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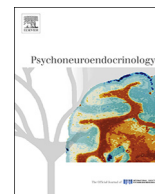
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Glucocorticoid receptor exon 1_F methylation and the cortisol stress response in health and disease

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ABSTRACT

Childhood trauma has been proposed to increase vulnerability to develop psychopathology in part through an altered cortisol stress response. Research in rats has suggested that this effect is mediated by methylation in the glucocorticoid receptor 1₇ region (GR-1₇ or GR-1_F in humans), with higher methylation after poor maternal care leading to an increased cortisol stress response in adulthood. In humans, the associations between childhood trauma and GR-1_F methylation or the cortisol stress response are equivocal. Remarkably, evidence for the relation between GR-1_F methylation and the cortisol stress response has been conflicting as well. To further explore this, we investigated the associations of peripheral GR-1_F methylation (52 CpGs) with the cortisol stress response (Trier Social Stress Test) and with childhood trauma in three independent studies (total N = 241) including healthy controls, patients with schizophrenia and bipolar disorder and unaffected siblings of patients with one of these disorders. We did not find any significant association between GR-1_F methylation and the cortisol stress response (areas under the curve) or childhood trauma, nor did we observe any group differences between patients, siblings and healthy controls. Our findings do not support GR-1_F methylation as a proxy for the cortisol stress response, nor its link with childhood trauma or psychopathology. These results suggest that multifactorial models for stress-related psychopathology are needed. Alternatively, future longitudinal studies may reveal GR-1_F methylation to be a useful parameter at an individual level.

1. Introduction

Stress is arguably the most common environmental factor leading to psychopathology (Smoller, 2016), and its effects are especially detrimental when occurring during childhood (Carr et al., 2013; Nanni et al., 2012). This persisting impact of stress during development is thought to be partially mediated by epigenetic mechanisms. A seminal study in 2004 proposed the 1_F region of the glucocorticoid receptor (GR) to be of crucial importance in this context (Weaver et al., 2004). Pups of low licking-grooming dams showed higher methylation in the rat ortholog of the 1_F region, which was linked to impaired feedback by corticosterone on the hypothalamus-pituitary-adrenal (HPA) axis in adulthood, irrespective of the genetic background (Weaver et al., 2004).

This appealing observation stimulated a considerable amount of

translational research in humans, where GR-1_F methylation was evaluated in relation to (childhood) trauma, HPA axis functionality and stress-related psychopathology (Daskalakis and Yehuda, 2014). Considering the ambiguous relationship between childhood trauma and HPA axis activity (e.g. Heim et al., 2000) and (Lovallo et al., 2012)), it is not surprising that childhood trauma can be positively (Van der Knaap et al., 2014), negatively (Tyrka et al., 2016) or not associated with GR-1_F methylation (Schür et al., 2017). Similarly, the relationship between GR-1_F methylation and stress-related psychopathology is not straightforward either, with conflicting findings in both major depressive disorder (MDD) (Na et al., 2014; Nantharat et al., 2015) and posttraumatic stress disorder (PTSD) (Perroud et al., 2014; Yehuda et al., 2015).

Remarkably, however, the link between GR-1_F methylation and

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HPA axis functionality in humans is not clear-cut either. Several studies confirmed impaired negative feedback on the HPA axis by elevated GR-1_F methylation. Two studies showed a positive relationship between GR-1_F methylation and cortisol levels after dexamethasone and/or CRH (Tyrka et al., 2016; Yehuda et al., 2015), even though the relation was not strong ($r < 0.2$). In line with this, two other studies found that higher GR-1_F methylation was linked to elevated cortisol levels following a social stress test, albeit either in the acute phase (Alexander et al., 2018) or in the recovery phase of the stress response (van der Knaap et al., 2015). In contrast, two other studies showed associations in the opposite direction, using the Trier Social Stress Test (TSST) and the dexamethasone/CRH test, respectively (Edelman et al., 2012; Tyrka et al., 2012). This equivocal evidence asks for large additional studies to evaluate whether GR-1_F methylation represents a useful proxy for HPA axis functionality or not.

In the present study, we used data from three independent cohorts to further explore the relationship between GR-1_F methylation and the cortisol stress response. We hypothesized that elevated methylation would be associated with an increased cortisol stress response and expected positive associations of GR-1_F methylation with childhood maltreatment. Moreover, as the association between GR-1_F methylation and psychotic disorders has not been thoroughly investigated yet, we explored this relationship in patients with either schizophrenia or bipolar disorder, siblings of such patients and controls.

