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Serotonin transporter gene methylation predicts long-term cortisol concentrations in hair

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ABSTRACT

Epigenetic signatures, such as DNA methylation (DNA_M), have been implicated in long-term dysregulation of the hypothalamus–pituitary–adrenal (HPA) axis and related health risks. Based on a wealth of neuroendocrine studies on genetic polymorphisms in the serotonin transporter gene (*SLC6A4*), this locus constitutes a key candidate to explore associations of DNA_M patterns and HPA-axis functioning. The few studies addressing this link so far exclusively relied on spot measurements of HPA-axis activity, which may not adequately reflect cortisol output over prolonged periods of time. To address this gap, hair cortisol concentrations (HCC), a valid measure of integrated long-term cortisol levels, were utilized to investigate endocrine correlates of *SLC6A4* DNA_M in 183 adults. Whole blood samples were drawn for DNA_M analyses of 83 CpG sites within a 799-bp promoter-associated CpG island of *SLC6A4* via bisulfite pyrosequencing. In addition, all participants were genotyped for the serotonin transporter polymorphism (5-HTTLPR). First, results revealed a significant negative association of *SLC6A4* DNA_M and HCC. Second, there was no significant main effect of 5-HTTLPR genotype on HCC when analyses were conducted on the basis of both bi-allelic classification and the 5-HTTLPR/rs25531 mini-haplotype. Third, the current data revealed a significant interaction of *SLC6A4* DNA_M and 5-HTTLPR genotype on HCC. Comparable to the pattern we had previously observed concerning cortisol stress reactivity, the S allele relates to increased HCC in individuals displaying low levels of *SLC6A4* DNA_M. By contrast, no such effect occurred under conditions of high *SLC6A4* DNA_M, indicating that epigenetic changes may compensate for genotype-dependent differences in long-term cortisol output. Together, respective findings support the idea of an epigenetic contribution to long-term HPA-axis activity and further highlight the usefulness of combining genetic and epigenetic information in future neuroendocrine studies.

1. Introduction

Epigenetic signatures, in particular DNA methylation (DNA_M), have been implicated in long-term dysregulation of the hypothalamus–pituitary–adrenal (HPA) axis and related health risks (Klengel and Binder, 2015). Based on clinical and experimental genetic association studies, the serotonin transporter gene (*SLC6A4*) constitutes a key candidate to investigate associations of DNA_M and HPA-axis functioning (Palma-Gudiel and Fananas, 2017). For example, a recent meta-analysis revealed a significant link between the *SLC6A4* 43-bp insertion/deletion polymorphism (5-HTTLPR) and increased HPA-axis reactivity to psychosocial stress, with homozygous carriers of the S allele displaying higher cortisol levels (Miller et al., 2013). However, the size of this effect was small, highlighting the need to explore additional sources of variance, such as DNA_M profiles.

Previous research identified a 799-bp promoter-associated CpG island in the *SLC6A4* gene, where DNA_M was found to reduce gene expression and appeared to be sensitive to early life stress (Palma-Gudiel and Fananas, 2017). To date, only few studies examined neuroendocrine correlates of epigenetic changes in *SLC6A4*. One study on 28 monozygotic twins discordant for bullying victimization found increased *SLC6A4* DNA_M at 1 out of the 12 CpG sites studied, which in turn was related to blunted HPA-axis reactivity (Ouellet-Morin et al., 2013). In another study by our group, *SLC6A4* DNA_M significantly moderated the association of 5-HTTLPR and cortisol stress reactivity (Alexander et al., 2014). For individuals displaying low levels of

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SLC6A4 DNA_M, the S allele related to increased cortisol stress reactivity, while no such effect occurred under conditions of high *SLC6A4* DNA_M. However, a third study could not replicate significant correlations between HPA-axis reactivity and SLC6A4 DNA_M for the sample overall or as a function of 5-HTTLPR genotype (Duman and Canli, 2015). While all prior studies rely on acute, dynamic markers of HPA-axis reactivity, such spot measurements might be sensitive to transient fluctuations and are further unable to capture long-term cortisol concentrations. To address this gap, the analysis of hair cortisol concentrations (HCC) has been established as a valid marker of long-term HPA-axis activity (Stalder et al., 2017). The present study is the first to utilize HCC in order to investigate endocrine correlates of *SLC6A4* DNA_M in a sample of 183 adults.

