biological stress systems.

Outlook

First, there is a strong need for prospective longitudinal human studies in behavioral epigenetics research. Although the prenatal and the early life stress study provide evidence that psychosocial stress early in life is associated with distinct epigenetic alterations, future studies should scrutinize whether these changes are caused by psychosocial stress exposure or are a simple biological correlate. For example, longitudinal studies could repeatedly assess epigenetic patterns, as well as the psychobiological and behavioral consequences of stress-related epigenetic alterations.

Second, future epidemiological studies could compare epigenetic patterns between exposed and non-exposed individuals who did or did not develop a specific disorder (e.g. see

Mehta et al., 2013). These epidemiological studies, especially in combination with a longitudinal study design, might shed light on epigenetic processes shaping the stress response systems, which could contribute to an individual's predispositions for mental disorders.

Third, future studies should replicate our findings in different populations. It might be interesting to include high-risk samples, e.g. with low socio-economic status or low levels of education. Successful replication could validate our findings and expand generalizability.

Last, an essential question that remains to be answered in future studies is the comparability of epigenetic patterns between different tissues. Up to date, there is evidence that tissue-specificity of epigenetic patterns depends on the gene investigated (Adalsteinsson et al., 2012; Davies et al., 2012). Correlating epigenetic patterns measured in blood cells with peripheral levels of proteins that are synthesized exclusively in the brain, e.g. hormones of the pituitary, could provide a potential approach for future research.

Overall Conclusions

DNA methylation of the *OXTR* could be sensitive to different psychosocial stressors during all periods of life. While stress-associated changes in DNA methylation of different *OXTR* target sequences seemed to be stressor-specific, we did not identify psychosocial stressors associated with DNA methylation of *BDNF* in the three papers presented here. The findings in *OXTR* could support our understanding of an epigenetic contribution in stress-adaptation. Notably, the results indicate that *OXTR* DNA methylation, which seems epigenetically sensitive to acute psychosocial stress, is associated with exposure to prenatal and early life stress. This could support our understanding of how stressful psychosocial experiences might contribute to an individual's potential for stress-adaptation and presumably the vulnerability or resilience to develop mental disorders later in life.

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Appendices A to D

Appendix A

Unternaehrer, E., Bolten, M., Nast, I., Staehli, S., Meyer, A. H., Dempster, E., Hellhammer, D. H., Lieb, R., Meinlschmidt, G. (submitted). Maternal Adversities during Pregnancy Predict Cord Blood Oxytocin Receptor (OXTR) DNA Methylation.

Appendix B

Unternaehrer, E., Meyer, A. H., Burkhardt, S. C. A., Dempster, E., Staehli, S., Theill, N., Lieb, R., Meinlschmidt, G. (submitted). Association of Maternal Care and DNA Methylation (*BDNF, OXTR*) in Human Peripheral Blood Cells.

Appendix C

Unternaehrer, E., Luers, P., Mill, J., Dempster, E., Meyer, A. M., Staehli, S., Lieb, R., Hellhammer, D. H., Meinlschmidt, G. (2012) Dynamic changes in DNA methylation of stress-associated genes (*OXTR, BDNF*) after acute psychosocial stress. *Translational Psychiatry, 2,* e150. doi: 0.1038/tp.2012.77

Appendix D

DNA Methylation Analysis

Appendix A:

Maternal Adversities during Pregnancy Predict Cord Blood Oxytocin Receptor (*OXTR*) DNA Methylation

Submitted to Biological Psychiatry

Titel Page

Maternal Adversities during Pregnancy Predict Cord Blood Oxytocin Receptor (*OXTR*) DNA Methylation

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ABSTRACT

Background

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*), a gene involved in maternal behavior and stress adaptation.

Methods

Cord blood of 39 newborns was collected from offspring of mothers recruited from a cross-sectional study including 100 pregnant women in Basel, Switzerland, conducted from 2007 to 2010. In the second (T2) and third (T3) trimester, mothers completed questionnaires on stressful life events during the past two years (Inventory of Life Events, T2), depressive symptoms during the past seven days (Edinburgh Postnatal Depression Scale, T3), and provided saliva samples for maternal cortisol awakening response (CAR) and diurnal cortisol profiles (T2). One to three weeks postpartum, mothers indicated chronic stress experience during pregnancy (Trier Inventory of chronic stress). Cord blood DNA methylation of *OXTR* was quantified using Sequenom EpiTYPER®. Statistical analyses were performed using mixed models.

Results

The number of stressful life events (Loglikelihood-Ratio(1)=4.606; p=0.032), depressive symptoms (Loglikelihood-Ratio(1)=7.183; p=0.007), and cortisol awakening response (Loglikelihood-Ratio(1)=5.027; p=0.025) but not chronic stress during the course of pregnancy (Loglikelihood-Ratio(1)=0.173; p=0.677) or diurnal cortisol profiles (Loglikelihood-Ratio(1)=3.011; p=0.083), were negatively associated with cord blood DNA methylation of *OXTR*.

Conclusions

Our findings suggest that maternal adverse experiences and cortisol awakening response during pregnancy predict DNA methylation of an *OXTR* sequence in cord blood. The results are in line with the assumption that adverse maternal life events and mood might prepare the offspring for the future environment by epigenetic stress-adaptation mechanisms.

Introduction

Maternal adverse psychosocial experiences, endogenous cortisol levels and exogenous glucocorticoid response during pregnancy are associated with an increased risk for poor birth outcome, behavioral and stress-related abnormalities and later physical and mental disorders in the offspring (1-17). Although the molecular mechanisms in these phenomena are not fully understood, a recent line of evidence suggests that the association between intrauterine environment and offspring's health could be epigenetically mediated (18). DNA methylation – the binding of a methyl-group to a CpG site – is commonly associated with transcriptional silencing of a gene and has been shown to be responsive to early life adversities and other environmental factors (19, 20). So far, several intrauterine predictors of cord blood DNA methylation in newborns have been identified, including maternal diet (21-26), maternal substance consumption (27-30) and maternal depression and stress experience (31-35). Although the identification of epigenetic patterns associated with maternal mood and her psychosocial environment could increase our understanding of how maternal psychosocial experiences during pregnancy affect offspring development, only few epigenetic studies addressed this issue: First, maternal depressed mood during pregnancy was related to increased cord blood DNA methylation in the cell growth-related Maternally Expressed Gene 3 (*MEG3*) (32, 36) and to decreased cord blood DNA methylation in the serotonin transporter gene (*SLC6A4*) (31). Second, maternal experience of pregnancy related anxiety and augmented maternal cortisol levels, particularly in the second trimester, predicted increased cord blood DNA methylation in several CpG sites of the human glucocorticoid receptor gene (*NR3C1*) promoter (35). Third, maternal exposure to war stress, material deprivation and daily hassles during pregnancy were found to be associated with increased cord blood DNA methylation in a *NR3C1* promoter region (34). Finally, intimate partner violence during pregnancy was positively associated with *NR3C1* DNA methylation in adolescent offspring

(37), suggesting long-term epigenetic adaptations in the offspring after intrauterine exposure to maternal psychosocial adversities. In sum, DNA methylation of some 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 relates to the offspring's epigenome. The oxytocin receptor (*OXTR*) is a potential candidate gene, due to the involvement of the oxytocin system in maternal (reproductive) behavior, mother-child bonding, and its interaction with the hypothalamic-pituitary-adrenal (HPA) axis to dampen the stress-response (38-46). Despite oxytocin's relevance for early neuronal and social development and mental health (47, 48), 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 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 (49), 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 whole course of pregnancy; and iii) maternal depressive symptoms during the third trimester. Additionally, we assessed iv) salivary cortisol levels (cortisol awakening response and diurnal cortisol profiles) during the third trimester as an indicator of HPA axis activity. 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 direction of the association.

Methods and Materials

Participants

The final sample consisted of 39 pregnant women representing a sub-sample of a larger cross-sectional study with multiple measuring time-points, which included 100 pregnant women and which was conducted in Basel, Switzerland from 2007 to 2010. Using different recruitment strategies, including promotion at local hospitals and advertisements in local newspapers and television stations, we recruited pregnant women between their 21th 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 1) no current mental disorder; 2) no severe medical complications; 3) no acute or chronic physical disease, such as metabolic disease or thyroid dysfunction; 4) no signs of fetal malformation, 5) pre-pregnancy body mass index below 32 kg/m^2; 6) no cigarette, alcohol or drug consumption beyond the 10th week of gestation; 7) good knowledge of the German language. A total of four women were excluded after study enrollment due to multiple outcome (i.e. 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 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. The subsets of participants providing versus those not providing cord blood for DNA methylation analyses did not differ with regard to birth parameters, socioeconomic status, pregnancy characteristics, scores on the maternal adversity questionnaires and cortisol profiles.

Procedure

At gestational week 21 to 32, participants completed a telephone screening to assess inclusion criteria and underwent the standardized DIA-X Munich-Composite International Diagnostic Interview (M-CIDI (50-52)). Between gestational week 20-24, included women completed questionnaires on stressful life events during the past two years (Inventory of Life Events, ILE (53)). Additionally, they provided saliva samples to assess morning and diurnal salivary cortisol levels. During gestational week 32 to 34 they completed a questionnaire on depressive symptoms during the past seven days (Edinburgh Postnatal Depression Scale, EPDS (54, 55)). At birth, cord blood samples were collected for DNA methylation analysis. One to three weeks post-partum, 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 (56)).

Questionnaires

Inventory of life events (ILE (53)). The ILE assesses the number of life-changing events during the last two years, such as being a victim or witness of assault, severe chronic illness or accident, or death in the family or close friends. A total of 32 life events are listed, including two open questions. Additionally, the ILE measures current strain caused by the respective life event on a 4-point Likert scale ranging from 1 to 4, whereby a high score indicates high levels of strain. The questionnaire provides information on three subscales: 1) number of life events (ILE life events); 2) total strain currently caused by all experienced life events (ILE total strain); and 3) average strain per experienced life event (ILE average strain per event). Reliability and criterion validity of the ILE are considered sufficient (53).

Edinburgh Postnatal Depression Scale (EPDS (54, 55)). The EPDS is a 10-item scale which assesses maternal depressive symptoms. Respondents have to indicate their mood during the

last seven days, such as the ability to experience joy, levels of anxiety, experience of excessive demands or sadness. Answers are given on a 4-point Likert scale. High scores on the EPDS indicate high 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 (55).

Trier Inventory of Chronic Stress – Short Version (TICS-K, (56)). The short version of the TICS consists of 30 items assessing chronic stress in different areas, including social life, working life or interpersonal communication. Respondents 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 possess good to very good reliability, the validity of the TICS-K scales is considered to be sufficient (56, 57). For the statistical analyses, we calculated an overall sum score.

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 body mass index (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 collected saliva six times a day during two consecutive normal workdays during gestational week 20-24. Samples had to be collected immediately after awakening, 30 minutes, 45 minutes and 60 minutes after awakening, at 1500h and at 2000h. We asked mothers to report sampling time, using written documentation, and excluded

samples from the analyses if they were collected outside a time frame before or after the scheduled sampling time of i) ±15 minutes for the first three samples; ii) ±30 minutes for the fourth sample and iii) ±60 minutes for the fifth and sixth sample. Participants received salivettes for saliva collection (Sarstedt, Nümbrecht, Germany) together with a detailed instruction sheet. Women stored the salivettes in a refrigerator until handing them in at the study center. Furthermore, we asked them not to collect saliva if they felt sick or ill and to avoid coffee, black tea and physical exercise on the day of saliva collection. Upon arrival at the study center, saliva samples were stored at –20°C until further analysis. Saliva samples were centrifuged at 2000 × *g* for 6 minutes before free salivary cortisol levels were measured using a time-resolved fluorescence immunoassay (DELFIA®, PerkinElmer Inc., Waltham, Massachusetts). All analyses were performed in duplicates and all intra- and inter-assay coefficients of variation were below 10%.

To analyze the cortisol awakening response within the first hour of awakening (CAR), we calculated the area under the curve with respect to ground (CAR AUCg: samples one to four). Since the AUCg provides information on total hormonal output (58) the AUCg was preferred to the AUCi because the CAR extenuates during pregnancy (59). For the diurnal cortisol profiles, we calculated the area under the curve with respect to increase (DAY AUCi: samples one to six) to assess HPA axis sensitivity (as suggested in 58). AUC values calculated for the two consecutive days separately and were then averaged to obtain one measure for CAR AUCg and one measure for DAY AUCi. In the case of missing data on one day, the AUC calculated for the other of the two days was used for further statistical analyses.

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 minutes. 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. 540ng of genomic DNA was bisulfite converted using EZ-96 DNA Methylation^{TM} Kit (Zymo Research, Irvine, California), according to manufacturer's protocol. Using the Sequenom EpiDesigner software, the *OXTR* target sequence (chromosome 3, p25, nt 8809275-8809534; 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 (60). 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^{TM} 1.0 (Sequenom Inc., San Diego, California). For each run, we included a fully methylated positive control (New England BioLabs® Inc., Ipswich, Massachusetts) and a blank control (destilled water).

Statistical Analyses

Data preparation included visual inspection for assumption of normality distribution (qq-plots, histograms, boxplots) of all predictors and the outcome variable (DNA methylation values, 5%meC). Sum score on the EPDS was the only predictor variable not normally distributed and was therefore transformed using the natural logarithm. To test whether the subsample of women who provided cord blood for DNA methylation analysis versus those who did not provide these samples differed with regard to sociodemographic data, pre-pregnancy BMI, pregnancy characteristics or birth outcomes, we used t-tests (continuously distributed data), Whitney U-test (ordinally distributed data) or χ^2-test (nominal data). To examine the inter-correlation between predictors we calculated Pearson's bivariate correlation coefficient.

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 analyzed due to high mass detection limit. CpG unit 4 (CpG site 9) was excluded because data was available for less than 10% of the participants.

For all statistical analyses on DNA methylation we used linear mixed models, with CpG unit as within subject factor. We specified an unstructured variances-covariance matrix for the within-subject factor as this pattern lead to the best model fit, based on the Akaike information criterion (AIC). Each model contained batch number as covariate to statistically control measurement bias due to batch effects. Then, each maternal predictor of adversity (ILE, EPDS, TICS-K, salivary CAR AUCg and DAY AUCi) was analyzed 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 maternal age, pre-pregnancy BMI, years of education, income category rating, (see questionnaire section), parity, length of gestation (days), mode of delivery (vaginal versus cesarean section) and birth weight (g) (61-65). To assess the effect of a specific predictor we used a Loglikelihood-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 analyzed its ten subscales, thereby adjusting for multiple testing using the Bonferroni-Holm method (66).

