DNA methylation profiles within the serotonin transporter gene moderate the association of 5-HTTLPR and cortisol stress reactivity

N Alexander1,4, M Wankerl1,4, J Hennig2, R Miller1, S Zänkert3, S Steudte-Schmiedgen, T Stalder1 and C Kirschbaum1

INTRODUCTION

Altered regulation of the hypothalamus–pituitary adrenal (HPA) axis, one of the body’s major stress systems, has been implicated as a correlate or even causal factor for a broad range of stress-related psychiatric disorders. While it is widely recognized that genetic factors substantially contribute to individual differences in HPA-axis reactivity, the search for robust associations with specific genetic variants has proven challenging. Among others, a large number of neuroendocrine studies have investigated a 43-bp insertion/deletion polymorphism (5-HTTLPR) in the serotonin transporter gene (SLC6A4) which comprises a short (S) and a long (L) allelic variant. This line of research is motivated by a strong biological and clinical rationale. First, the S allele has been repeatedly linked to a lower transcriptional efficiency of the SLC6A4 gene and thus potentially alters serotonergic modulation of HPA-axis activity. Second, the debate about whether the S allele conveys vulnerability to depression upon exposure to environmental adversity led to a growing interest in the biological pathways mediating genotype-dependent stress sensitivity. Consequently, several experimental studies have explored the association of 5-HTTLPR and cortisol stress reactivity to standardized laboratory stressors. In support of the stress sensitivity hypothesis, a recent meta-analysis confirms an overall increased HPA-axis reactivity in individuals carrying two copies of the S allele. However, the size of this effect was small with substantial variation between individual study outcomes, which highlights the need to explore additional sources of variance contributing to observed inconsistencies.

A more powerful and comprehensive approach in neuroendocrine candidate gene studies might be to account for functional variation within SLC6A4 both on a genetic and epigenetic level. Previous research has identified cytosine methylation within a 799-bp promoter-associated CpG island of the SLC6A4 gene as a functionally relevant marker of reduced serotonin transporter (SERT) expression. Given that SLC6A4 methylation profiles appear to be sensitive to early-life stress in several studies, they reflect a putative pathway explaining how environmental adversity might translate into stress and disease susceptibility. Indeed, initial evidence suggests an association of SLC6A4 methylation levels measured in peripheral cells and depression in one study dependent on 5-HTTLPR genotype. It is thus conceivable that in addition to genetic variation, epigenetic changes within SLC6A4 may also relate to differences in endocrine stress reactivity. To date, only one recent study on 28 monozygotic twin pairs discordant for bullying victimization has examined neuroendocrine correlates of epigenetic changes in SLC6A4. This study reports increased SLC6A4 methylation levels within the serotonin transporter gene as a correlate of bullying victimization.
methylation at 1 out of the 12 CpG sites studied in bullied twins which in turn was related to a blunted cortisol stress response. Such epigenetic recalibration of the neuroendocrine system presumably reflects a flexible adaption to environmental demands, but may also convey disease vulnerability if the programmed response is not adequate anymore.34

The current study is the first to investigate combined effects of functional genetic and epigenetic variation in the SLC6A4 gene on endocrine stress reactivity. Our major aim was to test whether SLC6A4 methylation modulates allele-specific cortisol response patterns and whether a larger portion of variance can be explained when both factors are taken into account. Respective findings are used to evaluate whether studying epigenetic marks might help to detect more robust effects in neuroendocrine and clinical studies investigating the 5-HTTLPR stress sensitivity hypothesis.

MATERIALS AND METHODS
Sample and procedure
The study sample comprised two hundred healthy participants (n = 100 females) aged 18–30 years. One hundred and thirty three participants from a previous study investigating associations between environmental adversity, SLC6A4 methylation and mRNA expression patterns were re-invited for the present study.12 This initial sample was extended by another sixty seven individuals who were exposed to exactly the same procedure. We included only Caucasians who were native German speakers. Exclusion criteria were current or past mental and/or physical diseases, medication intake (for example, psychotropic drugs, substances known to in

Hormone assays
Saliva samples were collected by means of cotton swabs (Salivettes, Sarstedt). Participants were instructed to chew on the swabs for 3 min to stimulate saliva flow. Cotton rolls were then transferred to plastic containers and stored at −20°C. For cortisol analyses, saliva samples were thawed and centrifuged at 3000 r.p.m. for 3 min. Salivary-free cortisol levels were measured using commercially available chemiluminescence-immunoassays (CLIA; IBL, Hamburg, Germany) with intra- and inter-assay precision of 3.0 and 4.2%.