2. Materials and methods

2.1. Sample characteristics and procedure

The initial study sample consisted of 200 healthy participants (n = 100 females) aged 18–30 years. Only Caucasian native German speakers were included. Exclusion criteria were current or past mental disorders and/or physical diseases, medication intake (e.g., psychotropic drugs, substances known to influence HPA-axis activity), pregnancy, an irregular menstrual cycle and a body mass index (BMI) of < 17 or > 30. After a structured telephone interview screening for exclusion criteria, participants were invited to a first session where the diagnostic interview for psychiatric disorders—short version (Mini-DIPS; (Margraf, 1994)) was used to assess lifetime prevalence of axis I disorders based on DSM IV criteria. Next, participants filled in a set of questionnaires, including a checklist on chronic diseases and medication status, the short form of the childhood trauma questionnaire (CTQ, Wingenfeld et al., 2010) for the assessment of childhood maltreatment and the life stressor checklist—revised (LSC-R, Ungerer et al., 2010) to obtain information on traumatic/stressful life events within the past 5 years. Hair-specific characteristics as well as sociodemographic, anthropometric and health-related variables were assessed through an in-house questionnaire. Finally, hair strands were collected and blood samples were drawn into EDTA tubes (Sarstedt, Nümbrecht, Germany) and stored at − 20 °C for no more than 6 months. A second experimental session included a standardized laboratory stress test (data reported elsewhere; Alexander et al., 2014). 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.

2.2. Hair cortisol analysis

Hair strands were cut as close as possible to the scalp from a posterior vertex position. HCC were analyzed from the 3 cm segment most proximal to the scalp, which captures the cumulated cortisol secretion over a 3-month period prior to sampling. Washing procedure and extraction followed a previously published protocol (Gao et al., 2013). Samples were analyzed by liquid chromatography coupled with tandem mass spectrometry. Three participants were excluded due to insufficient hair length (n = 2) and outliers (± 3 standard deviations from the mean, n = 1).

2.3. Bisulfite pyrosequencing

Quantitative methylation analysis of 83 CpG sites within a 799-bp CpG island in *SLC6A4* (Supplement Information 1) was performed by Varionostic GmbH (Ulm, Germany). Genomic DNA extracted from whole blood was bisulfite-treated using the EZ DNA Methylation Gold Kit (Zymo Research, Range, CA, USA). Subsequent pyrosequencing was performed on the Q24/ID System. A detailed protocol has been published elsewhere (Wankerl et al., 2014). Mean *SLC6A4* DNA_M levels across the CpG island were calculated for those participants for whom methylation values for at least 90% of the 83 CpG sites passed a strict quality control, which led to the exclusion of 14 individuals. For the remaining participants, missing data at specific CpG sites (less than 1.5% in total) were imputed using site-specific mean substitution.

2.4. 5-HTTLPR genotyping

DNA was extracted from whole blood samples by means of standard commercial extraction kits (High Pure PCR Template Preparation Kit; Roche) in a MagNA Pure LC System (Roche). Genotyping was performed according to a previously published protocol (Alexander et al., 2009). Participants were further genotyped for an A/G single-nucleotide polymorphism (rs25531) (Hu et al., 2006). This allows for conducting analyses based on the 5-HTTLPR/rs25531 mini-haplotyp comparing low [L_G/S] and high [LA] expressing variants.

2.5. 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 tests for potential confounders were assessed using Pearson correlations and t-tests. To investigate associations between quantitative *SLC6A4* DNA_M, 5-HTTLPR, and their interaction linear regression analyses were calculated. For illustrative purposes (Fig. 1) and post-hoc analyses, participants were divided into low vs. high *SLC6A4* DNA_M groups by median split.

3. Results

3.1. Sample characteristics

The final sample comprised 183 healthy participants with a mean age of 23.8 ± 2.8 years (47.5% women, 35.0% smokers, 31.0% oral contraceptive use within females, mean BMI: 22.4 ± 2.2). There was no significant deviation from Hardy Weinberg equilibrium using bi-allelic ($x^2_{(1)} = 0.82$, $p = 0.37$) or 5-HTTLPR/rs25531 mini haplotype ($x^2_{(3)} = 2.33$, $p = 0.13$) classification. *SLC6A4* DNA_M levels and HCC were unrelated to sex, smoking status, oral contraceptives use, alcohol consumption, childhood maltreatment and recent trauma (all p-values > 0.05). Mean *SLC6A4* DNA_M levels were comparable between 5-HTTLPR genotype groups ($F_{1,182} = 0.12$, $p = 0.89$). Moreover, HCC