The descriptive statistics, predictor correlations and subsample comparisons were conducted using IBM SPSS 20. All mixed model analyses were performed using R version 3.0.1 (2013-05-16) (67) using the gls function of the nlme package (68). A p-value less than 0.05 was considered as statistically significant.

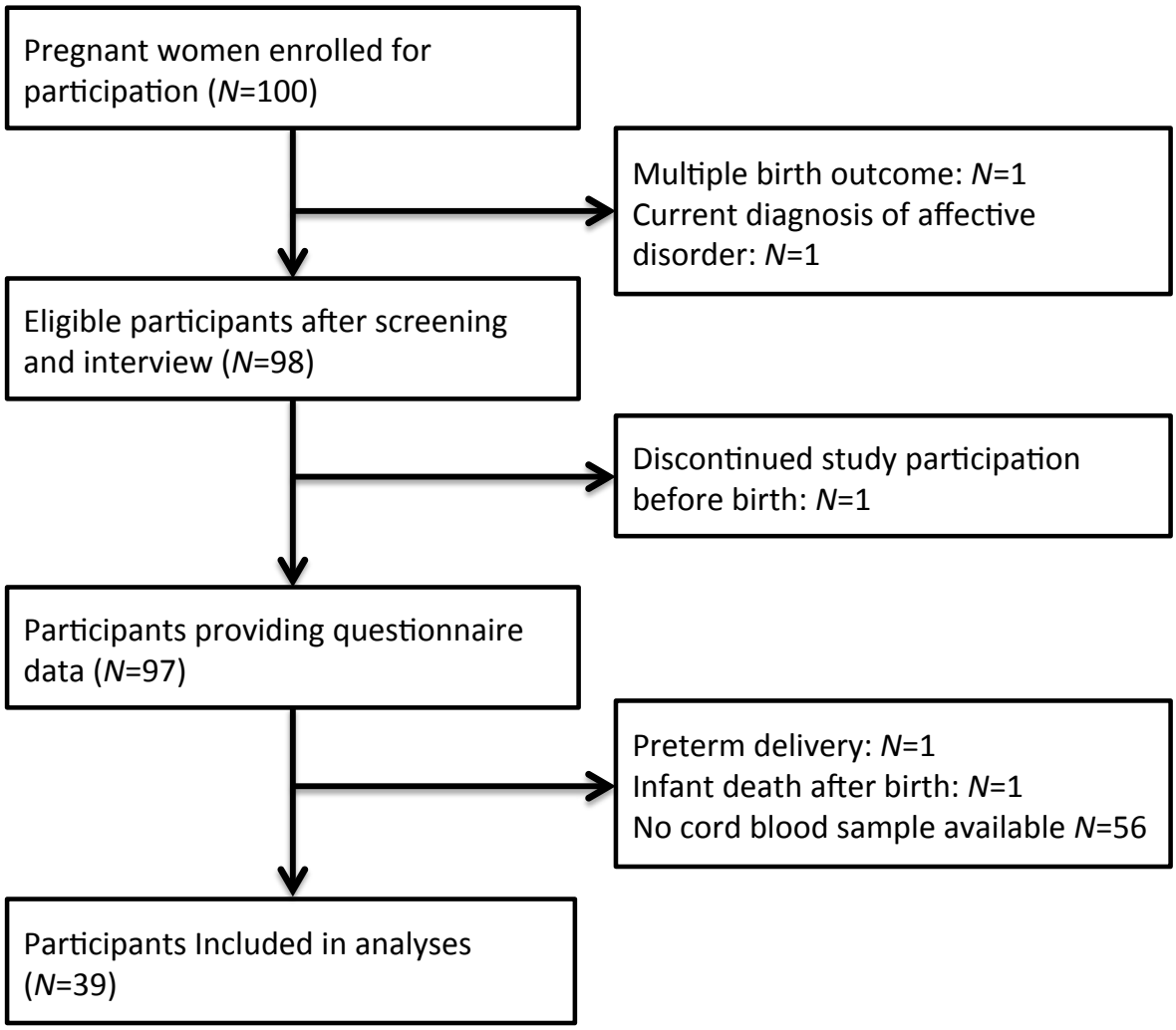

Figure 1. Flowchart of study participants.

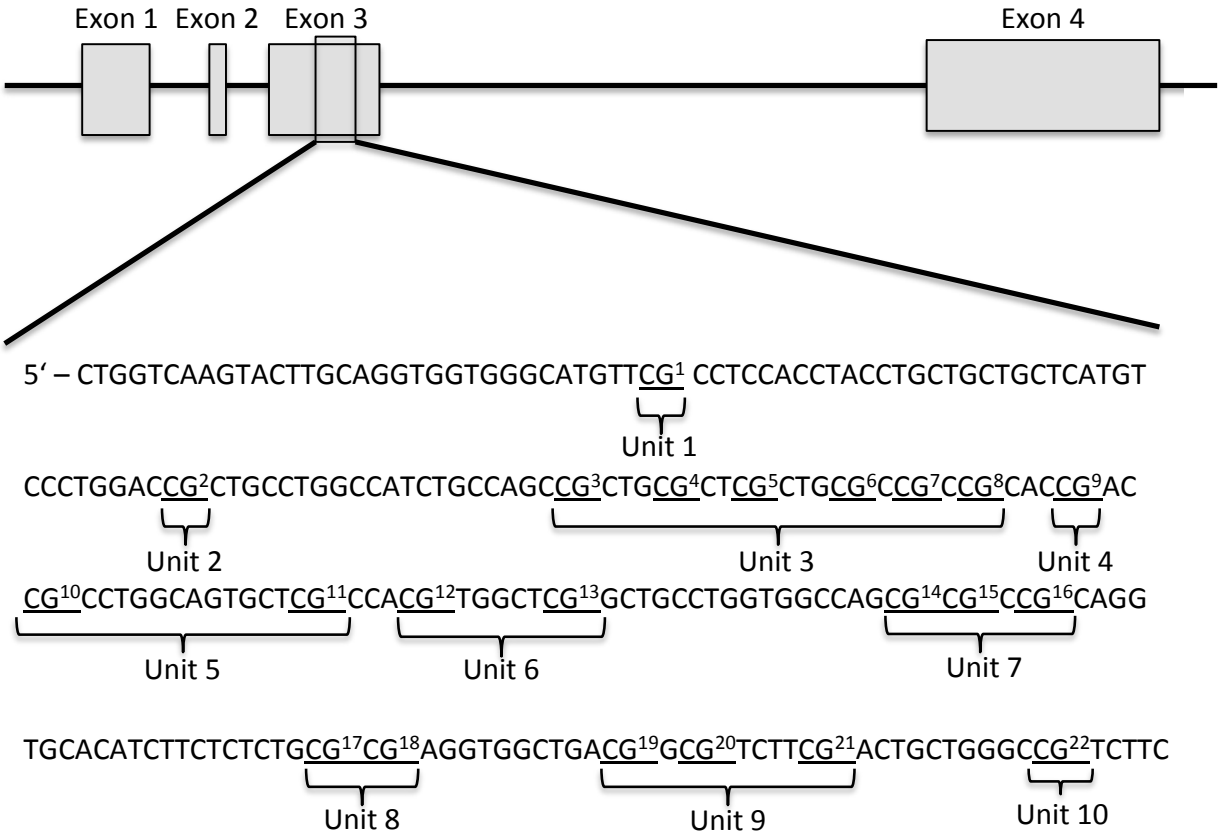

Figure 2. Schematic view of the oxytocin receptor gene (*OXTR*). CpG sites within the target sequence are underlined and numbered consecutively. Unit 1 to 10 indicate the CpG units based on resolution yielded by the Sequenom EpiTYPER.

Table 1. Characteristics of participants (*N*=39), pregnancy and birth outcomes and descriptive statistics (range, mean and standard deviation, *SD*) of predictors.

	Range	Mean	*SD*
Maternal age (years)	24 – 40	31.9	3.9
Education (years)	8 – 24	15.2	4.0
Pre-pregnancy BMI (kg/m2)	16.2 – 31.2	22.5	3.8
Length of gestation (days)	256 – 291	277	8.2
Birth weight (g)	2270 – 4440	3313	430
Life changing events (ILE score)			
Number of life events	1 – 15	7.08	3.67
Total strain	2 – 35	14.95	8.87
Average strain per life event	1 – 2.8	2.05	0.47
Depressive symptoms (EPDS score)	0 – 17	4.68	4.42
Chronic stress (TICS-K score)	16 – 60	34.08	11.00
Salivary cortisol CAR AUCg	537 – 1607	963	299
Salivary cortisol DAY AUCi	-138 – 745	269	237

	N	%
Household income rating[1]		
„Income is not enough“	0	0%
„Income is just enough“	6	17%
„Income is good to live with“	29	83%
Parity[2]		
First	25	66%
Second	11	29%
Third (or more)	2	5%
Delivery mode[3]		
Vaginal	24	63%
Caesarean section	14	37%

Abbrev: ILE: Inventory of Life Events; EPDS: Edinburgh Postnatal Depression Scale; TICS-K: Trier Inventory of Chronic Stress – Short Version; CAR AUCg: area under the curve with respect to ground of the cortisol awakening response; DAY AUCi: area under the curve with respect to increase for the diurnal cortisol profile; *SD*: standard deviation.

[1]Missing data (*N*=4); [2]Missing data (*N*=1); [3]Missing data (*N*=1).

Results

The EPDS sum score, the ILE score for number of life events and AUCg CAR were all negatively associated with cord blood DNA methylation, whereas the ILE scores for total strain, strain per life event, sum score on the TICS-K and diurnal cortisol profiles were not (table 2). Of the ten subscales of the TICS-K, only the subscale assessing social isolation was negatively associated with cord blood DNA methylation (L-Ratio=4.181; p=0.0409). However, this result was not significant after correction for multiple testing for the ten TICS-K subscales.

Table 2. Modell parameters and estimates from mixed model analysis (*N*=39) calculated for each predictor of *OXTR* DNA methylation. Shown is goodness of model fit (Akaike Information Criterion, *AIC*), degrees of freedom of the model (*df*), Loglikelihood-Ratio (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.

	Model Parameters				Parameter Estimates	
Predictors	***AIC***	***df***	**L-Ratio**	***p***	**Estimate**	***SE***
Model without predictors	1190					
Life changing events (ILE score)						
Number of life events	1135	1	5.047	0.025	-1.54E-01	6.03E-02
Total strain	1137	1	3.093	0.079	-5.24E-02	2.64E-02
Average strain per life event	1139	1	0.967	0.326	5.82E-01	5.07E-01
Depressive symptoms (EPDS score)*	1114	1	7.050	0.008	-7.04E-01	2.37E-01
Chronic stress (TICS-K score)	1097	1	0.173	0.677	-8.62E-03	2.02E-02
Salivary cortisol CAR AUCg	776	1	5.545	0.019	-1.90E-03	7.15E-04
Salivary cortisol DAY AUCi	777	1	4.117	0.043	-2.43E-03	9.83E-04

TICS: Trier Inventory of Chronic Stress; EPDS: Edinburgh Postnatal Depression Scale, ILE: Inventory of Life Events, AUCg: area under the curve with respect to ground, AUCi: Area under the curve with respect to increase; CAR cortisol awakening response; DAY: diurnal cortisol levels; *AIC*: Akaike Information Criterion; *df*: degrees of freedom; L-Ratio: Loglikelihood-Ratio; *SE:* standard error of the estimate.

* transformed using natural logarithm

None of the potential confounding (e.g. sociodemographic factors) or mediating factors (e.g. birth outcomes), were associated with *OXTR* DNA methylation. 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 supplemental table 1 (S1). The numbers of participants indicating a specific life event on the ILE are illustrated in supplemental figure 1 (S2). Graphical illustration of mean scores and standard deviations for the TICS-K subscales are shown in supplemental figure 2 (S3).

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 two 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 cortisol awakening response during the second trimester and maternal depressive symptoms were associated with decreased cord blood *OXTR* DNA methylation. In contrast, chronic stress during the course of pregnancy and diurnal cortisol profiles were not associated with cord blood *OXTR* DNA methylation.

Overall, our results suggest that more stressful life-events, higher levels of depressive symptoms, and an increased cortisol awakening response in mothers during pregnancy are linked to a decreased *OXTR* DNA methylation status in cord blood at parturition, even in a low-risk sample. These findings are intriguing, since the oxytocin system contributes to stress-adaptation and neuronal- and social development, and may thus be associated with mental wellbeing of the offspring later in life (47, 69).

This study has three main findings. First, the absolute number of maternal critical life events before the second trimester – rather than event-related strain or chronic stress that women experienced during pregnancy – predicted *OXTR* DNA methylation. Previous studies imply that maternal exposure to stressful life events even prior to conception increase the risk

for infant mortality and low birth weight (70, 71). The discrepancy that life changing events prior to the second trimester, but not stress experience during pregnancy *per se* predict cord blood *OXTR* DNA methylation seems surprising; however, they are in line with the finding that stressful war experiences (number of events, such as rape or death of a family member) were the strongest predictors of increased DNA methylation of the human glucocorticoid receptor gene (*NR3C1*), compared to more chronic stressors, such as material deprivation or daily hassles (34). However, the stress induction by war experiences might not be comparable with life changing events as assessed in our study with women from a generally secure environment.

Second, DNA methylation of *OXTR* seems 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 (31, 33). Regarding these bidirectional changes in DNA methylation – increase in *NR3C1* and decrease in *SLC6A4* or *OXTR* DNA methylation after maternal adversities during pregnancy – we speculate that offspring DNA methylation is sensitive to maternal depressive symptoms and mood during pregnancy in a gene-specific manner. Notably, maternal symptoms of depression during pregnancy and the first week post-partum are a predictor of negative maternal affect, hostility, or coercive behavior as well as maternal disengaged behavior (72). This association might be mediated, in part, by maternal oxytocin (73, 74). In addition, oxytocin signaling has important functions for bonding between mother and child and vice versa (45, 75, 76). 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 provide better oxytocin signaling in an environment with potentially restricted maternal caring behavior due to maternal depressive symptoms. Interestingly, a previous paper by our

group based on data from the same study, suggests that decreased maternal OXT levels in the third trimester predicted an increased risk to develop postpartum depression (77).

Third, a higher maternal CAR during pregnancy was associated with decreased *OXTR* DNA methylation. We suspect that maternal morning cortisol levels might serve as an intrauterine signal for an anticipated stressful extrauterine environment (4), in which improved accessibility of the *OXTR* might represent a beneficial mechanism for stress-adaptation. Furthermore, our results suggest that anticipated stress predicts cord blood *OXTR* DNA methylation rather than HPA axis sensitivity of the mother. To the best of our knowledge, this is the first study examining a connection between maternal cortisol levels and offspring DNA methylation of a gene, which is not directly but indirectly associated with the HPA axis.