2. Methods

2.1. Participants

The present study combined data from three different cohorts, of which previously different aspects have been described (Houtepen et al., 2015; Van Leeuwen et al., 2018; Vinkers et al., 2013; Zorn et al., 2017). Briefly, Vinkers et al. (2013) investigated time-dependent effects of stress on altruistic punishment in healthy controls, taking the cortisol stress response into account (see Table 1, cohort 1). Houtepen et al. (2015) evaluated medication effects on the cortisol stress response in euthymic patients with bipolar disorder, unaffected siblings of patients with bipolar disorder (unrelated to the patients with bipolar disorder) and healthy controls (see Table 1, cohort 2). Moreover, data from 13 schizophrenia patients were added to this sample (Zorn et al., 2017). Finally, Van Leeuwen et al. (Van Leeuwen et al., 2018) examined the

cortisol stress response in relation to functional brain activity in unaffected siblings of schizophrenia patients versus controls (see Table 1, cohort 3).

In all studies, lifetime DSM-IV diagnoses in healthy individuals were assessed with the Mini International Neuropsychiatric Interview (MINI) plus (Sheehan et al., 1998). Diagnosis in patients with bipolar disorder or schizophrenia was confirmed using the Structured Clinical Interview for DSM-IV (SCID) (First et al., 2002). The Young Mania Rating Scale (YMRS, range 0–7 (Tohen et al., 2000; Young et al., 1978)) and the 30-item Inventory of Depressive Symptomatology (IDS-C₃₀, range 0–24 (Rush et al., 1996)) were used to confirm euthymic mood in patients with bipolar disorder (Houtepen et al., 2015). All interviews were carried out by well-trained and independent raters.

Use of medication (lithium, antidepressants, anticonvulsants, antipsychotics and beta blockers) and drugs of abuse (including nicotine) was determined with a self-report questionnaire. In addition, multi-drug screening devices were employed to assess current use of psychoactive substances (benzodiazepines, cannabinoids, opiates, cocaine, amphetamines, and either MDMA and barbiturates (InstantView, cohorts 1 and 2) or methadone (Multi-line, cohort 3)). For female participants, information on contraceptive medication use and menstrual cycle was collected, as these variables are associated with altered HPA axis reactivity (Kirschbaum et al., 1999). The studies were approved by the ethical review board of the University Medical Center Utrecht (UMCU) and performed in accordance with the Declaration of Helsinki and the ICH guidelines for Good Clinical Practice. Prior to inclusion, all participants provided written informed consent.

2.2. Procedures

2.2.1. Trier social stress test

All participants were new to stress research and were subjected to the stress condition of the TSST (Kirschbaum et al., 1993), consisting of a public speaking test and a subsequent mental arithmetic test. All participants were fluent in Dutch and refrained from heavy exercise, heavy meals, or drinks other than water for at least 2 h prior to the TSST (caffeine use was not allowed within 4 h of the TSST). Patients with bipolar disorder or schizophrenia, siblings of patients with bipolar disorder and 99 healthy controls were subjected to the group version of the TSST (see for a more detailed description (von Dawans et al., 2011)). Participants enrolled in the same TSST (up to four in total) were

Table 1
Characteristics Table.

