

ORIGINAL ARTICLE

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

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The serotonin transporter gene-linked polymorphic region (5-HTTLPR) has been implicated in moderating vulnerability to stress-related psychopathology upon exposure to environmental adversity. A recent meta-analysis suggests a potential biological pathway conveying genotype-dependent stress sensitivity by demonstrating a small, but significant association of 5-HTTLPR and cortisol stress reactivity. An arguably more potent approach to detect larger effects when investigating the 5-HTTLPR stress sensitivity hypothesis is to account for both genetic and epigenetic variation in the serotonin transporter gene (*SLC6A4*). Here, we applied this approach in an experimental setting. Two hundred healthy adults were exposed to a laboratory stressor (Trier Social Stress Test) and cortisol response patterns were assessed as a function of 5-HTTLPR and DNA methylation profiles in *SLC6A4*. Specifically, we analyzed 83 CpG sites within a 799-bp promoter-associated CpG island of *SLC6A4* using a highly sensitive bisulfite pyrosequencing method. Our results suggest that *SLC6A4* methylation levels significantly moderate the association of 5-HTTLPR and cortisol stress reactivity. For individuals displaying low levels of *SLC6A4* methylation, the S allele relates to increased cortisol stress reactivity in a dose-dependent fashion accounting for 7–9% of the variance in the endocrine stress response. By contrast, no such effect occurred under conditions of high *SLC6A4* methylation, indicating that epigenetic changes may compensate for genotype-dependent differences in stress sensitivity. Studying epigenetic markers may advance gene–environment interaction research on 5-HTTLPR as they possibly capture the net effects of environmental influences relevant for stress-related phenotypes under serotonergic control.

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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.¹ While it is widely recognized that genetic factors substantially contribute to individual differences in HPA-axis reactivity,^{2–4} the search for robust associations with specific genetic variants has proven challenging. Among others,^{5,6} a large number of neuroendocrine studies^{7–14} 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.¹⁵ 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^{15,16} and thus potentially alters serotonergic modulation of HPA-axis activity.^{17,18} Second, the debate about whether the S allele conveys vulnerability to depression upon exposure to environmental adversity^{19–22} 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.^{7–14} 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.²³ 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.^{24–27} Given that *SLC6A4* methylation profiles appear to be sensitive to early-life stress in several studies,^{26,28–31} 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,^{25,30,32,33} in one study dependent on 5-HTTLPR genotype.²⁴ 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*.³¹ This study reports increased *SLC6A4*

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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 adaptation 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.²⁷ 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 influence HPA-axis activity), pregnancy, an irregular menstrual cycle and a body mass index (BMI) < 17 or > 30 . After a structured telephone interview screening for exclusion criteria, participants were invited to the first session, which comprised the diagnostic interview for psychiatric disorders—short version (Mini-DIPS)³⁵ to assess point and lifetime prevalence of axis I disorders on the basis of DSM IV criteria. Next, participants filled in a set of questionnaires, including a detailed checklist on chronic physical diseases and medication status, the Beck Depression Inventory (BDI-II)³⁶, the short form of the childhood trauma questionnaire (CTQ^{37,38}) for the assessment of childhood maltreatment and the life stressor checklist—revised (LSC-R^{39,40}) to obtain information on traumatic/stressful life events within the past 5 years. Finally, blood samples were drawn into EDTA tubes (Sarstedt, Nümbrecht, Germany) for DNA extraction and stored at -20°C for no more than 6 months. The experimental sessions were scheduled on a second appointment carried out within close succession. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Technische Universität Dresden. Participants provided written informed consent and received a monetary reward for participation.

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.

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.⁷ Participants were further genotyped for an A/G single-nucleotide polymorphism (rs25531) within the length polymorphism which results in the distinction between the variants L_A and L_G , with the latter being functionally similar to the S allele.¹⁶ This allows for conducting analyses based on the 5-HTTLPR/rs25531 mini-haplotype by comparing low [L_G/S] and high [L_A] expressing variants.

Bisulfite pyrosequencing

Quantitative methylation analysis of 83 CpG sites within a previously described⁴³ 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 $N=186$.