Statistical analyses
Statistical analyses were conducted using SPSS (Version 21.0, IBM, Chicago, IL, USA). All statistical tests were two-tailed with alpha set at P < 0.05. Initial group comparisons were conducted using χ²-tests and analyses of variance (ANOVA). Given that Kolmogorov-Smirnov testing indicated a deviation from a normal distribution for absolute cortisol values, all analyses were based on natural log-transformed values. For descriptive purposes, mean data in tables and figures are presented in original units. To test for effects of 5-HTTLPR, SLC6A4 methylation (mean methylation levels across the 83 CpG sites) and their interaction on cortisol reactivity, mixed-design analyses of covariance with repeated measures (cortisol samples) and baseline cortisol values as covariate were conducted. Regarding S-HTTLPR as between-subject factor, analyses of covariance were calculated on a genotype level (SS vs SL vs LL) and for completeness also according to an L

5-HTTLPR genotyping
DNA was extracted from EDTA whole blood samples by means of standard commercial extraction kits (High Pure PCR Template Preparation Kit; Roche, Mannheim, Germany) in a MagNA Pure LC System (Roche). Genotyping was performed according to a previously published protocol.2 The participants were further genotyped for an A/G single-nucleotide polymorphism (rs25531) within the length polymorphism which results in the distinction between the variants L₄ and L₅, with the latter being functionally similar to the 5 allele.13 This allows for conducting analyses based on the 5-HTTLPR/ rs25531 mini-haplotype by comparing low (L₄/S) and high (L₅) expressing variants.

Bisulfite pyrosequencing
Quantitative methylation analysis of 83 CpG sites within a previously described19 799-bp CpG island in SLC6A4 (Supplementary Information 1) was performed by Varionostic GmbH (Ulm, Germany, http://www.varionostic.de). Genomic DNA extracted from EDTA whole blood was bisulfite-treated using the EZ DNA Methylation Gold Kit (Zymo Research, Range, CA, USA) and subsequent pyrosequencing was performed on the Q24/ID System. A detailed protocol has been published elsewhere.27 Mean SLC6A4 methylation levels across the CpG island were calculated for those participants where methylation values for at least 90% of the 83 CpG sites passed a strict quality control, which led to the exclusion of 14 individuals. Consequently, all subsequent analyses on SLC6A4 methylation refer to a sample of N = 186.

Standardized laboratory stress test: TSST
All participants were exposed to the Trier Social Stress Test (TSST),41 a standardized protocol known to reliably elicit robust cortisol increases.42 The cortisol response to the TSST is characterized by substantial intraindividual stability across repeated test session along with a moderate heritability,2,3 which highlights the use of this procedure for (epi)genetic association studies. In short, the TSST consists of a public speaking (5 min) and a mental arithmetic (5 min) task performed in front of two evaluating panelists. During the experimental procedure, seven saliva samples were collected before onset of the TSST (after a 30 min resting period) as well as 1, 10, 20, 30, 45 and 60 min after stress exposure. Experimental sessions started between 1330 and 1500 hours to reduce variability in the circadian cortisol rhythm. Participants were instructed to refrain from physical exercising, smoking, eating and drinking anything but water 1 h before test sessions. For females, TSST appointments were scheduled during the second half of the menstrual cycle only (corresponding to the luteal phase in women free of oral contraceptives). To avoid creating a highly selective sample within this age group, smokers and oral contraceptive users were not excluded but these variables were treated as potential confounders in statistical analyses.