In sum, 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 (78). We speculate that relevant experiences of an adverse environment of the mother before and during pregnancy, signal this information to the unborn child (8, 35, 79). Decreased DNA methylation in the *OXTR* could result in changes in *OXTR* accessibility for transcription (60). If this results in an improved OXTR availability, it could be advantageous in a stressful environment, because oxytocin signaling is linked to social bonding and interferes with the HPA axis to dampen the stress response (38-41, 80, 81). In short, we hypothesize that the unborn child might get prepared for a potentially challenging environment by an epigenetic adaptation of the *OXTR*.

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 nutritional

intake or psychosocial experiences. Second, we used statistical mixed model analysis, which were 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 to statistical models investigating DNA methylation values averaged across a given target sequence. Last, we applied several instruments assessing different forms of maternal adversities, including maternal depressive symptoms and included cortisol awakening response and diurnal cortisol levels as indicators of HPA axis activity.

The study has several limitations: First, we were only able to collect cord blood from a subsample of the initial study, which is critical with regard to our mixed model calculations that would profit from more observations. Thus, the results should be 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. Second, the reported maternal adversities and levels of depression were rather moderate. It would be interesting to repeat the study in a sample of pregnant women with experiences of trauma before or during pregnancy or a mental disorder, to examine an interaction between severity of maternal adversities or socioeconomic status with the predictors investigated in this study. Third, we cannot draw conclusions about the functional relevance of the assessed target sequence, as we did not assess OXTR expression- or protein levels. Fourth, due to the small amount of cord blood available, we were not able to determine blood cell count and could therefore not statistically control for this potential mediator (82). Fifth, some of the assessed parameters of maternal adversities were inter-correlated (see supplemental materials S1), suggesting that some predictors were not independent from each other and could – at least in part – measure the same constructs. 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 and mental disorders, women having birth complications or pregnant women outside Switzerland.

In conclusion, increased maternal stress experience, depressive symptoms and cortisol awakening response 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 stressful environment. If 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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Supplemental Materials S1

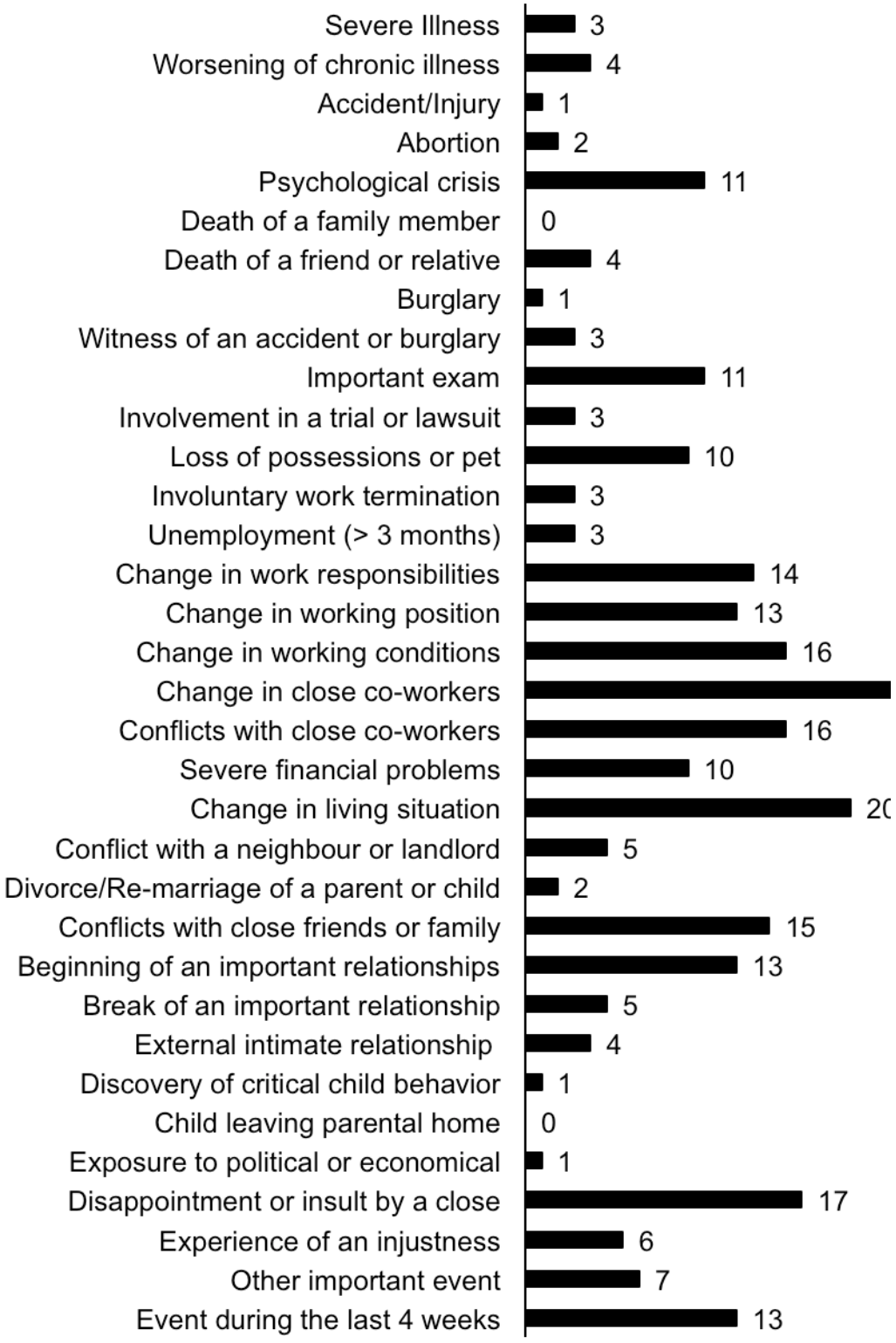
Table 1. Inter-correlation between measures of maternal adversities and maternal cortisol levels. Depicted are Pearson's correlation coefficients.

	EPDS	ILE1	ILE2	ILE3	CAR AUCg	DAY AUCi
TICS-K	0.293	0.241	.418*	.507*	-0.209	-.450*
EPDS	1	0.317	.361*	0.133	-0.01	-0.143
ILE number of life events (ILE1)	0.317	1	.942*	0.246	0.156	-0.04
ILE total strain (ILE2)	.361*	.942*	1	.522*	0.092	-0.171
ILE average strain per life event (ILE3)	0.133	0.246	.522*	1	0.022	-0.207
CAR AUCg	-0.01	0.156	0.092	0.022	1	.680*
DAY AUCi	-0.143	-0.04	-0.171	-0.207	.680*	1

Abbreviations: TICS-K: Trier Inventory of Chronic Stress - Short Version; EPDS: Edinburgh Postnatal Depression Scale; ILE: Inventory of life events; ILE1: ILE number of life events; ILE2: ILE total strain; ILE3: ILE average strain per life event; CAR: Cortisol Awakening Response; AUCg: Area under the curve with respect to ground; DAY: Diurnal cortisol profile; AUCi: Area under the curve with respect to increase.

* significant correlation with $p<0.05$

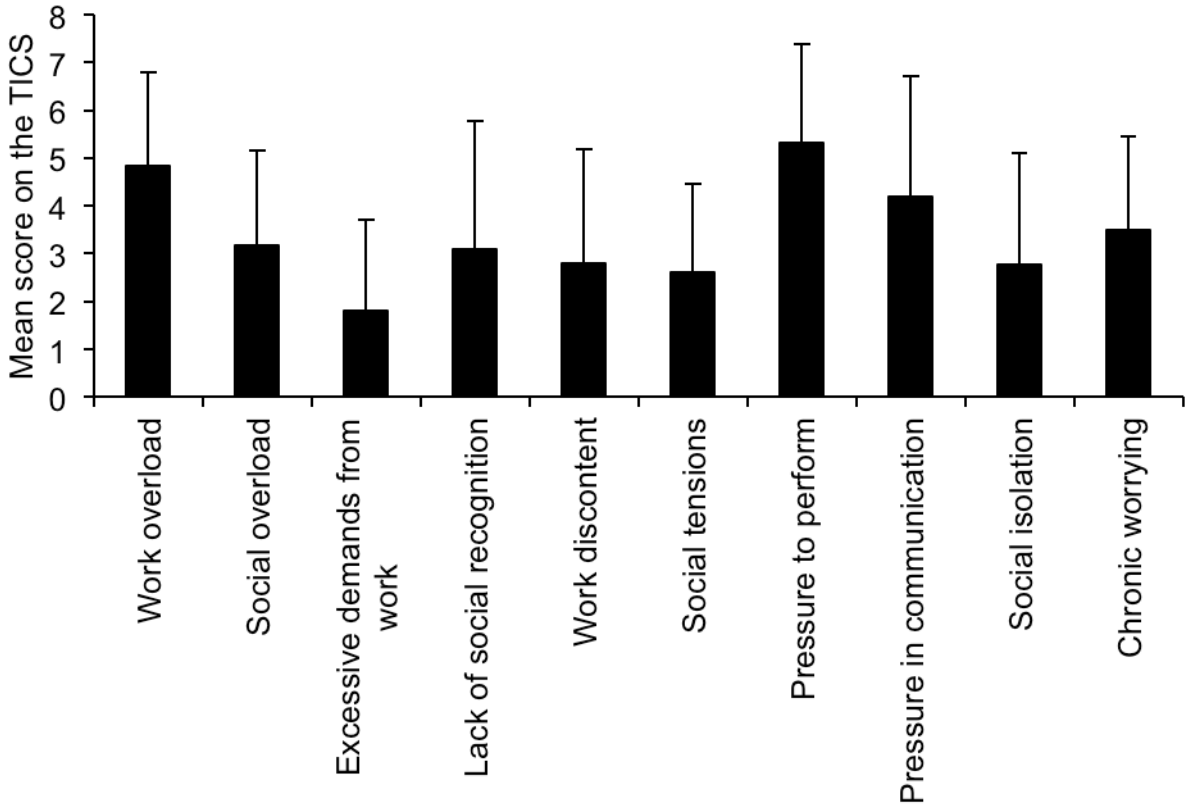
Supplementary Materials S2



Supplementary figure 1. Number of participants indicating a specific live

the Inventory of Life Events (ILE).

Supplementary Materials S3



Supplementary figure 2. Mean score on the subscales of the Trier Inventory of Chronic Stress (TICS). Error bars represent standard deviation

Appendix B:

Association of Maternal Care and DNA Methylation (*BDNF, OXTR*) in Human Peripheral Blood Cells

Submitted to Biological Psychiatry

Title Page

Association of Maternal Care and DNA Methylation (*BDNF, OXTR*) in Human Peripheral Blood Cells

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Abstract

Background

The aim of this study was to compare DNA methylation between participants reporting low and high maternal care during childhood in two stress-associated genes (two target sequences in the oxytocin receptor, *OXTR*; one target sequence in brain-derived neurotrophic factor, *BDNF*) in peripheral whole blood.

Methods

This cross-sectional study took place at the University of Basel, Switzerland between 2007 and 2008. We recruited 89 participants scoring lower than 27 or higher than 33 on the subscale maternal care of the Parental Bonding Instrument (PBI) at a previous assessment (N=709). At a personal appointment, 85 subjects provided blood samples for DNA methylation analyses (Sequenom^{R} EpiTYPER) and blood cell count (Sysmex PocH-100i^{TM}). Statistical analyses on whole blood DNA methylation were performed using mixed models.

Results

DNA methylation was decreased in the high maternal care group compared to the low maternal care group in one *OXTR* target sequence ($\chi^2(1)=4.45$; $p=0.035$). We found no differences in a second *OXTR* ($\chi^2(1)=0.010$; $p=0.920$) assay and in the *BDNF* target sequence ($\chi^2(1)=3.65$; $p=0.056$). Preliminary analyses indicated that blood cell count did not mediate the association between low maternal care and increased *OXTR* DNA methylation (estimate= -0.014, standard error=0.009; $p=0.104$).

Conclusions

This study provides first evidence that maternal care is associated with DNA methylation of an *OXTR* target sequence in peripheral whole blood. The findings could have implications for further elucidation of the epiphenotype of early life stress and for future research investigating DNA methylation in human peripheral blood cells.

Introduction

Parental care is crucial for the establishment of mental health for the offspring across life. Experiencing poor parental care during childhood increases the risk of abnormal psychological and endocrine responses to stress (1, 2), abnormal neuronal functioning (3) and mental disorders later in life (4-9). The molecular pathway underlying these associations is not yet fully elucidated. Increasing evidence suggests that early adverse experiences, especially low maternal care, alter DNA methylation with disadvantageous consequences for behavior and health later in life (for a review see 10).

DNA methylation is an epigenetic mechanism, by which a methyl-group usually binds to a cytosine followed by a guanine (CpG). This process regulates the accessibility of the DNA for the transcription machinery. Increased DNA methylation of promoter or exon regions often results in a less accessible DNA architecture with reduced mRNA expression (for a review see 11). Several rodent studies found aberrant DNA methylation of stress-related candidate genes in brain tissue of offspring reared in potentially stressful environments. The adverse environment commonly applied in these studies include low caring mothers or maternal separation (12-18). Recent human studies on epigenetic consequences of early life stress suggest similar processes: McGowan et al. (19, 20) found increased DNA methylation of the glucocorticoid receptor (*NR3C1*) and the ribosomal RNA gene in post-mortem brain tissue of suicide victims who experienced childhood abuse. Later, they extended their results on multiple promoter regions in hippocampal neurons of suicide victims who experienced childhood trauma (21). A few studies investigated DNA methylation in human peripheral blood after early life adversities and reported similar findings, including changes in DNA methylation after early life stress: i) Childhood sexual abuse and childhood maltreatment or -adversity predicted increased leukocyte DNA methylation of a target sequence in the *NR3C1*(22-24); ii) In CD3 T-cells, DNA methylation of several genes involved in the immune

system were either hyper- or hypomethylated in young adults who experienced parental adversities during childhood. Furthermore, the same study reported a negative association between maternal reports of warmth and affection towards their child and offspring DNA methylation of the *NR3C1* (25); iii) Brain-derived neurotrophic factor (*BDNF*) DNA methylation was positively associated with the number of childhood trauma in a sample of borderline personality patients (26). Taken together, these studies suggest that early life adversities are associated with long-term changes in DNA methylation of stress-associated genes, possibly with consequences for mental health later in life. Thus, investigating additional stress-related candidate genes could extend our understanding of an epiphenotype of early life stress.