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 χ^2 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 5-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 or S allele dominant model. Equivalent analyses were conducted on the basis of the 5-HTTLPR/rs25531 mini-haplotype by comparing the load of high (L_A) and low (L_G/S) expressing alleles (SS , SL_G , L_GL_G vs SL_A , L_GL_A vs L_AL_A). Regarding mean *SLC6A4* methylation as between-subject factor, participants were assigned to a 'low' and 'high' methylation group by median split to illustrate associations with cortisol response patterns over time in a repeated-measure design. Additional analyses were conducted using the area under the cortisol curve with respect to increase (AUC_I)⁴⁴ as the dependent variable to investigate Pearson correlations with *SLC6A4* methylation and to calculate linear regression analyses testing for 5-HTTLPR \times *SLC6A4* methylation interaction. In accordance with previous research,⁴⁵ we identified sex (males $>$ females), smoking status (nonsmoker $>$ smoker) and oral contraceptives (nonusers $>$ users) as variables associated with cortisol reactivity (all P -values ≤ 0.007), whereas no associations with age, BMI, depression score, childhood maltreatment and recent stress/trauma exposure were seen (all P -values ≥ 0.134). Although being equally distributed across genotype/methylation groups (Table 1), additional analyses were calculated controlling for sex, smoking status and oral contraceptives to ensure robustness of the current results.

Table 1. Sample characteristics (mean and s.d.) divided by 5-HTTLPR genotype and mean *SLC6A4* methylation levels

	5-HTTLPR genotype (N = 200)					SLC6A4 methylation (N = 186)		
	Total (N = 200)	SS (n = 33)	SL (n = 104)	LL (n = 63)	P	High (n = 93)	Low (n = 93)	P
Sex (% females)	50%	64.3%	42.9%	52.2%	0.11	52.7%	44.1%	0.24
Age (years)	23.72 ± 2.86	23.21 ± 3.01	24.00 ± 2.86	23.51 ± 2.76	0.30	24.08 ± 3.05	23.54 ± 2.65	0.20
Body mass index	22.37 ± 2.21	22.69 ± 2.41	22.36 ± 2.14	22.23 ± 2.34	0.63	22.12 ± 2.12	22.54 ± 2.25	0.18
Smokers (% yes)	33.5%	33.3%	30.8%	38.1	0.62	32.3%	34.4%	0.76
Oral contraceptives (% yes) ^a	52.0%	59.1%	44.2%	57.1%	0.39	59.2%	48.8%	0.40
Depression (BDI score)	6.45 ± 5.84	7.33 ± 5.57	5.80 ± 5.68	7.06 ± 6.18	0.25	6.44 ± 6.25	5.98 ± 5.18	0.58
Early trauma/stress (CTQ score)	33.38 ± 8.77	33.00 ± 7.77	33.22 ± 9.04	33.83 ± 8.91	0.88	33.43 ± 8.61	32.66 ± 7.25	0.51
Recent trauma/stress (LSC-R score)	1.44 ± 1.40	1.55 ± 1.28	1.37 ± 1.34	1.51 ± 1.56	0.73	1.52 ± 1.38	1.31 ± 1.45	0.33

Abbreviations: BDI, Beck Depression Inventory; CTQ, childhood trauma questionnaire; LSC-R, life stressor checklist-revised. ^aWithin the female sample.**Table 2.** Mean (± s.e.m.) salivary cortisol levels in response to the Trier Social Stress Test as a function of 5-HTTLPR genotype (5-HTTLPR/rs25531 mini-haplotype) and *SLC6A4* methylation levels

Cortisol (nmol l ⁻¹)	5-HTTLPR genotype (N = 200)				SLC6A4 methylation (N = 186)	
	Total	SS (SS, SL _G , L _G L _G)	SL (SL _A , L _G L _A)	LL (L _A L _A)	High	Low
Baseline	10.26 ± 0.40	11.14 ± 0.98 (10.64 ± 0.87)	10.17 ± 0.55 (9.96 ± 0.54)	9.93 ± 0.71 (10.61 ± 0.83)	9.98 ± 0.51	10.46 ± 0.65
Post TSST	15.51 ± 0.59	15.79 ± 1.45 (15.82 ± 1.29)	16.17 ± 0.82 (15.53 ± 0.79)	14.27 ± 1.05 (15.17 ± 1.22)	15.17 ± 0.79	15.63 ± 0.94
+10 min	21.88 ± 0.82	22.20 ± 2.02 (23.44 ± 1.80)	23.17 ± 1.14 (22.07 ± 1.11)	19.59 ± 1.46 (20.05 ± 1.70)	21.80 ± 1.19	22.00 ± 1.24
+20 min	21.61 ± 0.90	23.04 ± 2.19 (24.33 ± 1.95)	22.76 ± 1.24 (21.53 ± 1.20)	18.95 ± 1.59 (19.35 ± 1.84)	21.07 ± 1.22	21.82 ± 1.34
+30 min	17.82 ± 0.73	19.63 ± 1.80 (20.07 ± 1.60)	18.45 ± 1.01 (17.68 ± 0.98)	15.82 ± 1.30 (16.13 ± 1.51)	17.50 ± 0.99	17.73 ± 1.09
+45 min	13.52 ± 0.49	14.85 ± 1.20 (14.78 ± 1.07)	13.71 ± 0.68 (13.14 ± 0.66)	12.52 ± 0.87 (13.29 ± 1.01)	13.53 ± 0.69	13.22 ± 0.71
+60 min	11.19 ± 0.37	12.57 ± 0.95 (12.65 ± 0.84)	11.23 ± 0.53 (10.88 ± 0.52)	10.41 ± 0.69 (10.64 ± 0.79)	11.28 ± 0.56	10.90 ± 0.56
AUC _I	87.95 ± 3.85	93.75 ± 8.50 (96.85 ± 7.56)	91.84 ± 4.79 (87.33 ± 4.65)	78.27 ± 6.16 (81.18 ± 7.15)	86.57 ± 5.01	87.89 ± 5.01