Consequently, all subsequent analyses on SLC6A4 methylation refer to a sample of N = 186.
RESULTS

Sample characteristics

Table 1 shows sample characteristics for participants grouped by 5-HTTLPR genotype or SLC6A4 methylation level. There was no significant deviation from Hardy–Weinberg equilibrium using bi-allelic \((x_1^2) = 0.82, P = 0.37\) or the 5-HTTLPR/rs25531 mini-haplotype \((x_1^2) = 2.33, P = 0.13\) classification. As previously reported in a subsample, \(^7\) we observed substantial interindividual variation regarding absolute SLC6A4 methylation levels (Supplementary Information 1). Mean SLC6A4 methylation levels were comparable between groups separated by 5-HTTLPR genotype \((F_{1,182} = 0.12, P = 0.89)\). Participants grouped by 5-HTTLPR genotype or SLC6A4 methylation level did not differ regarding sex, age, BMI, smoking status, oral contraceptive use depression score, childhood maltreatment and recent trauma/stress exposure (all \(P\)-values \(>0.11\)).

Main effects of 5-HTTLPR and SLC6A4 methylation on cortisol stress reactivity

The TSST induced significant increases in salivary cortisol across the overall sample \((F_{6,1194} = 198.95, P < 0.001, \eta^2 = 0.50)\). Table 2 displays cortisol levels in response to the TSST as a function of 5-HTTLPR and SLC6A4 methylation levels. Despite a nominal trend for higher HPA-axis reactivity in S allele carriers, analyses of covariance revealed no significant effect of 5-HTTLPR on cortisol response patterns when comparing individuals on a genotype (genotype \(x_{time}: F_{2,196} = 0.98, P = 0.378\); genotype \(x_{time}: F_{12,1745} = 1.45, P = 0.137\) or allele-specific level \((S\) allele dominant model, genotype: \(F_{1,197} = 1.95, P = 0.165\), genotype \(x_{time}: F_{6,182} = 1.29, P = 0.26\); \(L\) allele dominant model, genotype: \(F_{1,197} = 0.90, P = 0.771\), genotype \(x_{time}: F_{6,182} = 1.32, P = 0.244\). When reanalyzing the data on the basis of the 5-HTTLPR/rs25531 mini-haplotype, we observed a trend for carriers of two low-expressing alleles to have higher cortisol responses compared with individual with at least one high-expressing allele (genotype \(F_{1,197} = 1.752, P = 0.187\); genotype \(x_{time}: F_{6,182} = 2.05, P = 0.056, \eta^2 = 0.01\)). The latter effect reached significance when controlling for sex, smoking status and use of oral contraceptives (genotype \(x_{time}: F_{6,182} = 2.34, P = 0.03, \eta^2 = 0.01\), however, explained variance in cortisol reactivity was small. Again, no differences in cortisol response patterns were found on a genotype level (genotype \(F_{2,196} = 1.03, P = 0.361\); genotype \(x_{time}: F_{12,1745} = 1.39, P = 0.162\) or when applying a dominant model for the high-expressing alleles (genotype \(F_{1,197} = 0.82, P = 0.366\); genotype \(x_{time}: F_{6,182} = 1.08, P = 0.373\)).

Regarding main effects of epigenetic modifications in SLC6A4, analyses of covariance revealed no significant differences in cortisol response patterns between individuals characterized by high or low mean SLC6A4 methylation levels (methylation: \(F_{1,183} = 0.41, P = 0.839\), methylation \(x_{time}: F_{6,1096} = 0.36, P = 0.905\)). Accordingly, mean SLC6A4 methylation levels were unrelated to the cortisol AUC \((r = 0.018, P = 0.805)\). We further conducted exploratory analyses on the level of individual CpG sites, which generally confirmed the absence of a significant association of SLC6A4 methylation and cortisol stress reactivity. Methylation levels at 3 out of 83 CpG sites \((CpG35: r = 0.14, P = 0.045, CpG51: r = -0.19, P = 0.008, CpG64: r = 0.16, P = 0.031)\) were nominally associated with cortisol AUC \((\text{before Bonferroni adjustment})\). However, these associations are likely to result from chance \((P < 0.05, n = 83) = 0.79\).
Interaction of genetic and epigenetic variation in the SLC6A4 gene on cortisol stress reactivity