In our study, we chose the oxytocin receptor (*OXTR*) as a first candidate gene, because i) oxytocin signaling modulates the stress response by interacting with the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system to dampen the stress-response (27-30) and ii) plays a crucial role in mother-child bonding (31) and a wide range of additional maternal behaviors (32, 33). Furthermore, the oxytocin system seems to be sensitive to stress experiences: i) Adult women suffering from early life adversities such as abuse had decreased oxytocin levels in cerebrospinal fluid (34); ii) men who experienced early parental separation showed reduced oxytocin sensitivity in adulthood (35) and iii) a previous study by our group indicated that DNA methylation of an *OXTR* target sequence was sensitive to acute psychosocial stress (36). Furthermore, a recent fMRI study suggests that *OXTR* DNA methylation in the periphery could have a functional relevance for social cognition and behavior (37). Finally, Kumsta and colleagues suggested to examine DNA methylation of genes involved in the oxytocin pathway after early life adversities, since epigenetic regulation of the oxytocin system might mediate the association between early adverse experiences and socio-behavioral outcomes (38).

BDNF, our second candidate gene, encodes for a neuronal growth factor involved in neuronal development and plasticity (39, 40) and in neuro-protective functions (41). Some of these BDNF-related functions were shown to be affected by early maternal care (42-45). Several immune cells, including peripheral human T and B cells, secrete bioactive BDNF and thereby support neuronal survival (41). Animal studies found altered BDNF levels in the rodent central nervous system and in the periphery of rhesus macaques after early life stress (46-48). Furthermore, central BDNF mRNA and -protein levels were increased in rat offspring raised by high compared to those raised by low caring mothers (45, 49). Epigenetic studies in rodents have shown that early life stress resulted in a higher methylation status of *BDNF* in neuronal tissue (16, 50), a result confirmed in an animal model of posttraumatic stress disorder (51). In addition, several human studies reported a negative association between psychopathology and peripheral BDNF concentration (52-55), suggesting that BDNF expression in the periphery is related to mental health. Finally, results from clinical studies suggest an involvement of increased *BDNF* DNA methylation in the etiology of stress-related mental disorders in humans (56-58).

Based on these findings, the aim of this study was to examine an association between maternal care during childhood and DNA methylation in the *OXTR* and *BDNF* in human peripheral blood. Therefore, we compared DNA methylation in two target sequences in *OXTR* (referred to as $OXTR_1$ and $OXTR_2$) and one target sequence in *BDNF* between adults reporting high versus low maternal care during childhood and adolescence. Because DNA methylation was shown to be blood cell type specific for certain loci – which could affect the interpretation of DNA methylation measured in whole blood cells (59) – and early life adversities are associated with changes in blood cell distribution (60), we estimated the effect of relative blood cell count as a potential mediator in the association between maternal care and DNA methylation. As both, increases and decreases in DNA methylation were reported

after early life stress in previous studies (for instance 25), we tested our hypotheses that maternal care during childhood and adolescence is associated with changes in DNA methylation of *OXTR* and *BNDF* in either direction.

Methods and Materials

Participants and Procedure

The sample of this cross-sectional study consisted of 89 adults. They were selected from an ad-hoc sample comprising 709 university students, who were recruited after a preliminary screening conducted at the University of Basel in 2007. All participants completed the Parental Bonding Instrument (PBI) (61) and gave written informed consent. Those participants who scored below a cut-off of 27 or above 33 on the subscale maternal care of the PBI were invited to a personal appointment. All appointments took place at the facilities of the University of Basel, Switzerland, from late 2007 to early 2008. Participants arrived fasting at the laboratory between 0800h and 0900h. After written informed consent was obtained, a study nurse took two blood samples of 2.7ml each, from brachial vein, at room temperature using EDTA Monovette (Sarstedt, Nümbrecht) for DNA extraction and blood cell count. Thereafter, participants completed questionnaires on sociodemographic data. The study was approved by the local ethics committee Basel (Ethikkommission beider Basel, EKBB) and was carried out in accordance with the latest version of the declaration of Helsinki.

Blood samples of four participants could not be analyzed due to lack of blood material and thus these participants were excluded from all subsequent analyses. The participants' characteristics are depicted in table 1 and a flow chart of study participation is given in figure 1. The resulting sample consisted of 85 participants: 45 reported low, 40 high maternal care. Women (n=67) and men (n=18) were about equally distributed between the maternal care

groups ($\chi^2(1)=0.612$, $p=0.594$). Age ranged from 19 to 66 years with a mean age of 27.5 years (*SD*=8.4).

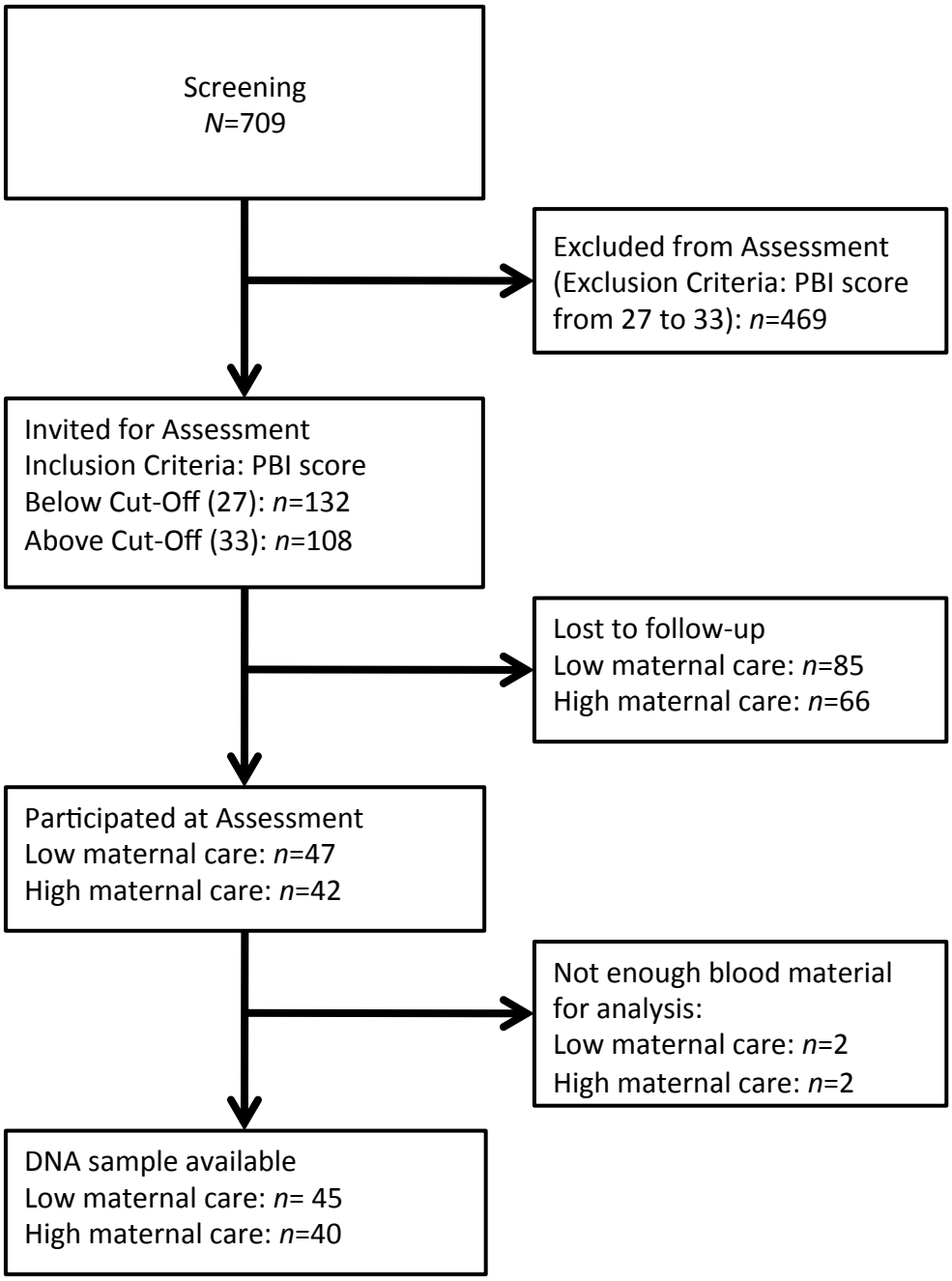

Figure 1. Flow Chart of study partcipants included in data analysis (*N*=85).

Table 1. Group characteristics for participants reporting low or high maternal care on the Parental Bonding Instrument (PBI). Displayed are means (*M*) and 95% confidence intervalls (95% – CI).

	Low maternal care		High maternal care	
	M	95% – CI	*M*	95% – CI
Age (years)*	30.3	27.3 - 33.2	24.3	22.7 - 25.9
Sum score on PBI	16.5	14.7 - 18.2	35.1	34.9 - 35.3
Blood cell count				
Lymphocytes (%)	33.5	31.2 - 35.9	31.7	29.6 - 33.9
Neutrophils (%)[2]	58.3	55.6 - 61.0	60.4	57.6 - 63.1
Mixed[1] (%)[2]	11.1	6.7 - 15.5	7.9	6.5 - 9.3

[1] monocytes, eosinophils and basophlis; [2] missing values: n=2

Abbreviations: PBI: Parental Bonding Instrument; *M:* Mean; CI: Confidence Interval

* significant group difference with p<0.05

Maternal Care

Parenting was assessed using the *PBI* (61) measuring parental bonding until the age of 16 retrospectively on the two subscales "care" (12 items) and "overprotection" (13 items). Psychometric studies suggested a satisfactory validity and reliability (62) and indicated a high stability over a 20 year period (63). Participants responded on a 4-point Likert scale to which extent different statements about perceived maternal behavior applied (0 = not true; 3 = very true). High scores on the two PBI subscales indicate high care or high levels of overprotection, respectively. We focused on the dimension of maternal care and recruited

participants when scoring below a cut-off of 27 or above a cut-off of 33 on the respective subscale in a preliminary screening. We chose this subscale due to earlier studies identifying maternal care as a strong risk factor for later mental health (5) and due to previous experiments investigating animal models of maternal care (17, 64). Furthermore, a similar recruitment strategy was successfully applied in a previous study on maternal care in humans (65). For all analyses, participants were stratified into a high and a low maternal care group according to recruitment strategy.

Blood and DNA Methylation Analysis

DNA was extracted from whole blood using Puregene (Qiagen, Venlo) according to the manufacturer's protocol. Samples were stored at –80°C for subsequent DNA methylation analysis. 540ng of genomic DNA was treated with sodium bisulfite using the EZ-96 DNA Methylation™ Kit (Zymo Research, Irvine, USA) according to the manufacturer's standard protocol. Bisulfite PCR amplification of one target sequence in *BDNF* and two target sequences in *OXTR* (*OXTR$_1$*, *OXTR$_2$*) was conducted using Hot Star *Taq* DNA polymerase (Qiagen, UK). The *OXTR$_1$* target sequence is located in the protein-coding region of *OXTR* exon III; the *OXTR$_2$* target sequence partly covers the non-coding and protein-coding regions of *OXTR* exon III. Both target sequences were designed to cover the *OXTR* CpG island – a region with a high density of CpG sites – comprising exons I to III, which was previously described to be associated with transcriptional regulation (66). The *BDNF* target sequence around the 3'-end of *BDNF* exon VI is situated mainly within a CpG island that covers *BDNF* exons V, Vh, and VI (67). *BDNF* exon VI is expressed in the non-neuronal tissue of the periphery (67). PCR products were prepared according to the manufacturer's instructions for quantitative DNA methylation analysis using EpiTYPER 1.0 (Sequenom Inc., San Diego, USA). For each run, a fully methylated positive control (New England BioLabs® Inc.) and a

blank control (distilled water) were included. The assays for the amplicons were designed using the Sequenom EpiDesigner software (for target sequences see supplementary information S1).

We analyzed blood cell count using Sysmex PocH-100i^{TM}. As only leukocytes have a nucleus containing DNA compared to other blood cells (red blood cells, platelets), we considered the relative number of leukocytes as potential mediators in the association between maternal care and DNA methylation, more specifically percentage of lymphocytes (small leukocytes), neutrophil granulocytes (large leukocytes) or mixed cell types, consisting of monocytes, eosinophils and basophils (middle sized leukocytes).

Statistical analysis

The resolution of EpiTYPER yielded CpG units consisting of one to six individual CpG sites: 11 CpG units for *OXTR$_1$*, 28 CpG units for *OXTR$_2$* and 12 CpG units for *BDNF*. Two CpG units in *OXTR$_1$*, one CpG unit in *OXTR$_2$* and one CpG unit in *BDNF* could not be measured due to upper or lower detection limits of Sequenom EpiTYPER. CpG units with > 20% missing data were excluded, which left 8 CpG units for *OXTR$_1$*, 27 CpG units for *OXTR$_2$* and 10 CpG units for *BDNF* for statistical analysis. All CpG methylation values were compared with the values of the fully methylated positive control: if the value of the sample exceeded the value of the positive control, the value was set missing. All blank controls were negative. We identified suspicious samples by setting outlier values as missing data (≥3 standard-deviations from mean methylation of the respective CpG unit). By conducting missing values analyses separately for each gene, participants with >20% missing data were identified and excluded from the statistical analyses of the respective gene (*OXTR$_1$*: n=2; *OXTR$_2$*: n=1; *BDNF*: n=1). Methylation values of *OXTR$_2$* and *BDNF* were ln-transformed to meet

assumptions of normality (tMethylation = ln (5%meC methylation + 0.02)+4). The distribution of untransformed data for each CpG is shown in figure 2.