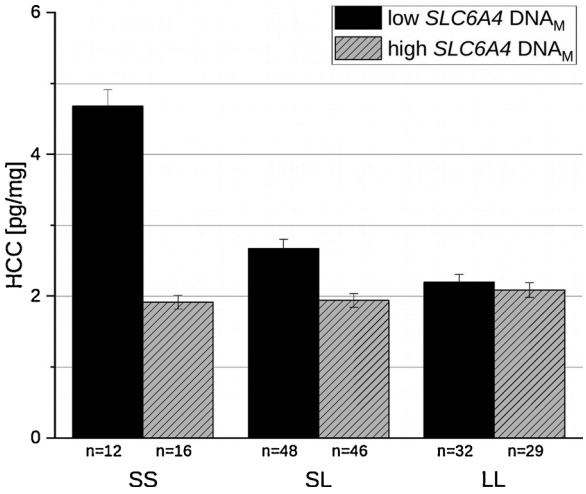

Fig. 1. Hair cortisol concentrations (HCC) as a function of 5-HTTLPR genotype (comparing SS vs SL/LL) and mean DNA methylation levels within a 799-bp CpG island in the serotonin transporter gene *SLC6A4* (comparing individuals with high and low *SLC6A4* methylation by median split).

Table 1
Linear regression of hair cortisol concentrations (HCC) on mean *SLC6A4* DNA_M, 5-HTTLPR genotype and their interaction.

	Dependent variable: hair cortisol concentrations (HCC)			
Model	Variable	β	T	*p*
Main effects	*SLC6A4* DNA_M[1]	−0.15	−2.02	0.045*
	5-HTTLPR[2]	−0.12	−1.70	0.091
Interaction effect	*SLC6A4* DNA_M[1]	−0.50	−2.61	0.010*
	5-HTTLPR[2]	−0.86	−2.28	0.024*
	SLC6A4 DNA_M[1] x 5-HTTLPR[2]	0.83	1.98	0.049*

Note: [1]*SLC6A4* DNA_M was entered as a continuous variable into the model. [2]Analysis were conducted according to an l-allele dominant model comparing SS vs SL/LL based on the meta-analysis by Miller et al. (Miller et al., 2013).
* p < .05.

levels were unaffected by potential hair-related confounds such as hair washes per week, curls, coloration and permanent waves (all *p*-values > 0.05). Consequently, none of these variables were included as potential confounds in subsequent analyses.

3.2. Associations of SLC6A4 DNA_M, 5-HTTLPR genotype and HCC

Results of the linear regression of HCC on mean *SLC6A4* DNA_M, 5-HTTLPR and their interaction are presented in Table 1. A first analysis revealed a negative association of *SLC6A4* DNA_M and HCC (β = -0.15, p = 0.045). Additional exploratory analyses (Supplement 2) revealed 8 specific CpG sites where DNA_M levels were found to negatively correlate with HCC (before Bonferroni adjustment). Notably, this cluster nicely corresponds to those sites with largest interindividual variation in overall DNA_M levels (Supplement 1). Second, no significant main effect of 5-HTTLPR on HCC was observed when analysis were conducted based on both bi-allelic (β = -0.12, p = 0.091) and the 5-HTTLPR/rs25531 mini-haplotype classification comparing carriers of two low-expressing alleles to those with at least one high-expressing allele (β = -0.12, p = 0.093). Only a nominal trend for higher HCC in homozygous carriers of the low-expressing (bi-allelic classification: M = 3.10 ± 3.8 pg/mg, HTTLPR/rs25531 mini-haplotype: M = 3.00 ± 3.5 pg/mg) compared to individuals with two copies of the high-expressing alleles was observed (bi-allelic classification: M = 2.25 ± 2.2 pg/mg, HTTLPR/rs25531 mini-haplotype: M = 2.11 ± 2.3 pg/mg)

Third, regression analysis revealed a significant interaction of *SLC6A4* DNA_M and 5-HTTLPR on HCC (β = 0.83, p = 0.049, Table 1, Fig. 1). Comparable to the pattern observed in our cortisol stress reactivity data (Alexander et al., 2014), post-hoc analysis revealed a significant effect of 5-HTTLPR when *SLC6A4* DNA_M was low (R^2 = 0.06, $F_(1,89_)$ = 5.3, p = 0.023), with homozygous S allele carriers displaying higher HCC levels compared to individuals with the SL and LL genotype. No such effect was observed under conditions of high *SLC6A4* DNA_M where a moderate HCC occurred across all genotype groups, indicating that high *SLC6A4* DNA_M prevents genotype specific effects (R^2 < 0.01, $F_(1,90_)$ = 0.4, p = 0.842). Together, *SLC6A4* DNA_M, 5-HTTLPR and the interaction of *SLC6A4* DNA_M x 5-HTTLPR explained 6 % of the variance in HCC (R^2 = 0.06, $F_(3,179_)$ = 3.6, p = 0.014).