The major study finding suggests a significant interaction of 5-HTTLPR and SLC6A4 methylation status on cortisol stress reactivity (genotype x methylation: $F_{2,179} = 3.66, P = 0.028, \eta^2 = 0.04$; genotype x methylation x time: $F_{12,1074} = 2.59, P = 0.002, \eta^2 = 0.03$; Figure 1). Post hoc analysis revealed a significant effect of 5-HTTLPR when SLC6A4 methylation was low, characterized by a dose-dependent effect of the S allele being associated with higher cortisol levels (genotype: $F_{2,39} = 4.17, P = 0.019, \eta^2 = 0.09$; genotype x time $F_{12,334} = 3.41, P \leq 0.001, \eta^2 = 0.07$, Figure 1a). No such effect could be observed in the high methylation group where a moderate cortisol response occurred across all genotype groups, indicating that high SLC6A4 methylation prevents genotype-specific effects (genotype: $F_{2,49} = 0.72, P = 0.492$, genotype x time: $F_{12,334} = 0.74, P = 0.711$, Figure 1b). We further conducted linear regression analyses using cortisol AUC as the dependent variable which confirmed a significant 5-HTTLPR x SLC6A4 methylation interaction ($t = 2.94, \beta = 0.95, P = 0.004$). Including this 5-HTTLPR x SLC6A4 methylation interaction term in the regression model ($F_{2,182} = 3.10, P = 0.028, R^2 = 0.049$) incrementally increased the portion of variance explained by an additive model ($F_{2,183} = 0.32, P = 0.730, R^2 = 0.003$).

A comparable 5-HTTLPR x SLC6A4 methylation interaction occurred when analyses were conducted on the basis of the 5-HTTLPR/rs25531 mini-haplotype (genotype x methylation: $F_{2,179} = 3.21, P = 0.043, \eta^2 = 0.04$, genotype x methylation x time: $F_{12,1074} = 1.89, P = 0.032, \eta^2 = 0.02$, Figure 2). Again, a dose-dependent association between the low-expression alleles and cortisol stress reactivity was found when SLC6A4 methylation was low (genotype: $F_{2,39} = 4.00, P = 0.022, \eta^2 = 0.08$; genotype x time $F_{12,334} = 2.86, P = 0.001, \eta^2 = 0.06$, Figure 2a), whereas no genotype-dependent differences occurred in the high methylation group (genotype: $F_{2,89} = 0.25, P = 0.778$, genotype x time: $F_{12,334} = 0.43, P = 0.935$, Figure 2b). Accordingly, linear regression analysis revealed a significant 5-HTTLPR x SLC6A4 methylation interaction on cortisol AUC ($t = 2.25, \beta = 0.72, P = 0.025$). Again, inclusion of this interaction term in the regression model ($F_{2,182} = 2.30, P = 0.412, R^2 = 0.037$) incrementally increased the portion of variance in cortisol AUC explained by an additive model ($F_{2,183} = 0.89, P = 0.412, R^2 = 0.010$).

Comparable results regarding a significant interaction of 5-HTTLPR and SLC6A4 methylation were achieved when reanalyzing the data by including sex, smoking status or oral contraceptives intake as covariates in the statistical model.

**DISCUSSION**

This is the first study to report a significant interaction of functional genetic and epigenetic variation at the SLC6A4 locus on endocrine stress reactivity in a sample of healthy, young adults. Specifically, methylation patterns within a promoter-associated CpG island of the SLC6A4 gene were found to moderate the association of 5-HTTLPR and cortisol reactivity to psychosocial stress. For individuals displaying low levels of SLC6A4 methylation, the S allele relates to increased cortisol stress reactivity in a dose-dependent fashion, whereas no such effect occurred when SLC6A4 methylation was high. As expected from meta-analytic results, the effect of 5-HTTLPR on cortisol stress reactivity appeared to be small and mostly falls below significance in the overall sample, while explaining 7–9% of the variance in case of low SLC6A4 methylation. Accounting for epigenetic modifications in SLC6A4 might thus allow for a more robust detection of effects when evaluating the 5-HTTLPR stress sensitivity hypothesis in neuroendocrine and clinical studies.