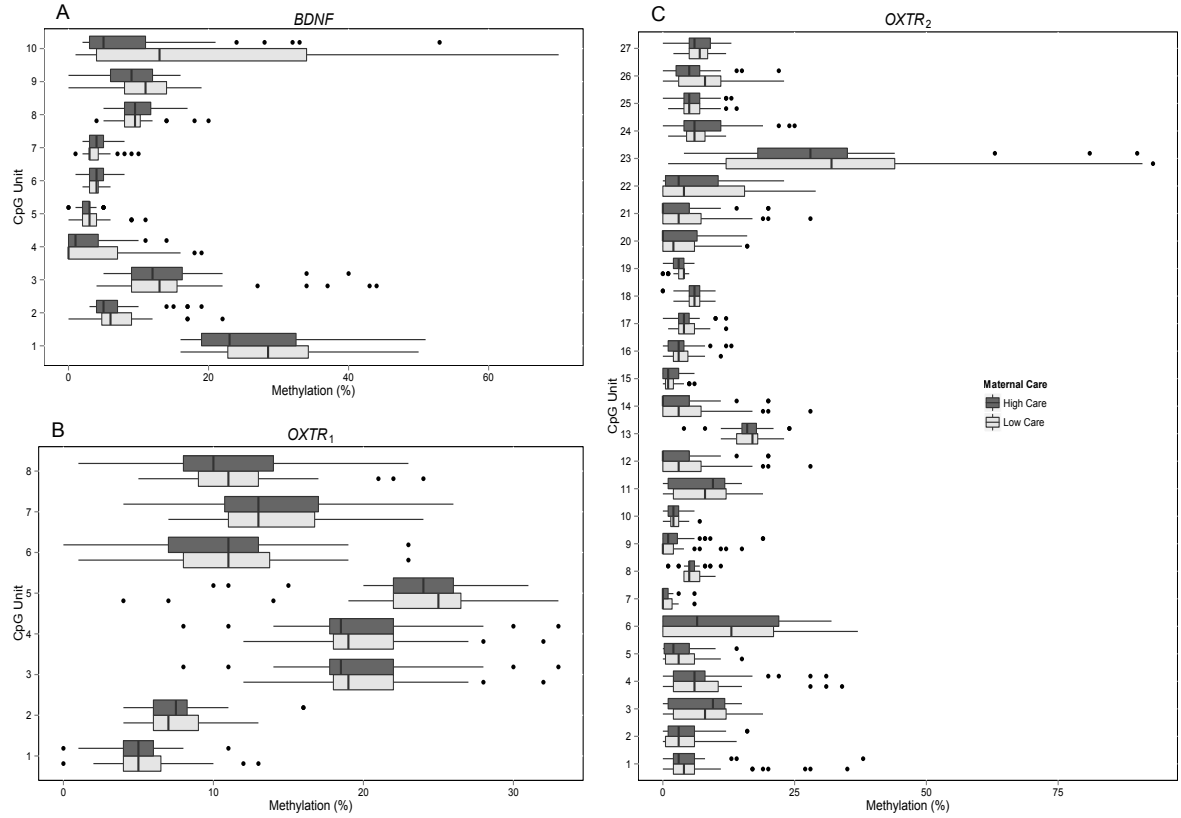

Figure 2. Box plots showing the distribution of untransformed DNA methylation (%5meC) of the *BDNF, OXTR$_1$ and OXTR$_2$* target sequences by high and low maternal care groups. CpG units yielded by Sequenom EpiTYPER are numbered consecutively. Black lines: median, box: percentiles 25 – 75, whiskers: percentiles 2.5 – 97.5, dots: outliers.

Abbreviations: *BDNF*: brain-derived neurotrophic factor gene; *OXTR*: oxytocin receptor gene.

In *OXTR$_1$*, CpG unit 3 and 4 had identical DNA methylation values. In *OXTR$_2$,* DNA methylation values were identical for CpG unit 3 and 11, and for CpG unit 12, 14 and 21. Repeated values of the duplicate and triplicate CpG units had to be excluded in order to

perform the mixed model analyses. We used a covariance pattern model (68), a type of mixed model, to analyze associations between maternal care and DNA methylation for each target sequence separately. In covariance pattern models, the covariances among the repeated measures are modeled directly. The choice of the specific covariance pattern was based on the best model fit as indicated by the Akaike Information Criterion (AIC) and was "unstructured" for all target sequences (i.e. all variances and covariances were estimated independently).

We were particularly interested in the association of maternal care and DNA methylation of the target sequences, given repeated measures of CpGs and controlling for batch number, sex and age. These variables were included in all analyses due to their previously described associations with DNA methylation and parental care (69-72). Therefore, our main model contained maternal care, age, sex and batch number as predictors and CpG unit as repeated measure variable. Effects were tested using Chi-square tests, that is by comparing the fits of nested models including or excluding maternal care group, sex, and age.

In subsequent analyses, we investigated relative amount of blood cell type as a potential mediator, using a multilevel mediation model as suggested by Preacher and collegues (73). Thereby, we only examined those blood cell types that were significantly associated with DNA methylation of the respective target sequence (identified using the mixed model described above).

Data preparation and data check were conducted using IBM SPSS 20. Covariance pattern models were analyzed using R, version 2.15.2 (74), including the package lme4 (75). Mediation models were calculated using the software Mplus6 (76). An alpha level of <0.05 was considered significant. DNA methylation values are presented as percent of 5' cytosine methylation (%5meC).

Results

In *OXTR*$_{2,}$ we found higher DNA methylation in the low compared to the high maternal care group ($\chi^2(1)=4.48$; $p=0.035$) (table 2). Mean DNA methylation values and magnitude of change in DNA methylation between the maternal care groups for each CpG unit, as well as corresponding standard errors (*SE*) are shown in supplementary table 1 (S2). Additionally, we found a sex difference with men having lower DNA methylation in *OXTR*$_2$ compared to women ($\chi^2(1)=10.4$; $p= 0.001$) but no association of DNA methylation with age ($\chi^2(1)=0.105$; $p=0.746$). In the mediation model, we examined relative number of lymphocytes as a potential mediator in the association between maternal care and DNA methylation of *OXTR*$_2$, as this blood cell type proved to be strongest associated with DNA methylation ($\chi^2(1)=22.444$; $p< 0.001$) compared to the other blood cell types. The indirect effect of maternal care on *OXTR*$_2$ DNA methylation via relative number of lymphocytes was thereby not significant (estimate=–0.014; *SE*=0.009; $p=0.104$), nor was the direct effect (estimate=–0.028; *SE*=0.016; p=0.086).

In *OXTR*$_1$, DNA methylation did not differ between maternal care groups ($\chi^2(1)=0.010$; $p=0.920$). We found no effects for sex ($\chi^2(1)=1.113$; $p=0.291$) or age ($\chi^2(1)=0.189$; $p=0.664$).

In *BDNF*, DNA methylation did not differ between maternal care groups ($\chi^2(1)=3.648$; $p=0.056$). We also found no effects for age ($\chi^2(1)=2.111$; $p= 0.146$) and sex ($\chi^2(1)=0.945$; $p=0.331$).

Table 2. Results obtained from mixed model analysis for each target sequence. Estimated effects maternal care group include the values for estimated parameter effects of maternal care on DNA methylation and standarderrors (*SE*). χ^2 and *p*-values refer to the improvement in model fit, if the respective predictor (high care versus low care) was included in the model. Additionally, number of participants (*N*) and number of observations are shown. All models included sex, age and batch number as covariates. DNA methylation values for $OXTR_2$ and *BNDF* were transformed using natural logarithm transformation

	Maternal care group		Modell characteristics			
	Value[1]	*SE*	χ^2	*p*-value	*N*	Observations
$OXTR_1$	-0.374	3.617	0.010	0.920	83	577
$OXTR_2$	-0.035	0.015	4.447	0.035	81	1885
BDNF	-0.051	0.025	3.648	0.056	78	764

[1] Value corresponds to the difference in average DNA methylation between the low and high maternal care group, whereby low maternal care represents the reference group

Abbreviations: *OXTR*: oxytocin receptor; *BDNF*: brain-derived neurotrophic factor; *SE*: standard error

Discussion

We examined DNA methylation of two stress-related candidate genes – two target sequences in *OXTR* and one target sequence in *BDNF* – in peripheral blood of adults reporting high or low maternal care during childhood and adolescence. We found that low maternal care was associated with higher whole blood DNA methylation of the $OXTR_2$ target sequence

compared to high maternal care. We did not find any indication that this association between maternal care and *OXTR$_2$* DNA methylation was mediated by blood cell count. We did not find any maternal care-related differences in DNA methylation in *OXTR$_1$* or *BDNF*.

Higher DNA methylation in the *OXTR$_2$* target sequence in subjects reporting low compared to those reporting high maternal care provides evidence for epigenetic changes related to early adverse experiences. Since the oxytocin system is involved in the dampening of the stress response (27-30), we speculate that a low maternal care-related increase in DNA methylation of the *OXTR* could provide a molecular mechanism contributing to the higher stress-reactivity (1, 2) and the increased risk to develop a mental disorders later in life, which is often observed after insufficient experience of parental care (4-6, 9). Similar studies in neuronal tissue (i.e. in human post-mortem brain tissue or animal models) could translate our results to oxytocin signaling in the brain or give information about tissue-specificity of experience-related changes in *OXTR* DNA methylation. This could have implications for the understanding of central processes associated with early life experiences and oxytocin-related behaviors (77-80). The indirect and direct effect from the mediation model were both non-significant (with p values around 0.1) suggesting that relative number of lymphocytes is neither a mediator in the association between maternal care and DNA methylation of *OXTR$_2$* nor does maternal care simply have a direct impact on DNA methylation, despite a significant total effect. This finding stresses the need to scrutinize potential mediators further, using a greater sample. Finally, it seems that changes in DNA methylation in the *OXTR* is target sequence specific, as we did not find any maternal care-related differences in *OXTR$_1$*. Whether these specific target sequences exhibit distinctive functional characteristics should be investigated in future studies.

Low maternal care did not predict DNA methylation of *OXTR$_1$* or *BDNF*. In a previous study by our group, DNA methylation changes were related to acute psychosocial stress in the

same assessed *OXTR1* target sequence (36), which was independent from stress-related changes in blood cell composition. Therefore, we hypothesize that DNA methylation in this target sequence could be more sensitive to acute psychosocial stress as compared to early life stress. In *BDNF*, we did neither observe any differences between the high and low maternal care group, nor dynamic changes in DNA methylation after acute psychosocial stress in the previous study investigating the identical *BDNF* target sequence (36). Whether DNA methylation in this *BDNF* target sequence measured in human peripheral blood cells is sensitive to other psychosocial stress experiences or environmental factors should be addressed in future studies. Taken together, findings from this and other studies imply that changes in DNA methylation are stressor- and target sequence specific, even within the same gene.

Finally, we found higher *OXTR2* DNA methylation in women compared to men. However, whether DNA methylation in the examined target sequence exhibits a functional relevance with regard to sex differences has to be elucidated in future studies.

Although the purpose of this study was not to analyze specific CpG units individually, we would additionally like to point out the strong DNA methylation difference between the maternal care groups in CpG unit 10 of *BDNF* (figure 2), which is located at an mRNA polymerase 2 binding site and could therefore have direct functional consequences for *BDNF* transcription (81, 82). Future studies could investigate this particular candidate CpG unit in more detail.

In sum, previous studies have indicated epigenetic alterations by early life stress in several stress-related genes across the whole genome in animal and human studies. In this study, we extended these findings to altered DNA methylation in an *OXTR* target sequence measured in human peripheral blood cells. Several questions should be addressed in future studies: i) is DNA methylation in this target sequence associated with peripheral OXTR

mRNA levels, and ii) what is the specific functional relevance of this sequence with special regard to maternal care?

This study has several strengths. First, this is the first study investigating changes in DNA methylation of *BDNF* and *OXTR* in the human periphery of subjects experiencing low maternal care compared to high maternal care. Second, we considered blood cell count as a potential mediator in the association between maternal care and DNA methylation. Third, due to the inclusion of two candidate genes and two different target sequences within one of the genes, the findings suggest that maternal care-related differences in DNA methylation may be target sequence specific, even within the same gene. Last, since the present data are hierarchical, with CpG units nested within genes, the use of covariance pattern models appears to represent a valuable extension to simpler models in which methylation values are first aggregated across CpGs and then analyzed using standard models like ANOVA or ANCOVA, which do not consider the internal consistency of CpG units. Indeed, our results suggest that CpG units exhibited different variances and covariances.

This study has the following limitations: First, due to the cross-sectional nature of the study, differences in DNA methylation between high and low maternal care cannot be interpreted causally. Second, an adverse maternal environment often comes with additional potentially stressful environmental factors, which could be linked to changes in DNA methylation. Third, the primary care person is not necessarily the child's mother. Therefore, future studies should also investigate care provided by fathers or other primary care persons. Fourth, maternal care was assessed retrospectively and the association between age and ratings of maternal care could indicate a bias. Fifth, *BDNF* and *OXTR* DNA methylation was measured in peripheral blood and findings cannot be translated into neuronal or other tissue types, although there is evidence that DNA methylation of some genes might be correlated across tissue (83). Sixth, the method applied to measure DNA methylation cannot distinguish

between DNA methylation and DNA hydroxymethylation, which is another cytosine modification with potentially regulatory functions (84). However, this modification is more abundant in neuronal cells and embryonic stem cells, as compared to other tissues (85, 86). Seventh, the sample was derived from an ad-hoc student sample and might not be representative of the general population. Last, mediation analyses should be replicated in a study with a greater sample size, as our data set produced instable results (i.e. we found only a trend for the direct effect of maternal care on $OXTR_2$ DNA methylation in the mediation models). Additionally, a further benefit for future studies with respect to the statistical analysis of this type of data could be the use of multilevel structural equation models explicitly taking measurement error into account (73).

In conclusion, DNA methylation in one *OXTR* target sequence was increased in peripheral blood of adults reporting low compared to those reporting high maternal care during childhood and adolescence. These results could improve the biological understanding of how maternal care influences the epiphenotype in humans.