4. Discussion

This is the first study to investigate associations of genetic and epigenetic variation in the serotonin transporter gene with long-term cortisol concentrations in hair. In line with meta-analytical findings from studies of acute cortisol stress reactivity (Miller et al., 2013), a nominal, albeit non-significant, trend for increased HCC was observed in homozygous 5-HTTLPR S allele carriers compared to individuals with the SL/LL genotype. In addition, the present study revealed a significant negative correlation between DNA_M within a promoter-associated CpG island of the *SLC6A4* gene and HCC, highlighting the predictive value of epigenetic modulation of the serotonergic system for HPA-axis functioning. This finding concurs with one earlier study demonstrating blunted cortisol stress responses in individuals with increased *SLC6A4* DNA_M (Ouellet-Morin et al., 2013), but conflicts with two other studies reporting no direct effect of *SLC6A4* DNA_M on acute HPA-axis functioning (Alexander et al., 2014; Duman and Canli, 2015).

From a functional perspective, the current study raises the question why the 5-HTTLPR S allele and *SLC6A4* DNA_M exert opposing effects on HCC, although both relate to reduced SLC6A4 expression (Palma-Gudiel and Fananas, 2017). One possible explanation refers to the observation that disruptions in serotonin transporter functioning induce highly variable effects depending on the individuals' developmental stage. For example, animal models suggest that pre- and early postnatal blockage of the serotonin transporter by selective serotonin reuptake inhibitors (SSRI) promote sustained depressogenic effects (Homberg et al., 2010). This so-called 'SSRI paradox' stimulated the assumption that deficient serotonin transporter functioning in S allele carriers potentially disrupts normal maturation of stress-related brain circuits during very early neurodevelopment (Homberg et al., 2010). In contrast, interindividual variation in *SLC6A4* DNA_M partly evolves with exposure to specific environmental conditions (Palma-Gudiel and Fananas, 2017), thereby possibly exerting its maximum effects later in life. Unlike the detrimental effects observed during early neurodevelopment, pharmacological blockade of the serotonin transporter is known to effectively reduce symptoms of depression and HPA-axis hyperactivity later in life (Nickel et al., 2003). It is thus tempting to speculate that genetic and epigenetic changes in *SLC6A4* operate during different time windows, thereby conveying divergent effects on HPA-axis functioning.

The current data further suggest a significant interaction of 5-HTTLPR genotype and *SLC6A4* DNA_M on HCC. Precisely, the S/S genotype was found to predict increased HCC only in those individuals with low *SLC6A4* DNA_M, while no genotype-dependent effect occurred when *SLC6A4* DNA_M was high. This result pattern strikingly parallels our previously observed 5-HTTLPR by *SLC6A4* DNA_M interaction effect on HPA-axis reactivity to acute stress (Alexander et al., 2014). While these findings highlight a comparable interplay of genetic and epigenetic variation in the *SLC6A4* gene on phasic and tonic HPA-axis activity, the small number of S/S individuals with low *SLC6A4* DNA_M constitutes a limitation of the current study. As discussed elsewhere (Alexander et al., 2014), one might speculate that DNA_M may compensate for genotype-dependent differences in stress sensitivity and potentially promotes an adaptive fine-tuning of the HPA-axis. In support of this idea, increased *SLC6A4* DNA_M was uniformly associated with moderate HCC across 5-HTTLPR genotype groups, which is commonly considered as an adaptive phenotype in the light of the detrimental effect of both hyper- and hypocortisolism (Stalder et al., 2017). In line with our endocrine data, a first clinical study has provided evidence that the combination of 5-HTTLPR S/S genotype and lower *SLC6A4* DNA_M may indeed predict increased stress sensitivity on a behavioral/psychological level (van IJzendoorn et al., 2010). Consequently, a combined investigation of genetic and epigenetic information in candidate genes may allow the detection of more robust associations in future neuroendocrine and clinical studies.

Conflicts of interest

The authors declare no financial or other conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.03.033.

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