Citation: Transl Psychiatry (2012) 2, e150; doi:10.1038/tp.2012.77

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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.
Translational Psychiatry (2012) **2,** e150; doi:10.1038/tp.2012.77; published online 14 August 2012

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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Keywords: acute psychosocial stress; brain-derived neurotrophic factor gene (*BDNF*); dynamic DNA methylation; epigenetics; oxytocin receptor gene (*OXTR*)
Received 9 April 2012; revised 18 June 2012; accepted 4 July 2012

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

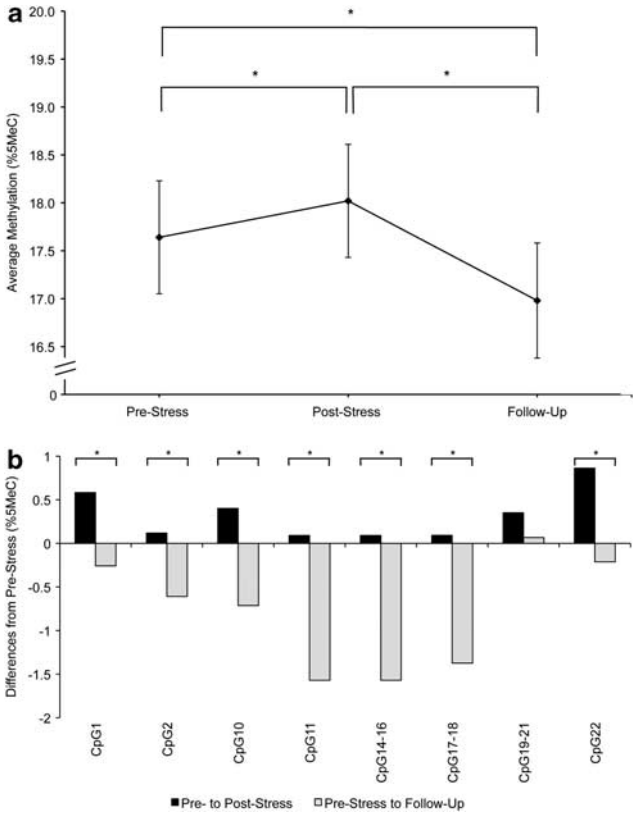

Figure 1 (**a**) Estimated mean DNA methylation level (%5MeC) in *OXTR*$_1$ amplicons averaged across CpGs at pre-stress, post-stress, and 90 min follow-up stress assessments. Error bars are s.e. of the estimated mean. (**b**) Differences in individual CpG mean methylation (%5MeC) from pre-stress to post-stress and from pre-stress to follow-up. All estimates obtained from the unadjusted model. *$P<0.05$.

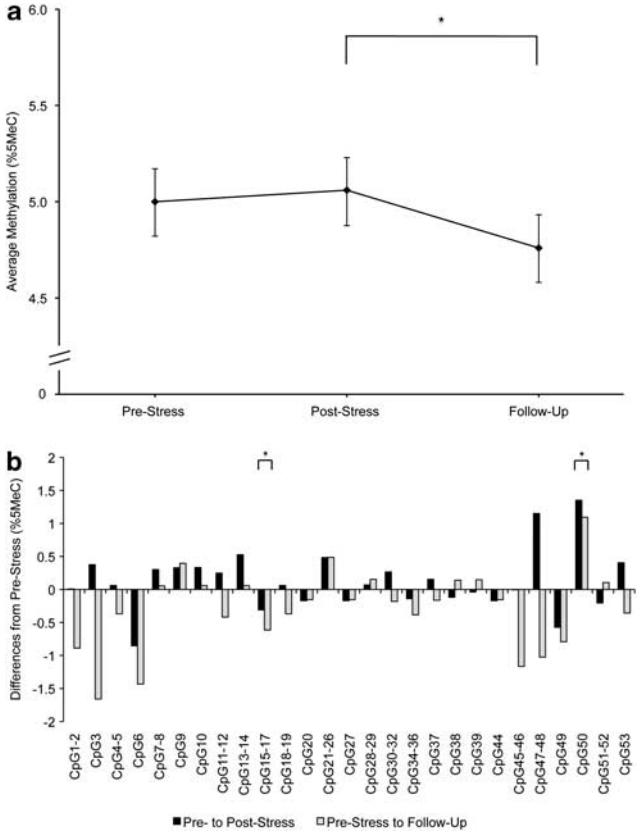

Figure 2 (**a**) Estimated mean DNA methylation level (%5MeC) in *OXTR*$_2$ amplicons averaged across CpGs at pre-stress, post-stress and 90 min follow-up stress assessments. Error bars are s.e. of the estimated mean. (**b**) Differences in individual CpG mean methylation (%5MeC) from pre-stress to post-stress 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.

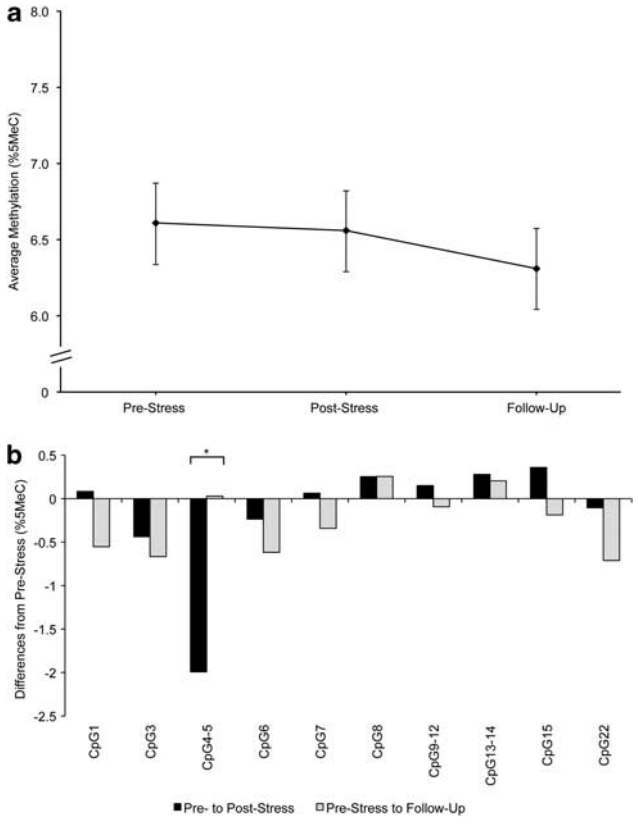

Figure 3 (**a**) Estimated mean DNA methylation level (%5MeC) in *BDNF* amplicons averaged across CpGs at pre-stress, post-stress and 90 min follow-up stress assessments. Error bars are s.e. of the estimated mean. (**b**) Differences in individual CpG mean methylation (%5MeC) from pre-stress to post-stress 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.

Acknowledgements. This work was supported by a grant of the German Research Foundation to DH (DFG grant number HE 1013/21-1 and HE 1013/21-2) and by grants of the Swiss National Science Foundation to GM and RL (project number 100014-135328 and project number 51A240-104890). We thank all people involved in data collection and entry, especially Malgorzata Kaszynska, Stephanie Hartmann, Dr Andrea Gierens and Celine Pleimling from the Trier lab and research team, everyone supporting the biological analyses in Basel, as well as the staff in the laboratories of the 'MRC Social, Genetics and Developmental Psychiatry center' of the King's College in London.

Disclaimer. The authors alone are responsible for the content and writing of the manuscript. The funders had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

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