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Supplemental information accompanying the manuscript

Association of Maternal Care and DNA Methylation (*BDNF, OXTR*) in Human Peripheral Blood Cells

Supplemental information S1

Target primer sequences

$OXTR_1$: (chromosome 3, nt 8809275-8809534)

CTGGTCAAGTACTTGCAGGTGGTGGGCATGTTCGCCTCCACCTACCTGCTGCTGCT
CATGTCCCTGGACCGCTGCCTGGCCATCTGCCAGCCGCTGCGCTCGCTGCGCCGC
CGCACCGACCGCCTGGCAGTGCTCGCCACGTGGCTCGGCTGCCTGGTGGCCAGCG
CGCCGCAGGTGCACATCTTCTCTCTGCGCGAGGTGGCTGACGGCGTCTTCGACTG
CTGGGCCGTCTTCATCCAGCCCTGGGGACCCAAGGCCTA

$OXTR_2$: (chromosome 3, nt 8809510-8809993)

GTGGAGTCTCCAGGAGTGGAGCCCCGGGCGCCCCTACACCCTCCGACACGCCGG
ATCCGGCCCAGCCGCGCCAAGCCGTAAAGGGCTCGAAGGCCGGGGCGCACCGCT
GCCGCCAGGGTCATGGAGGGCGCGCTCGCAGCCAACTGGAGCGCCGAGGCAGCC
AACGCCAGCGCCGCGCCGCCGGGGGCCGAGGGCAACCGCACCGCCGGACCCCCG
CGGCGCAACGAGGCCCTGGCGCGCGTGGAGGTGGCGGTGCTGTGTCTCATCCTGC
TCCTGGCGCTGAGCGGGAACGCGTGTGTGCTGCTGGCGCTGCGCACCACACGCCA
GAAGCACTCGCGCCTCTTCTTCTTCATGAAGCACCTAAGCATCGCCGACCTGGTG
GTGGCAGTGTTTCAGGTGCTGCCGCAGTTGCTGTGGGACATCACCTTCCGCTTCTA
CGGGCCCGACCTGCTGTGCCGCCTGGTCAAGTACTTGCAGGTGGTGG

BDNF: (chromosome 11, nt 27721543-27721857)

GGGGGAGAAAACTCCCCAAGAGTAACTCCAAATCGTCCCTTCTACCGGAGGGGA

GGAAAGAAGGAGACTGGCCTCGTCCCACAACTTTGGGGTGGGGGATCCCCCAGT

CAACTCTCTCCCGCGGACGGGCAGCTCCTGCACCAAGCCCCATTCCCAGCGCTTG

CCTACCTCGGGGTCCACACAAACCTCACGGGTCCCCGGCGGCGGAGTCACATCGT

GGTTCCGATTCTGGCTCCAGCGCCCAGCCCCGGTCCCCGTCGCGGTGCTGCTCCC

CGCCGGCCCCACAGCAGCGGTGGGTGTCTCATTAAAGCCCCC

Supplemental information S2

Supplemental table 1. Mean DNA methylation values and standard errors (*SE*) for all participants and differences between the maternal care groups (low - high maternal care). The final column shows the CpG sites corresponding the anaylzed CpG units yielded by Sequenom EpiTYPER.

		Methylation (%5meC)		Difference Methylation (%5meC)		
Gene	unit	Mean	*SE*	Mean Difference	*SE* of Mean Difference	Corresponding CpG sites
OXTR$_1$	1	5.11	0.256	0.649	0.511	1
	2	7.61	0.254	-0.117	0.512	2
	3	19.75	0.497	0.525	0.999	10, 11
	4	23.83	0.541	0.114	1.090	14 to 16
	5	10.85	0.491	0.202	0.988	17 to 18
	6	14.04	0.489	0.120	0.985	19 to 21
	7	11.37	0.487	0.866	0.977	22
OXTR$_2$	1	5.90	0.838	1.957	1.679	1, 2
	2	3.91	0.455	-0.163	0.919	3
	3	7.36	0.613	0.724	1.234	4, 5
	4	7.46	0.857	0.257	1.729	6
	5	3.78	0.412	0.375	0.829	7, 8
	6	12.07	1.399	2.220	2.813	9
	7	0.70	0.148	0.191	0.297	10
	8	5.60	0.198	0.346	0.397	11, 12
	9	2.33	0.484	-0.897	0.973	13, 14
	10	2.16	0.160	0.098	0.322	15 to 17
	11	4.77	0.824	2.074	1.650	20
	12	16.27	0.354	0.512	0.712	21 to 26
	13	1.63	0.185	0.195	0.373	28, 29
	14	3.38	0.302	-0.201	0.608	30 to 32
	15	4.43	0.275	0.036	0.555	34 to 36
	16	6.08	0.226	0.349	0.455	37
	17	3.16	0.167	0.352	0.335	38
	18	3.91	0.572	0.571	1.153	39
	19	7.05	0.907	1.648	1.826	45, 46
	20	31.41	2.426	3.935	4.877	47, 48
	21	7.16	0.537	-2.011	1.059	49
	22	5.67	0.322	0.017	0.649	50
	23	6.83	0.649	2.278	1.283	51, 52
	24	6.59	0.301	0.918	0.597	53

Supplemental table 1 (continuation). Mean DNA methylation values and standard errors (*SE*) for all participants and differences between the maternal care groups (low - high maternal care). The final column shows the CpG sites corresponding the anaylzed CpG units yielded by Sequenom EpiTYPER.

		Methylation (%5meC)		Difference Methylation (%5meC)		
Gene	CpG unit	Mean	*SE*	Mean Difference	*SE* of Mean Difference	Corresponding CpG sites
BDNF	3	14.19	0.969	1.632	1.943	4, 5
	4	3.37	0.584	1.497	1.163	6
	5	3.00	0.239	0.667	0.476	7
	6	3.92	0.136	0.004	0.274	8
	7	4.10	0.200	-0.528	0.399	9 to 12
	8	9.69	0.326	-0.343	0.654	13 to 14
	9	9.63	0.614	2.021	1.214	15
	10	15.43	2.020	11.289	3.853	22

Abbreviations: SE: Standard error; %5meC: Percent 5' methyl-cytosine methylation; *OXTR:* oxytocin receptor gene; *BDNF*: brain-derived neurotrophic factor gene

Appendix C:

Dynamic Changes in DNA Methylation of Stress-Associated Genes (*OXTR*, *BDNF*) after Acute Psychosocial Stress

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Dynamic changes in DNA methylation of stress-associated genes (*OXTR*, *BDNF*) after acute psychosocial stress

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Environmentally induced epigenetic alterations are related to mental health. We investigated quantitative DNA methylation status before and after an acute psychosocial stressor in two stress-related genes: oxytocin receptor (*OXTR*) and brain-derived neurotrophic factor (*BDNF*). The cross sectional study took place at the Division of Theoretical and Clinical Psychobiology, University of Trier, Germany and was conducted from February to August 2009. We included 83 participants aged 61–67 years. Thereof, 76 participants completed the full study procedure consisting of blood sampling before (pre-stress), 10 min after (post-stress) and 90 min after (follow-up) the Trier social stress test. We assessed quantitative DNA methylation of whole-blood cells using Sequenom EpiTYPER. Methylation status differed between sampling times in one target sequence of *OXTR* ($P<0.001$): methylation increased from pre- to post-stress ($P=0.009$) and decreased from post-stress to follow-up ($P<0.001$). This decrease was also found in a second target sequence of *OXTR* ($P=0.034$), where it lost statistical significance when blood cell count was statistically controlled. We did not detect any time-associated differences in methylation status of the examined *BDNF* region. The results suggest a dynamic regulation of DNA methylation in *OXTR*—which may in part reflect changes in blood cell composition—but not *BDNF* after acute psychosocial stress. This may enhance the understanding of how psychosocial events alter DNA methylation and could provide new insights into the etiology of mental disorders.
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Introduction

DNA methylation is an epigenetic mechanism related to mental and physical health and disease.[1–4] Aberrant DNA methylation has been implicated in the etiology of various mental disorders including, depression,[5–9] psychotic disorders,[10–15] post-traumatic stress disorder,[16,17] autism,[18,19] eating disorders[20,21] and substance dependence (for review see[22]), but also has an important role in the pathology of physical illnesses, such as cancer.[23] Thereby DNA methylation provides a biological basis for gene–environment interactions relevant to mental health[24]: animal and human studies have found that early life experiences can alter DNA methylation and affect gene expression and behavior.[25–32] Similarly, experiences later in life can modify the epigenome.[33,34] However, changes in DNA methylation immediately after adverse experiences, such as acute psychosocial stress, have not yet been investigated. Insight into how acute psychosocial stress affects DNA methylation may further elucidate our understanding of etiological mechanisms in mental health. Therefore, we investigated DNA methylation of two stress-related candidate genes—oxytocin receptor (*OXTR*)[35] and brain-derived neurotrophic factor (*BDNF*)[35,36]—before and after an acute psychosocial stressor.

We included the *OXTR* because the oxytocin system interacts with the hypothalamic-pituitary-adrenal axis[35,37–40] and cardiovascular stress reactivity.[41,42] To the best of our knowledge, there have been no studies investigating methylation of *OXTR* with reference to stress in humans or animals. A study on patients suffering from autism spectrum disorder revealed aberrant DNA methylation in an *OXTR* region in peripheral mononuclear blood cells; similar results were found for brain tissue.[43]

BDNF, the second candidate gene, encodes a neuronal growth factor involved in neuronal development, cell differentiation and synaptic plasticity.[44,45] In addition to its pivotal role in the central nervous system, *BDNF* is also expressed in the periphery where it shows neuro-protective action.[46] Peripheral BDNF concentration is decreased in various stress-related mental disorders[47] including depression[48] and post-traumatic stress disorder.[49] Previous work has also shown that early life- and chronic stress resulted in a higher methylation status of *Bdnf*,[32] and a decrease in *Bdnf* mRNA

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and BDNF protein levels in several rodent brain areas.[32,50–54] Animal studies have examined dynamic changes in DNA methylation of *Bdnf* associated with memory, learning and physical activity,[55–57] but not following a psychosocial stressor.

The aim of this study was to investigate dynamic changes in DNA methylation in stress-related genes after an acute psychosocial stressor.

Materials and methods

Participants and procedure. The sample of this cross-sectional study consisted of 76 adults. All participants underwent three sequential study parts:

In the first study part (2006–2007), we contacted Trier inhabitants born between 1942 and 1947. This population experienced war adversities early in life and was chosen as the overall goal of the initial project was to assess long-term consequences of early adversities. Of 2117 contacted adults, 365 completed psychological and medical questionnaires. In the second study part (2007–2008), we invited participants from the first study part for a psychological interview. Thereby, exclusion criteria were medical conditions potentially interfering with planned biological measures: impaired general health status, signs of acute infection, untreated hypertension (blood pressure $>$160/95 mm Hg during unstimulated conditions) or diabetes mellitus, intake of glucocorticoid-, immunosuppressive-, anti-depressant- or antidiabetic-medication, current therapy for a mental disorder and previous participation in a study applying the Trier social stress test (TSST).[58,59] We invited 274 persons, of whom 179 completed the interview. The third study part (2009) consisted of a laboratory session at the Division of Theoretical and Clinical Psychobiology, University of Trier, Germany. Those participants of the second study part who did not suffer from clinically relevant symptoms of depression (assessed by the German Version of the Center for Epidemiological Studies Depression Scale[60]) and did not meet the above-described exclusion criteria were invited for the third study part. Of the 127 invited participants, 83 took part in the third study part. Blood samples of seven participants did not contain enough blood for analysis and had to be excluded from the statistical analyses. Thus the final sample consisted of 76 adults—43 women and 33 men—aged between 61 and 67 years (mean age: 64.11 years; s.d.: 1.65 years). Participants gave written informed consent in accordance with the Declaration of Helsinki and received financial compensation. The Chamber of Physicians (Landesärztekammer Rheinland-Pfalz, Germany) approved the study protocol.

For the laboratory session of the third study wave, we requested participants to abstain from heavy sports and alcohol the evening before and on the day of testing. In addition, they were asked to have a regular meal on the testing day and to avoid eating and drinking 2 hours before arriving at the laboratory. Upon arrival, we acquainted the participants with the staff and informed them about the general procedures. A study physician undertook a medical examination and placed a peripheral venous catheter into the antecubital vein of the nondominant arm for multiple blood draws. A study assistant then conducted two memory tests before starting with the TSST, which took place in a remote room, equipped with a standing microphone and a video camera in front of two desks. The TSST consisted of a 3-min anticipation period and a 10-min test period, during which the participants had to undergo a fictitious job interview and perform mental arithmetics in front of one male and one female expert, trained in behavioral observation techniques, as well as in abstaining from giving any positive or negative social cues. The experts were of about the same age as the participants themselves, wore white doctor coats and used stop watches in order to check time. Study participants were informed that they would be video- and speech-taped during the whole test period for later evaluation of his or her performance and behavior. After the TSST, we accompanied the participants back to a study room, where they were asked to complete two additional memory tests and to fill in several questionnaires. The whole study session took 3.5 h.

Blood sampling, pre-analytics and blood cell count. At each blood sample collection, a study physician drew 5.5-ml blood from a peripheral venous catheter (Vasofix safety, Braun Melsungen AG, 18G, Melsungen, Germany) in EDTA-coated S-Monovettes (Sarstedt, Nuembrecht, Germany). Blood was taken 1 min before the TSST (pre-stress sampling), 10 min after the TSST (post-stress sampling) and 90 min after the TSST (follow-up sampling). To avoid acute orthostatic influences on pre-stress blood analyses, we asked persons to stand up and remain standing 10 min before the TSST until we collected the pre-stress blood sample.

EDTA samples collected for later blood counts were stored without centrifugation in a refrigerator until the end of the testing session. We delivered these samples to an external laboratory (SynLab Trier, Trier, Germany) the same day. Complete blood cell counts were obtained using an automated haematology analyzer (Sysmex XE2100i, Norderstedt, Germany).

Immediately after collection, EDTA samples for DNA methylation analysis were put on ice and centrifuged within 5 min (4000 rpm at $+6\,^{\circ}$C for 10 min) before freezing at –80 $^{\circ}$C. The QIAamp DNA Blood Midi (Qiagen, Hilden, Germany) was used to extract DNA, following the manufacturer's protocol. Samples were stored at $-20\,^{\circ}$C for subsequent DNA methylation analysis.

DNA methylation analysis. Genomic DNA (540 ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research, CA, USA) according to the manufacturers' standard protocol. Bisulfite PCR amplification of two target sequences in *OXTR* (*OXTR*$_1$, *OXTR*$_2$) and one target sequence in *BDNF* was conducted using Hot Star *Taq* DNA polymerase (Qiagen). The *OXTR*$_1$ target sequence is located in the protein-coding region of *OXTR* exon III; the *OXTR*$_2$ target sequence partly covers the noncoding and protein-coding promoter regions of *OXTR* exon III. Both target sequences were designed to cover the *OXTR* promoter region and the CpG island comprising exons I–III.[61] The *BDNF* target sequence around the 3′ end of *BDNF* exon VI is situated mainly within a CpG island that covers *BDNF* exons V, Vh and VI.[62] *BDNF* exon VI is frequently expressed especially in non-neuronal tissue of the periphery.[62] PCR products were prepared according to the

manufacturer's standard protocol for quantitative DNA methylation analysis using EpiTYPER 1.0 (Sequenom, CA, USA). For each run, a fully methylated positive control (New England BioLabs) and a blank control (distilled water) were included. The assays for the amplicons were designed using the Sequenom EpiDesigner software (for target sequences see Supplementary information).