Abbreviations: TSST, Trier Social Stress Test; AUC_I, area under the response curve with respect to increase.

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 ($\chi^2_{(1)} = 0.82$, $P = 0.37$) or the 5-HTTLPR/rs25531 mini-haplotype ($\chi^2_{(3)} = 2.33$, $P = 0.13$) classification. As previously reported in a subsample,²⁷ 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 ($F_{6,1194} = 198.95$, $P \leq 0.001$, $\eta^2 = 0.50$). 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: $F_{2,196} = 0.98$, $P = 0.378$; genotype \times time: $F_{12,1176} = 1.45$, $P = 0.137$) or allele-specific level (S allele dominant model, genotype: $F_{1,197} = 1.95$, $P = 0.165$, genotype \times time $F_{6,1182} = 1.29$, $P = 0.26$; L allele dominant model, genotype: $F_{1,197} = 0.90$, $P = 0.771$,

genotype \times time: $F_{6,1182} = 1.32$, $P = 0.244$). 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 \times time: $F_{6,1182} = 2.05$, $P = 0.056$, $\eta^2 = 0.01$). The latter effect reached significance when controlling for sex, smoking status and use of oral contraceptives (genotype \times time: $F_{6,1164} = 2.34$, $P = 0.03$, $\eta^2 = 0.01$), 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 \times time: $F_{12,1176} = 1.39$, $P = 0.162$) or when applying a dominant model for the high-expressing alleles (genotype: $F_{1,197} = 0.82$, $P = 0.366$; genotype \times time: $F_{6,1182} = 1.08$, $P = 0.373$).

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 \times time: $F_{6,1098} = 0.36$, $P = 0.905$). Accordingly, mean *SLC6A4* methylation levels were unrelated to the cortisol AUC_I ($r = -0.018$, $P = 0.805$). 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_I (before Bonferroni adjustment). However, these associations are likely to result from chance ($P(i \geq 3 | \alpha = 0.05, n = 83) = 0.79$).