Cohort	1	2	3		All		
Group status	<i>Control</i>	<i>BD pt</i>	<i>SCZ pt</i>	<i>BD sib</i>	<i>Control</i>	<i>SCZ sib</i>	<i>Control</i>
N	51	43	13	24	48	30	32
Sex	47	49	54	29	50	100	100
<i>% male</i>							
Age	22.6 (2.5)	43.9 (12.9)	40.1 (15.0)	55.8 (7.1)	43.4 (15.9)	32.6 (8.9)	32.9 (8.0)
<i>mean (sd)</i>							
Childhood trauma	30.5 (6.4)	37.1 (10.7)	30.5 (6.4)	35.0 (11.3)	33.2 (10.0)	34.2 (7.8)	35.3 (12.2)
<i>mean (sd)</i>							
Emotional abuse	6.8 (2.5)	8.6 (4.0)	6.6 (3.2)	7.8 (3.1)	7.2 (3.8)	6.9 (1.9)	7.1 (3.3)
<i>mean (sd)</i>							
Physical abuse	5.2 (0.5)	5.3 (0.7)	5.2 (0.6)	5.5 (1.6)	5.3 (0.8)	5.4 (1.1)	6.0 (3.7)
<i>mean (sd)</i>							
Sexual abuse	4.1 (0.3)	5.1 (3.3)	4.2 (0.8)	4.9 (3.1)	4.5 (1.3)	5.4 (2.2)	5.3 (0.9)
<i>mean (sd)</i>							
Emotional neglect	9.0 (3.8)	11.8 (5.0)	9.4 (4.5)	10.6 (3.1)	10.2 (4.8)	10.3 (3.7)	9.8 (4.4)
<i>mean (sd)</i>							
Physical neglect	5.8 (1.5)	6.7 (1.9)	6.2 (1.8)	6.7 (2.8)	6.4 (2.6)	6.1 (1.8)	7.1 (2.4)
<i>mean (sd)</i>							
N no drugs of abuse or medication	51	0	0	19	36	30	32
N TSST	49	42	13	24	46	13	17

Pooled data from three cohorts were used in the present study (cohort 1 (Vinkers et al., 2013), cohort 2 (Houtepen et al., 2015; Zorn et al., 2017), cohort 3 (Van Leeuwen et al., 2018)). BD = bipolar disorder; pt = patients; SCZ = schizophrenia; sib = siblings; TSST = Trier Social Stress Test.

unfamiliar to each other. The remaining participants performed the individual TSST (17 siblings of schizophrenia patients and 15 healthy controls, for more details see (Van Leeuwen et al., 2018)).

2.2.2. Salivary cortisol samples

Cortisol levels were measured in saliva, which was collected in salivettes (Sarstedt, Nümbrecht, Germany). Sampling was carried out at 7–8 time points around the start of the TSST, slightly differing between the original studies (–10, 8, 16, 35, 50, 65, 90 and 125 min (Vinkers et al., 2013); –10, 8, 20, 40, 50, 65, 80 and 85 min (Houtepen et al., 2015); –10, 5, 20, 30, 65, 90 and 120 min (Van Leeuwen et al., 2018)). All samples were stored at –80 °C immediately after the TSST and analyzed as previously described (Vinkers et al., 2013). Briefly, an in house competitive radio-immunoassay was used to measure cortisol without extraction. In case of a missing saliva sample, the average of two surrounding time points was used (Houtepen et al., 2015). All participants were tested between 12:00 h and 20:30 h to reduce variation in baseline cortisol levels due to the diurnal rhythm. In total, data for methylation and the cortisol stress response were available for 204 individuals; 32 individuals of cohort 3 and 2 individuals of cohort 1 were only exposed to a control condition of the TSST, and three individuals in cohort 2 were excluded as more than one salivette did not have enough saliva for analysis.

The trapezoidal rule was employed to calculate areas under the curve of the cortisol stress response. We examined both the AUC with respect to increase (AUCi) and ground (AUCg), as these measures hold different information (Pruessner et al., 2003). Cohort was added as a covariate in our statistical models, as AUCs were based on three sets of slightly different time points (cohort 1: mean AUCi = 199, mean AUCg = 1383; cohort 2: mean AUCi = 174, mean AUCg = 905; cohort 3: mean AUCi = 30, mean AUCg = 947).

2.2.3. GR-1_F methylation

Methylation levels were determined at all 47 CpGs in the GR-1_F region, as well as at 5 adjacent CpGs relevant for GR-1_F exon transcription (see (Schür et al., 2017)).

Saliva (cohorts 1) and whole blood EDTA (cohorts 2 and 3) samples were collected at the day of testing and standard procedures were used to extract DNA (salting for blood and Qiagen extraction kits for saliva). DNA integrity and concentration were determined using BioAnalyser (Agilent Technologies, Santa Clara, CA) and riboGreen (Thermo Fisher Scientific, Waltham, MA), respectively. Quantification of GR-1_F methylation was carried out in two batches by EpigenDx (EpigenDx Inc, MA, USA (Brakensiek et al., 2007; England and Pettersson, 2005; Liu et al., 2007)) and procedures of quantification are more extensively described elsewhere (Schür et al., 2017). In short, the percentage of methylation per CpG was determined by considering the CpG site as an artificial C/T SNP using QCpG software (Qiagen, Valencia, CA), where % C equals % methylation as calculated by the equation below:

$$C\% = \text{RLU (C peak)} / \text{RLU (C peak} + \text{T peak)}$$

All 241 samples yielded sufficient pyrosequencing signals and good quality data (for assays, sensitivity, coefficients of variance, numbers of CpG sites, and chromosomal regions targeted by the primers, see Table S1 in the Supplemental Material). In accordance with our previous study (Schür et al., 2017), we examined mean methylation across all CpGs, the number of methylated loci (the number of CpGs with > 0% methylation) and mean methylation at 17 CpGs where methylation change was significantly associated with GR-1_F expression change (termed functional methylation (Schür et al., 2017)). In our measure of functional methylation, CpG 1 was not included for 179 subjects (cohorts 1 and 2) as these data were not available. See for mean methylation per CpG Supplemental Table S2.

2.2.4. Childhood trauma

The Childhood Trauma Questionnaire (CTQ) was used in all participants to retrospectively determine childhood adversity (Bernstein et al., 2003). The CTQ comprises the following five subscales: sexual, physical and emotional abuse, as well as physical and emotional neglect.

2.3. Statistical analyses

Linear regression models were used to investigate the association between GR-1_F methylation (mean methylation, number of methylated sites and functional methylation) and the cortisol stress response (AUCi and AUCg). To adjust for between-cohort differences in cortisol assessment protocol, tissue type, GR-1_F methylation batch (cohort 3 was measured separately from cohort 1 and 2) and TSST version (individual or group), cohort was included as factorial covariate. In addition, age, sex, childhood trauma and group status (two patient groups, two sibling groups and controls, discussed below) were included as covariates, yielding the following model: $AUC \sim \text{methylation} + \text{age} + \text{sex} + \text{childhood trauma} + \text{cohort} + \text{group status}$

This model was also used to investigate the association between childhood trauma and the cortisol stress response (without methylation as a covariate).

In secondary analyses, linear regression models were used to focus on the relation between GR-1_F methylation and childhood trauma (total CTQ score and scores on the five subscales), adjusting for the same covariates: $\text{methylation} \sim \text{age} + \text{sex} + \text{childhood trauma} + \text{cohort} + \text{group status}$

Residual plots of the main models were normally distributed, variance was homogeneous and there was no indication of outliers (all values of Cook's Distance < 1).

In separate analyses, GR-1_F methylation differences were evaluated between the following five groups: healthy controls (n = 131), patients with bipolar disorder (n = 43), schizophrenia patients (n = 13), siblings of patients with bipolar disorder (n = 24) and siblings of schizophrenia patients (n = 30). First, to remove unwanted covariation, residuals of the following model were obtained: $\text{methylation} \sim \text{age} + \text{sex} + \text{childhood trauma} + \text{cohort}$ Subsequently, ANOVAs were used with these residuals as the dependent variable and group status as the independent variable. As Bartlett's tests revealed heteroscedasticity among the groups for mean GR-1_F methylation, Welch's ANOVA for unequal variances was used for analysis. For the number of methylated sites non-parametric analyses were done using Kruskal-Wallis tests.

We included results of the analyses per CpG for the cortisol stress response and childhood trauma in the Supplemental Material as a resource for future studies. P-value significance in these analyses was set at < 0.00096 (0.05/52 CpGs), as opposed to < 0.05 in our main analyses.

2.4. Sensitivity analyses

To rule out possible confounding effects of drugs (including nicotine) or medication on the associations between our three GR-1_F methylation measures and the cortisol stress response, childhood trauma or group status, 93 subjects (among which all patients with schizophrenia or bipolar disorder) were excluded in sensitivity analyses. Moreover, separate sensitivity analyses focusing on the association between GR-1_F methylation and the cortisol stress response were carried out in women (n = 96), adjusting for oral contraceptive use, stage of the menstrual cycle and menstrual status. Finally, for the subset of participants from whom blood was available (n = 117), white blood cell types were inferred using DNA methylation signatures (Houseman et al., 2012), resulting in fractions of natural killer cells, granulocytes, CD4+ or CD8+ T-lymphocytes, monocytes and B-lymphocytes. To rule out confounding by cell type, the associations of these fractions with the main methylation measures (mean methylation, number of methylated

sites and functional methylation) were examined. In addition, these fractions were included as covariates to examine their effects on the main results.