Statistical analysis. The resolution of EpiTYPER yielded CpG units consisting of 1–6 individual CpG sites: 11 CpG units for *OXTR*$_1$, 28 CpG units for *OXTR*$_2$ and 12 CpG units for *BDNF*. Two CpG units in *OXTR*$_1$, one CpG unit in *OXTR*$_2$ and one CpG unit in *BDNF* could not be measured because of upper and lower detection limits of Sequenom EpiTYPER. CpG units with $>20\%$ missing data were excluded, which left eight CpGs units for *OXTR*$_1$, 27 for *OXTR*$_2$ and 10 for *BDNF* for statistical analyses. All sample CpG methylation values were compared with the values of the fully methylated positive control: If the value of the sample exceeded the value of the positive control, the value was set as missing data. All blank controls were negative. We identified suspicious samples by setting outlier values as missing data ($\geqslant 3$ s.d.'s from mean methylation of the respective CpG unit). By conducting missing analyses separately for each gene, samples with $>20\%$ missing data were identified and excluded from the statistical analyses of the respective gene. Methylation values (*OXTR*$_2$ and *BDNF*) were log-transformed to meet assumptions of normality and homoscedasticity. We analyzed time-associated changes in mean DNA methylation (averaged across CpG units) using multilevel models.[63] The three hierarchical levels were subjects, CpGs within subjects, and time within CpGs within subjects. Methylation values were allowed to vary across time for individual CpGs within subjects as this improved model fit. In a first step, we examined overall effects of sampling time and analyzed differences between the three sampling time points using *post-hoc* contrasts. In a second step, we tested the same overall effect of sampling time while including blood cell counts as covariates.[64] In both models, we included the identities of the bisulfite conversion plates and the Sequenom plates as covariates to negate laboratory batch effects. As we did not find any gender differences in DNA methylation, we did not include gender as potential confounder in the final models. We considered an alpha level of <0.05 as significant. All analyses were conducted using SPSS 20. DNA methylation values are presented as percent of cytosine methylation (%5MeC).

Results

Estimated means from the multilevel model of methylation (%5MeC) averaged across all CpG units of a target sequence and descriptive values of blood cell count are shown in Table 1.

Methylation of *OXTR*$_1$. We found an overall effect for sampling time on *OXTR*$_1$ mean methylation status. All *post-hoc* contrasts between sampling times were significant, with the greatest difference in mean methylation between post-stress and follow-up (Figure 1a, Table 2). Moreover, seven of eight individual CpG units within *OXTR*$_1$ revealed significant time effects (Figure 1b). Notably, when adjusting for blood cell counts, the overall effect of sampling time on *OXTR*$_1$ methylation averaged across CpG units remained significant. However, of the three *post-hoc* contrasts, the one between pre-stress and post-stress was no longer significant.

***Methylation of OXTR*$_2$.** We found a trend effect for sampling time in *OXTR*$_2$. *Post-hoc* contrast analyses indicated a difference in *OXTR*$_2$ mean methylation between post-stress and follow-up (Figure 2a, Table 2). Time effects were significant in two of 27 CpG units (Figure 2b). After adjustment for blood cell count, the overall effect for sampling time remained nonsignificant; the contrast between post-stress and follow-up was no longer significant.

Methylation of *BDNF* (Figure 3, Table 2). The analysis revealed no overall effect for sampling time on *BDNF* mean methylation and no *post-hoc* contrast was significant

Table 1 Estimated means and 95% confidence intervals (CI) from the multilevel model of methylation (%5MeC) averaged across CpG units of each target sequence and descriptive values of blood cell count for each sampling time (pre-stress, post-stress and follow-up)

	Sampling time					
	Pre-stress		***Post-stress***		***Follow-up***	
	M	***95% CI***	***M***	***95% CI***	***M***	***95% CI***
Methylation (%5MeC)						
OXTR$_1$	17.64	16.48 – 18.81	18.02	16.85 – 19.19	16.98	15.79 – 18.16
OXTR$_2$[a]	5	4.67 – 5.35	5.06	4.73 – 5.42	4.76	4.42 – 5.11
BDNF[a]	6.61	6.10 – 7.15	6.56	6.05 – 7.10	6.31	5.80 – 6.84
Blood cell count						
Leukocytes[b]	6.53	6.25 – 6.80	7.01	6.69 – 7.32	6.74	6.41 – 7.06
Lymphocytes[c]	30.59	28.98 – 32.21	32.61	30.85 – 34.36	26.74	25.09 – 28.40
Monocytes[c]	8	7.57 – 8.43	8.24	7.75 – 8.74	7.54	7.06 – 8.01
Granulocytes[c]	62.12	60.63 – 63.60	59.60	58.05 – 61.16	65.45	63.55 – 67.34

Abbreviations: *BDNF*, brain-derived neurotrophic factor; 95% CI, 95% confidence interval; M, mean; *OXTR*, oxytocin receptor.
[a]Estimates were re-transformed from natural logarithm to %5MeC; [b]Number $\times 10^3/\mu$l; [c]% of leukocytes.

(Figure 3a, Table 2). However, analysis of individual CpG units revealed a significant time effect in 1 of 10 CpG units (Figure 3b). The inclusion of blood cell count as covariates did not change these results.

Discussion

The aim of this study was to investigate immediate changes in DNA methylation in stress-related genes after acute

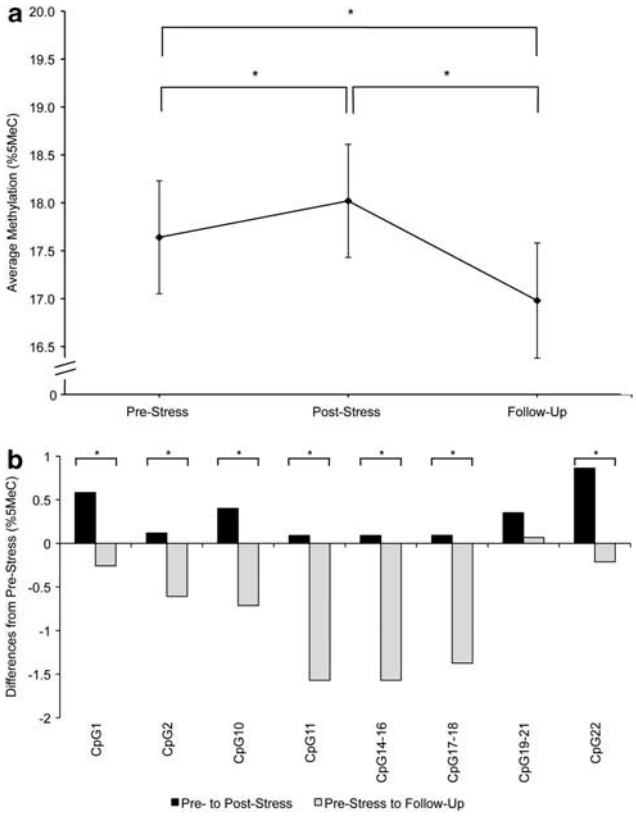

Figure 1 (**a**) Estimated mean DNA methylation level (%5MeC) in $OXTR_1$ amplicons averaged across CpGs at pre-stress, post-stress, and 90 min follow-up stress assessments. Error bars are s.e. of the estimated mean. (**b**) Differences in individual CpG mean methylation (%5MeC) from pre-stress to post-stress and from pre-stress to follow-up. All estimates obtained from the unadjusted model. $^*P<0.05$.

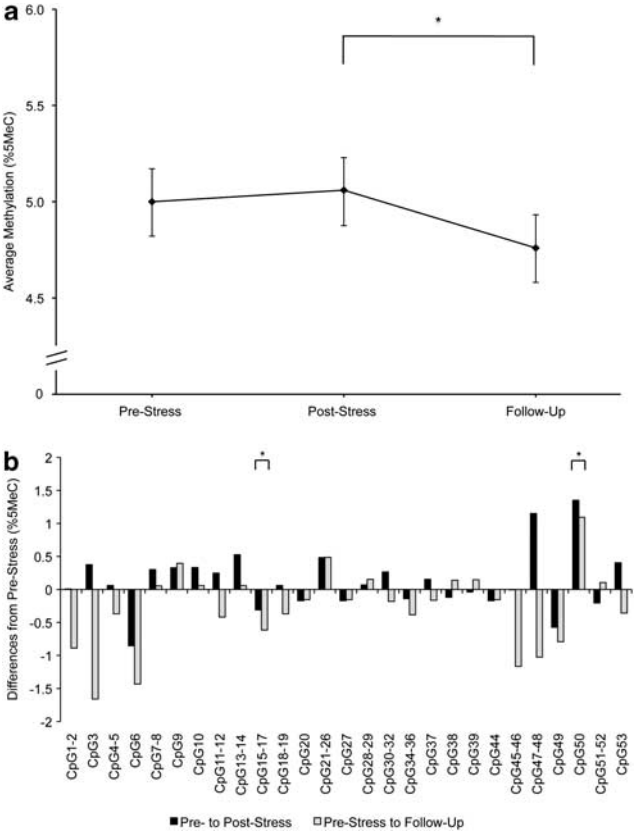

Figure 2 (**a**) Estimated mean DNA methylation level (%5MeC) in $OXTR_2$ amplicons averaged across CpGs at pre-stress, post-stress and 90 min follow-up stress assessments. Error bars are s.e. of the estimated mean. (**b**) Differences in individual CpG mean methylation (%5MeC) from pre-stress to post-stress and from pre-stress to follow-up. All estimates obtained from the unadjusted model. $^*P<0.05$.

Table 2 Overall effects of sampling time on mean methylation of $OXTR_1$, $OXTR_2$[a] and *BDNF*[a] without and with adjustment for blood cell count as covariates; *post-hoc* contrasts between sampling times pre-stress, post-stress and follow-up. Results based on multilevel analysis

	Main effects			*Contrasts*			*Number of observations*
	df[b]	**F**	**p**	***C1*** **p**	***C2*** **p**	***C3*** **p**	***N***
$OXTR_1$							
Overall model	2; 802	25.84	<0.001	0.009	<0.001	<0.001	600
Adjusted for blood cell count	2; 1133	10.70	<0.001	0.278	<0.001	<0.001	600
$OXTR_2$[a]							
Overall model	2; 2998	2.46	0.086	0.672	0.034	0.099	2045
Adjusted for blood cell count	2; 1368	1.92	0.146	0.536	0.058	0.137	2044
BDNF[a]							
Overall model	2; 1098	1.31	0.271	0.780	0.184	0.139	747
Adjusted for blood cell count	2; 1523	0.87	0.418	0.536	0.518	0.191	737

Abbreviations: *BDNF*, brain-derived neurotrophic factor; C1, contrast pre-stress versus post-stress; C2, contrast post-stress versus 90 min after stress; C3, contrast pre-stress versus 90 min after stress; *OXTR*, oxytocin receptor.
[a]Natural logarithm transformed; [b]Numerator; denominator.

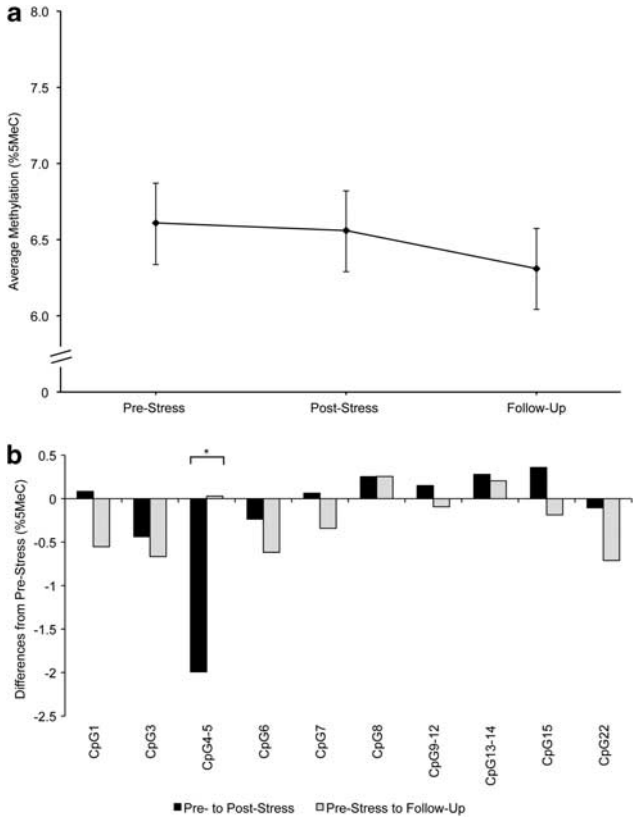

Figure 3 (**a**) Estimated mean DNA methylation level (%5MeC) in *BDNF* amplicons averaged across CpGs at pre-stress, post-stress and 90 min follow-up stress assessments. Error bars are s.e. of the estimated mean. (**b**) Differences in individual CpG mean methylation (%5MeC) from pre-stress to post-stress and from pre-stress to follow-up. All estimates obtained from the unadjusted model. $^{*}P<0.05$.

psychosocial stress. We found stress-associated DNA methylation changes in one of two *OXTR* target sequences but not in the assessed target sequence of *BDNF*, suggesting a considerable variation in the sensitivity of short-term DNA methylation responses among different stress-related genes. For $OXTR_1$, we found an increase in DNA methylation from pre-stress to post-stress and a decrease from post-stress to follow-up. In $OXTR_2$, methylation decreased from post-stress to follow-up only. Notably, in $OXTR_1$ the time-associated changes, as well as the difference from post-stress to follow-up, remained significant even after controlling for blood cell count. The changes from pre-stress to post-stress in $OXTR_1$ and from post-stress to follow-up in $OXTR_2$ may have been secondary to stress-associated changes in blood cell composition.[65]

Although (i) methylation increase in *OXTR* is associated with decreased *OXTR* expression[61] and (ii) the oxytocin system antagonizes the short-term stress response,[37,41] methylation increase from pre- to post-stress in $OXTR_1$ could constitute a part of the immediate stress response, which relies on rapid autonomic sympathetic activation to mobilize resources and increase performance.[66] After the stressor had passed, DNA methylation of the *OXTR* not only receded back to pre-stress baseline, but also fell below pre-stress levels. This could indicate an overcompensating mechanism in *OXTR* methylation after acute psychosocial stress, allowing for an upregulation of the oxytocin system as a middle-term physiological buffer of the acute stress response. Previous studies have shown that the oxytocin system has an essential role in the regulation of blood pressure and volume, heart rate and cardiovascular homeostasis, as well as in the cardiovascular response to stress.[42,67,68] Therefore, a decrease in DNA methylation of the *OXTR* and the subsequent increase in expression[43] may indeed be a potential mechanism to support physiological recovery after acute stress on an epigenetic level.