Given that epigenetic modifications are discussed as a key process mediating long-term changes of HPA-axis regulation in response to environmental exposure, the present findings may reflect a molecular substrate of G x E interaction. In line with G x E research on depression, accumulating evidence suggests that heightened neural and endocrine stress sensitivity associated with the 5-HTTLPR S allele is most pronounced upon environmental adversity. More generally, these findings concur with twin studies indicating that heritability of cortisol levels at rest and in response to stress substantially varies as a function of early adversity. Although genetic factors were found to account for a considerable amount of variance in HPA-axis reactivity when familiar adversity was low, no heritable component could be detected in a high stress environment. Our finding of a stress-relevant genetic variant being less important upon epigenetic modifications could be discussed as a specific example and mechanism here, given that individual methylation profiles partly result from differential environmental exposure. One intriguing advantage of investigating functional epigenetic markers in SLC6A4 could be that they presumably reflect the net effects of a broad range of those environmental influences relevant for long-term changes in outcome measures under serotonergic control.
This is of particular importance as the inability to capture the relevant type and timing of adversity (with the appropriate method) has been identified as a crucial source of inconsistencies in previous G × E research. A series of studies have recently started to link several types of early adversity with increased SLC6A4 methylation, however, no specific CpG site has yet been consistently identified across individual studies. As reported elsewhere, we could not replicate such associations for prenatal stress and childhood maltreatment, indicating that these specific stressors are unlikely to account for the moderating role of SLC6A4 methylation in our study. Although the precise environmental (and genetic) correlates of SLC6A4 methylation are largely unknown, future studies should elucidate whether the use of epigenetic markers instead of specific life events will yield more consistent results in G × E research.

The finding of SLC6A4 methylation compensating for increased cortisol reactivity in S allele carriers also raises the possibility that epigenetic modifications reflect an adaptive fine-tuning of the genetically influenced stress response. In favor of this assumption, our study reveals that increased SLC6A4 methylation was associated with a relatively uniform expression of a normal, moderately sized cortisol stress response across different S-HTTLPR genotype groups. Such moderate cortisol reactivity is often considered as an adaptive response preparing the organism to successfully cope with environmental challenges, while both hyper- and hypoactivity of the HPA-axis are frequent correlates of psychopathology. Indeed, this hypothesis closely concurs with the assumption that 5-HTTLPR primarily exerts its unfavorable effects on stress-relevant brain circuits. In contrast, individual differences in SLC6A4 methylation might gradually evolve as a function of environmental exposure, thereby possibly exerting maximum impact during a later time period. Unlike the unfavorable effects on stress sensitivity observed during early brain maturation, inhibition of serotonin transporter functioning (for example, via SSRIs) is known to effectively reduce symptoms of depression and HPA-axis hyperactivity later in life. This led to the assumption that 5-HTTLPR primarily exerts its unfavorable effects during early neurodevelopment when deficient serotonin transporter functioning in S allele carriers might disrupt maturation of stress-relevant brain circuits. In contrast, individual differences in SLC6A4 methylation might gradually evolve as a function of environmental exposure, thereby possibly exerting maximum impact during a later time period. Unlike the unfavorable effects on stress sensitivity observed during early brain maturation, inhibition of serotonin transporter functioning (for example, via SSRIs) is known to effectively reduce symptoms of depression and HPA-axis hyperactivity later in life. This led to the assumption that 5-HTTLPR primarily exerts its unfavorable effects during early neurodevelopment when deficient serotonin transporter functioning in S allele carriers might disrupt maturation of stress-relevant brain circuits.

Several limitations should be acknowledged for the present study. First, the current results rely on peripheral measures of SLC6A4 methylation, which may not generalize to the central nervous system. Nonetheless, the usefulness of peripheral epigenetic biomarkers has been increasingly recognized in psychiatric research, given that methylation profiles, as well as interindividual variation in those epigenetic signatures, are significantly correlated across peripheral and neural cells. Furthermore, accumulating evidence suggests that environmentally induced epigenetic changes appear to be system-wide. For SLC6A4 in particular, a recent imaging study indicates peripheral SLC6A4 methylation to be an informative marker of human brain serotonin synthesis. Second, we chose to analyze whole blood as this procedure does not require transformations known to modify DNA methylation, however, the heterogeneous mixture of cell.
types may constitute a potential confound here. Finally, our finding of SLC6A4 methylation profiles moderating the link between 5-HTTLPR and cortisol stress reactivity remains preliminary until independent replication is available.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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