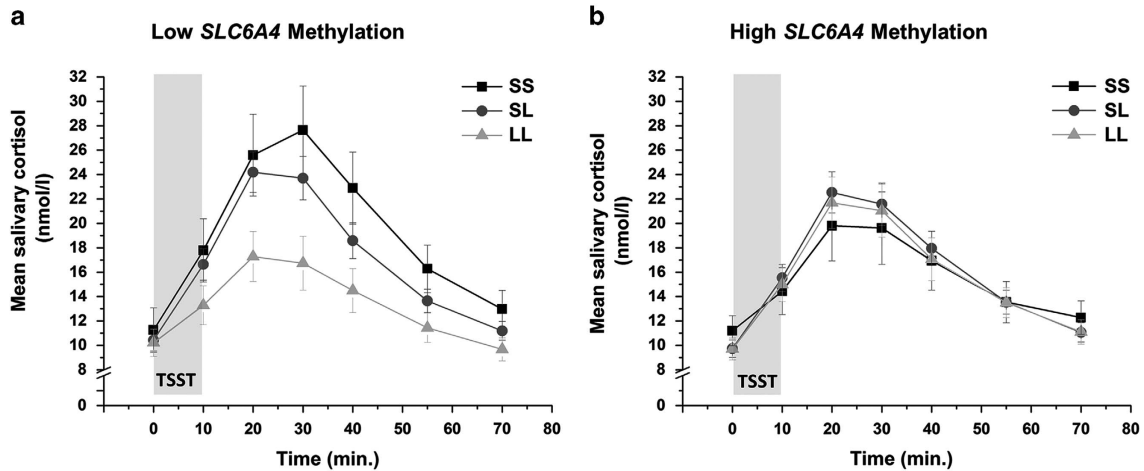


Figure 1. 5-HTTLPR (bi-allelic classification) \times *SLC6A4* methylation interaction on cortisol stress reactivity. (a) Mean (\pm s.e.m.) salivary cortisol levels in response to the Trier Social Stress Test as a function of 5-HTTLPR genotype (bi-allelic classification) in individuals displaying low levels of mean *SLC6A4* methylation. (b) Mean (\pm s.e.m.) salivary cortisol levels in response to the Trier Social Stress Test as a function of 5-HTTLPR genotype (bi-allelic classification) in individuals displaying high levels of mean *SLC6A4* methylation.

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 \times methylation: $F_{2,179} = 3.66$, $P = 0.028$, $\eta^2 = 0.04$; genotype \times methylation \times 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: $F_{2,89} = 4.17$, $P = 0.019$, $\eta^2 = 0.09$; genotype \times time $F_{12,534} = 3.41$, $P \leq 0.001$, $\eta^2 = 0.07$, 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: $F_{2,89} = 0.72$, $P = 0.492$, genotype \times time: $F_{12,534} = 0.74$, $P = 0.711$, Figure 1b). We further conducted linear regression analyses using cortisol AUC₁ as the dependent variable which confirmed a significant 5-HTTLPR \times *SLC6A4* methylation interaction ($t = 2.94$, $\beta = 0.95$, $P = 0.004$). Including this 5-HTTLPR \times *SLC6A4* methylation interaction term in the regression model ($F_{3,182} = 3.10$, $P = 0.028$, $R^2 = 0.049$) 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 \times *SLC6A4* methylation interaction occurred when analyses were conducted on the basis of the 5-HTTLPR/rs25531 mini-haplotype (genotype \times methylation: $F_{2,179} = 3.21$, $P = 0.043$, $\eta^2 = 0.04$, genotype \times methylation \times 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: $F_{2,89} = 4.00$, $P = 0.022$, $\eta^2 = 0.08$; genotype \times time $F_{12,534} = 2.86$, $P \leq 0.001$, $\eta^2 = 0.06$, Figure 2a), whereas no genotype-dependent differences occurred in the high methylation group (genotype: $F_{2,89} = 0.25$, $P = 0.778$, genotype \times time: $F_{12,534} = 0.43$, $P = 0.953$, Figure 2b). Accordingly, linear regression analysis revealed a significant 5-HTTLPR \times *SLC6A4* methylation interaction on cortisol AUC₁ ($t = 2.25$, $\beta = 0.72$, $P = 0.025$). Again, inclusion of this interaction term in the regression model ($F_{3,182} = 2.30$, $P = 0.412$, $R^2 = 0.037$) incrementally increased the portion of variance in cortisol AUC₁ 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,²³ 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,³⁴ the present findings may reflect a molecular substrate of G \times E interaction. In line with G \times E research on depression,^{19–21} accumulating evidence suggests that heightened neural and endocrine stress sensitivity associated with the 5-HTTLPR S allele is most pronounced upon environmental adversity.^{7,46,47} More generally, these findings concur with twin studies indicating that heritability of cortisol levels at rest⁴⁸ and in response to stress⁴ 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 familial adversity was low, no heritable component could be detected in a high stress environment.⁴ 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.^{34,49} 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.