Regarding *BDNF*, our results suggest that in the periphery, DNA methylation in *BDNF* remains stable after a short and non-recurring psychosocial stressor. Previous studies found lifelong and transgenerational perpetuation of changes in *BDNF* methylation after early-life adversity.[32] Fuchikami *et al*.[69] recently suggested DNA methylation of *BDNF* in peripheral blood as a diagnostic biomarker of major depression. These results and our finding implicate that *BDNF* methylation has a long-term, rather than a short-term, role in stress adaptation.

This study has several strengths: First, the TSST is a highly established and robust standardized protocol to induce psychosocial stress and a robust hypothalamic-pituitary-adrenal axis activation.[70] Various biological markers of acute stress have been investigated in relation to the TSST. Here we extend previous findings, by adding DNA methylation changes in *OXTR* as an additional biomarker of acute psychosocial stress, especially from post-stress to 90 min after the stressor. Second, we included blood cell count as a time-varying covariate into the analyses to ensure that DNA methylation changes were not the result of alterations in blood cell composition in response to stress.[64] Indeed, our results highlight the necessity to consider blood cell count in the analyses while investigating DNA methylation in the periphery. Third, DNA methylation was not only assessed at pre- and post-stress, but also after a time interval of 90 min, which provided insight into methylation changes after stress recovery. Fourth, the focus on not only one, but on multiple genes (*OXTR* and *BDNF*) and target sequences revealed remarkable specificity of the short-term DNA methylation response of individual stress-related genes.

Several limitations of this study should also be noted: First, we measured DNA methylation in peripheral blood, which does not allow us to directly draw conclusions about processes in the central nervous system. To what degree DNA methylation in the periphery corresponds to DNA methylation in the brain remains to be elucidated, although some studies suggest certain consistency across tissues.[43,71,72] Second, we did not apply an unstressed control group and can therefore not completely exclude that DNA methylation changes were due to factors unrelated to the psychosocial stress experience. Third, we analyzed DNA methylation changes after acute psychosocial stress in a study population with high likelihood of early experiences of war-related adversities, who may have been sensitized to stress. As a consequence, study subjects might have been especially susceptible to changes in *OXTR* DNA methylation after acute psychosocial stress. Therefore, generalizability of our results to populations without early adversities may be limited.

Fourth, it should be noted that differences in mean DNA methylation between time points were small, and the functional effects of such modest alterations are not known. In this context, however, the following should be considered: (i) We did not compare different study groups, but assessed changes in DNA methylation over time within the same individuals. Therefore mean values in methylation are not independent of each other and differences are expected to be smaller in contrast to between-group comparison. (ii) Changes in DNA methylation were larger for several individual CpG units than for averaged target sequences (Figures 1–3). (iii) The absolute change of 1% in methylation of $OXTR_1$ (%5MeC) from post-stress to follow-up represents a relative change of 5–6%. (iv) DNA methylation changes may accumulate and increase in magnitude in case of repeated psychosocial stress experience.

Future studies should replicate our findings for *OXTR* and *BDNF*, but also include additional stress-related candidate genes. Furthermore, we suggest shortening blood-sampling intervals to identify the time point of greatest DNA methylation changes. In addition, future studies could assess DNA methylation not only after different stressors, but also after positive experiences to determine whether DNA methylation is sensitive not only to aversive but also to positive psychosocial experiences. Moreover, subjects from other populations (such as cohorts without increased likelihood of early adversities) should be studied to scrutinize the generalizability of our results. Finally, future studies should assess DNA methylation after repeated psychosocial experiences to elucidate possible long-term modifications in DNA methylation. Identifying and studying short- and long-term effects of psychosocial experiences—which for example could reverse aberrant DNA methylation—could become an important goal in the development of new treatment approaches.

Conclusion

To the best of our knowledge, this is the first study in humans investigating dynamic short-term changes in DNA methylation related to a specific life event, namely a psychosocial stressor. We found different DNA methylation states in the *OXTR* when comparing pre-stress, post-stress and 90-min follow-up stress measurement. These findings contribute to the understanding of epigenetic mechanisms in general, but may also have clinical significance in the future: We found that psychosocial experiences are linked to immediate epigenetic modifications in a sample of subjects with early adverse experiences. This could have clinical implications regarding the etiology of mental and stress-related disorders, as well as of general medical conditions.

Conflict of Interest

The authors declare no conflict of interest.

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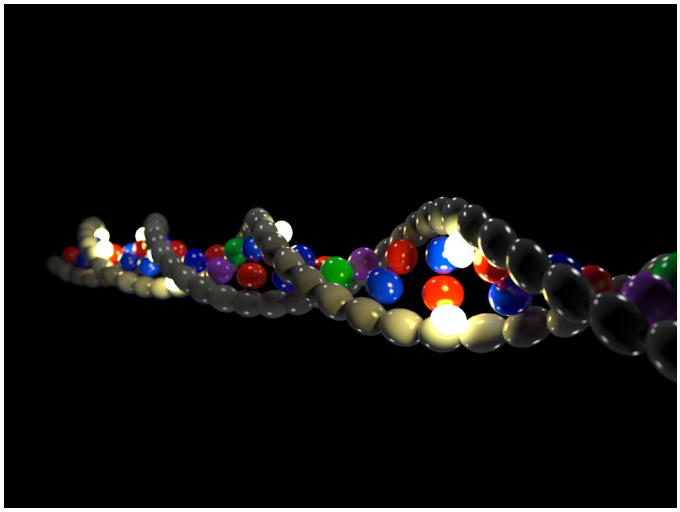
Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

Appendix D:



DNA Methylation Analysis

DNA Methylation Analysis

The aim of Appendix D is to document the methods applied to measure DNA methylation in whole blood samples. While sample collection and DNA extraction differed between studies, DNA methylation analysis was performed identically for all three studies reported here. Blood samples were collected in cord blood at birth or from brachial vein (study 2 and 3). Blood was frozen at –20°C until DNA extraction, which was performed using different standardized protocols for DNA extraction from whole blood: (1) prenatal stress: Gentra Puregene Cell Kit (Qiagen, Hilden, Germany); (2) low maternal care: Puregene (Qiagen, Venlo, The Netherlands); (3) acute psychosocial stress: QIAamp DNA Blood Midi (Qiagen, Hilden, Germany). Extracted DNA was frozen at –20°C and sent to the labs of Prof. Jonathan Mill at the King's College in London. DNA was i) bisulfite converted, ii) amplified by bisulfite PCR and iii) DNA methylation was quantified using Sequenom EpiTYPER. The following sections are aimed at documenting the three steps.

Bisulfite Conversion

All DNA samples were diluted to obtain 50µl of DNA solutions with concentration of 12ng DNA per µl. Dilutions were prepared in 96-well plates. Subsequently, DNA was treated with sodium-bisulfite, which converts unmethylated cytosine into uracil, while methylated cytosine is "protected" against this substitution. We applied the EZ-96 DNA methylation (ZYMO research, CA, USA) standard protocol (table D1). In each run, we included one well containing a negative control (destilled water) and one well containing a fully methylated positive control.

Table D1
Protocol for Bisulfite Conversion using EZ-96 DNA Methylation Kit (ZYMO Research)

Step	Quantity	Reagent/Material	Process
Preparation Day 1: Preparation			
	1 Bottle	CT Conversion Reagent	
	7.5ml	H2O	mix @ room temp with vortex
	2.1ml	M-Dilution Buffer	*10 minutes*
Protocol Day 1			
1	5ul	M-Dilution Buffer	96-well plate
	45ul	DNA sample	add, mix, close
2			incubate at 37°C *15 minutes*
3	100ul	CT-Conversion Reagent	add, mix, close with alu lid
4	96-Well	plate	incubate on heat block *over night*
Protocol Day 2: Preparation			
	1 Bottle	M-Wash Buffer	
	144ml	100% Ethanol	Add, mix
Protocol Day 2			
5	96-Well	plate	put on ice *10 minutes*
6	400ul	M-Binding Buffer	silicon-A binding plate on collection
7	150ul	DNA sample	add, mix (pipette)
8	96-Well	plate	centrifuge at 2000g for 5 minutes empty collection plate and dry
9	500ul	M-Wash Buffer	centrifuge at 2000g for 5 minutes empty collection plate and dry
10	200ul	M-Desulphonation Buffer	add incubate at room temp. *20 minutes* centrifuge at max 5 minutes
11-a	500ul	M-Wash Buffer	centrifuge at 2000g for 5 minutes empty collection plate and dry
11-b	500ul	M-Wash Buffer	centrifuge at 2000g for 10 minutes empty collection plate and dry
12	96-Well	Silicon-A Binding Plate	put on elution plate
12-a	25ul	M-Elution Buffer	add, centrifuge at 2000g for 3 minutes
12-b	25ul	M-Elution Buffer	add, centrifuge at 2000g for 3 minutes
13	96-Well	Elution plate	put on ice

For each run, four test samples of bisulfite converted DNA (single stranded) were measured using NanoDrop (Thermo Scientific, Wilmington, USA).

Bisulfite PCR

2.5µl of bisulfite converted DNA was pipetted into a 96-well plates and mixed with 7.5 µl matrix mix solution (table 2). Selected target sequences (*$OXTR_1$*: chromosome 3, nt 8809275-8809534; *$OXTR_2$*: chromosome 3, nt 8809510-8809993; *BDNF*: chromosome 11, nt 27721543-27721857) were amplified using bisulfite polymerase chain reaction (PCR, Qiagen Hotstart Taq Polymerase). During this process, nucleotides containing uracil become thymine, which can be detected in DNA sequencing and compared to the template sequence. Tagged primers were the following:

BDNF left: aggaagagagGGGGGAGAAAATTTTTTAAGAGTAA

BDNF right: cagtaatacgactcactatagggagaaggctAAAAACTTTAATAAAACACCCACC

$OXTR_1$ left: aggaagagagTTGGTTAAGTATTTGTAGGTGGTGG

$OXTR_1$ right: cagtaatacgactcactatagggagaaggctTAAACCTTAAATCCCCAAAACTAAA

$OXTR_2$ left: aggaagagagGTGGAGTTTTTAGGAGTGGAGTTT

$OXTR_2$ right: cagtaatacgactcactatagggagaaggctCCACCACCTACAAATACTTAACCAA

Table D2
Recipes for Matrix Solution and Instructions for Bisulfite PCR

Reagent	*BDNF*	*OXTR$_1$*	*OXTR$_2$*
H_2O (µl/sample)	5.0	5.2	5.2
PCR-Buffer (µl/sample)	1.0	1.0	1.0
dNTPs (µl/sample)	0.2	0.2	0.2
$MgCl_2$ (µl/sample)	0.2	-	-
Primer F (µl/sample)	0.5	0.5	0.5
Primer R (µl/sample)	0.5	0.5	0.5
H-Taq (µl/sample)	0.1	0.1	0.1
PCR program duration			
15:00 minutes (Start)	95°C	95°C	95°C
00:30 Minutes	95°C	95°C	95°C
00:30 Minutes	56°C	56°C	57°C
01:00 Minutes	72°C	72°C	72°C
Repeats	44	44	44
10:00 Minutes (Finish)	72°C	72°C	72°C

Notes. PCR=Polymerase chain reaction; dNTP=deoxyribonucleoside triphosphate; F=forward, R=reverse, H-Taq=Hotstart Taq Polymerase; *BDNF*=brain-derived neurotrophic factor; *OXTR*=oxytocin receptor

PCR products (4 µl) were checked by gel-electrophoresis using orange-G (2 µl) (Sigma-Aldrich Co., St. Louis, U.S.A.) on bromide containing 1.5% agarose gel. Bisulfite treated DNA samples (4 µl) were mixed with orange-G (2 µl). The PCR run was considered successful if i) the PCR product accumulated nicely in a thin stripe; ii) PCR products were more distinct than primer-dimers; and iii) the negative controls (distilled water) was not contaminated (no signal). Figure D1 shows an example of a successful and an unsuccessful PCR run.

A) B)

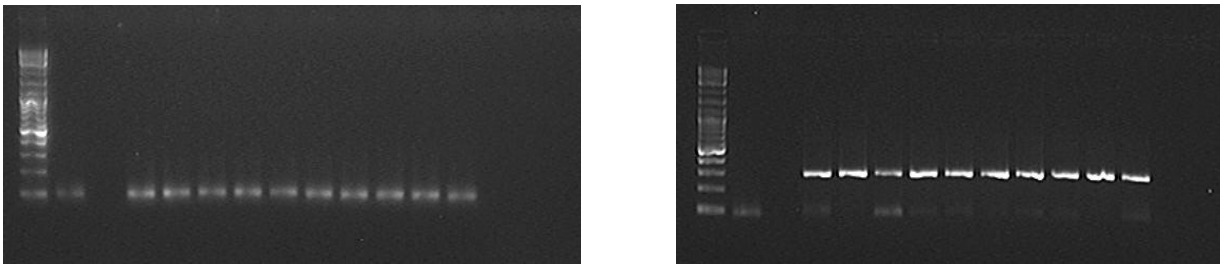


Figure 1. Results from gel-electrophoresis. An example of an A) unsuccessful and an B) successful PCR run.

Sequenom EpiTYPER

DNA methylation was quantified using the standard protocol for Sequenom EpiTYPER, which applies base-specific cleavage and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). This method detects differences in signal intensity between masse signals derived from methylated versus non-methylated DNA (EpiTYPER 1.0 Software User's Guide).

There is no single CpG resolution of Sequenom EpiTYPER, but some CpGs are combined into CpG units (table D3). Often, CpG units containing a high number of CpG sites could not be analyzed due to high or low detection limits. Furthermore, DNA methylation values with more than 20% missing data were excluded from the analysis. Samples with > 50% missing data or outlier methylation values (> 3 standard deviations from mean DNA methylation values of respective CpG unit) were repeated.

Table D3
Gene-Specific CpG Units with Corresponding CpG Sites

Gene	CpG unit	Corresponding CpG sites	Gene	CpG unit	Corresponding CpG sites
OXTR$_1$	1	1	*OXTR*$_2$	1	1, 2
	2	2		2	3
	3	10, 11		3	4, 5
	4	14 to 16		4	6
	5	17 to 18		5	7, 8
	6	19 to 21		6	9
	7	22		7	10
				8	11, 12
BDNF	1	1		9	13, 14
	2	3		10	15 to 17
	3	4, 5		11	20
	4	6		12	21 to 26
	5	7		13	28, 29
	6	8		14	30 to 32
	7	9 to 12		15	34 to 36
	8	13 to 14		16	37
	9	15		17	38
	10	22		18	39
				19	45, 46
				20	47, 48
				21	49
				22	50
				23	51, 52
				24	53

Notes. *BDNF*=brain-derived neurotrophic factor; *OXTR*=oxytocin receptor; CpG=cytosine-guanine dinucleotide