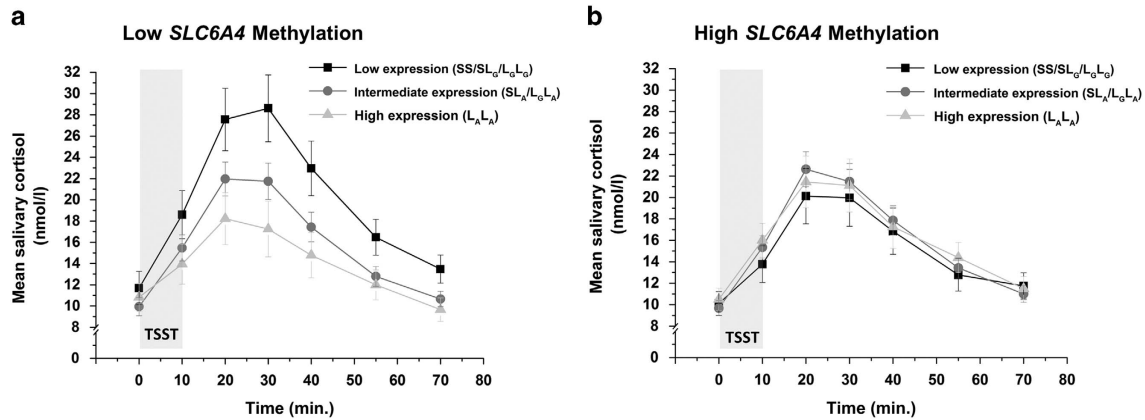


Figure 2. 5-HTTLPR/rs25531 \times *SLC6A4* methylation interaction on cortisol stress reactivity. **(a)** Mean (\pm s.e.m.) salivary cortisol levels in response to the Trier Social Stress Test as a function of 5-HTTLPR/rs25531 genotype in individuals displaying low levels of mean *SLC6A4* methylation. **(b)** Mean (\pm s.e.m.) salivary cortisol levels in response to the Trier Social Stress Test as a function of 5-HTTLPR/rs25531 genotype in individuals displaying high levels of mean *SLC6A4* methylation.

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 $G \times E$ research.^{50,51} A series of studies have recently started to link several types of early adversity with increased *SLC6A4* methylation,^{26,28–31} 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,²⁷ 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 $G \times E$ 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 5-HTTLPR genotype groups. Such moderate cortisol reactivity is often considered as an adaptive response preparing the organism to successfully cope with environmental challenges,⁵² while both hyper- and hypoactivity of the HPA-axis are frequent correlates of psychopathology.^{53,54} Indeed, this hypothesis closely concurs with a recent report suggesting that vulnerability of *S/S* carriers for developing psychological problems in response to stress may be reduced by increased *SLC6A4* methylation.⁵⁵ In this study, the *S/S* genotype was associated with more unresolved responses to traumatization, but only in the case of lower methylation within the promoter-associated *SLC6A4* CpG island.⁵⁵ Seemingly conflicting evidence for *SLC6A4* methylation being an adaptive process is provided by first studies linking epigenetic modifications in *SLC6A4* to vulnerability for stress-related psychopathology.⁵⁶ Most of these studies found higher mean or site-specific *SLC6A4* methylation in depressed patients compared with healthy controls,^{25,30,33} however, opposite results have also been obtained.³² Still, it remains difficult to draw firm conclusions about whether increased *SLC6A4* methylation either reflects the body's attempt to adapt to environmental adversity or constitutes a risk factor for psychopathology. As (specific) trauma exposure *per se* may relate to higher *SLC6A4* methylation^{26,28–31} and is also more frequently observed in depressed patients,^{22,57} including

both traumatized and non-traumatized healthy controls might help to resolve this question.

Future studies should provide further insights into the underlying mechanisms by which genetic and epigenetic modification in the *SLC6A4* gene interact to shape stress-relevant pathways. From a molecular perspective, one might have expected that *SLC6A4* methylation further promotes increased stress sensitivity in *S* allele carriers, given that both the *S* allele^{15,16,27} and *SLC6A4* methylation^{24–27} relate to lower serotonin transporter expression. Although our findings are not supportive of such additive effects with regard to cortisol reactivity, it is important to note that a disruption of serotonin transporter functioning may have very distinct impact on the organism during different developmental stages.⁵⁸ One example refers to the 'SSRI paradox', where early pre- and postnatal blockage of the serotonin transporter by SSRI (selective serotonin reuptake inhibitors) was found to induce sustained depressogenic effects in rodents.^{59–61} 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.⁵⁸ 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 SSRI) is known to effectively reduce symptoms of depression and HPA-axis hyperactivity later in life.^{62,63} Despite comparable effects on gene expression, it is thus conceivable that genetic and epigenetic changes in *SLC6A4* operate during different time windows to exert divergent effects on the stress response system.

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 research⁵⁶ given that methylation profiles,⁶⁴ as well as interindividual variation in those epigenetic signatures,⁶⁵ are significantly correlated across peripheral and neural cells. Furthermore, accumulating evidence suggests that environmentally induced epigenetic changes appear to be system-wide.^{66,67} For *SLC6A4* in particular, a recent imaging study indicates peripheral *SLC6A4* methylation to be an informative marker of human brain serotonin synthesis.⁶⁸ Second, we chose to analyze whole blood as this procedure does not require transformations known to modify DNA methylation,⁶⁹ 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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