2.5. Power analysis

GPower (Erdfeiler et al., 1996) was used to calculate the power of our primary analyses. With the current sample size and significance level of 0.05 we have 88% percent power to detect similar effect sizes as reported by Yehuda et al. ($r = 0.198$) (Yehuda et al., 2015). However, for the smaller effect sizes reported by Tyrka et al. ($r = 0.148$) (Tyrka et al., 2016) the current study has 68% power.

3. Results

3.1. General

Sample characteristics, including age, sex, childhood trauma and numbers of subjects in main and secondary analyses are presented in Table 1, stratified by cohort and group status.

Of note, childhood trauma was not associated with the cortisol stress response (AUCi: $p = 0.101$, $\beta = -4.6$; AUCg: $p = 0.141$, $\beta = -4.2$)

3.2. GR-1_F methylation and the cortisol stress response
No significant associations were found between our three main GR-1_F methylation measures (mean methylation, number of methylated sites and functional methylation) and the cortisol stress response (AUCi and AUCg; all p -values > 0.3, see Table 2 and Fig. 1). Moreover, after multiple comparisons correction, there were no significant associations between the cortisol stress response and GR-1_F methylation at the 52 single CpGs (all p -values > 0.00096, see Supplementary Material, Table S2).

3.2. GR-1_F methylation and childhood trauma

GR-1_F methylation was not significantly associated with the total level of childhood trauma (all p -values > 0.4, see Table 2 and Fig. 2), nor with any of the five subscales: emotional abuse, physical abuse, sexual abuse, emotional neglect and physical neglect (all p -values > 0.4, see Supplemental Table S3). In addition, analyses at individual CpGs did not reveal any associations surviving multiple comparisons correction (all p -values > 0.00096, see Supplementary Material, Table S2).

3.3. GR-1_F methylation in patients, siblings of patients and controls

There were no differences in GR-1_F methylation between any of the five groups, after adjusting for age, sex, childhood trauma and cohort (all p -values > 0.4, see Table 2 and Fig. 3).

3.4. Sensitivity analyses

Excluding subjects using drugs (including nicotine) or medication did not yield significant associations between GR-1_F methylation and the cortisol stress response, childhood trauma, or group status (all p -

values > 0.1, $n = 122$, $n = 148$ and $n = 148$, respectively; results not shown). Furthermore, adding the variables oral contraceptive use (yes: $n = 37$; no: $n = 18$), stage of the menstrual cycle (luteal phase: $n = 26$; follicular phase: $n = 5$) or menstrual status (premenopausal: $n = 59$; postmenopausal: $n = 33$) to the analyses investigating the association between GR-1_F methylation and the cortisol stress response in women did not render these associations significant (all p -values > 0.1, results not shown). Finally, none of the white blood cell type fractions were associated with any of the main methylation measures (all p -values > 0.1). Moreover, inclusion of the white blood cell type fractions in the main analyses did not yield significant associations of GR-1_F methylation with the cortisol stress response, childhood trauma, or group status (all p -values > 0.2, $n = 116$, $n = 117$ and $n = 117$, respectively; results not shown).

4. Discussion

The main goal of the present study was to investigate the relation between GR-1_F methylation and the cortisol stress response, as this link is presumed to be crucial in how (childhood) trauma may increase the risk to develop psychopathology later in life. To this end, we investigated the relation between GR-1_F methylation in the complete GR-1_F region (52 CpGs) and the cortisol stress response. We did not find any significant associations between our main methylation measures (mean methylation, number of methylated sites and functional methylation) or individual CpGs and the cortisol stress response. In secondary analyses, we evaluated the relation between GR-1_F methylation and childhood trauma, as well as its link to bipolar disorder and schizophrenia. We found no significant associations between childhood trauma and any of our methylation measures. Moreover, there were no group differences in our main methylation measures between patients with schizophrenia or bipolar disorder, siblings of such patients and healthy controls.

4.1. GR-1_F methylation in relation to HPA axis functionality

The focus of previous studies has been primarily on the relation between (childhood) trauma and GR-1_F methylation, with less consideration for its functional implications. Although GR-1_F methylation is assumed to be positively correlated to HPA axis activity through diminished GR-mediated negative feedback (Weaver et al., 2004), results of studies examining this relation have been surprisingly mixed (for a review see (Palma-Gudiel et al., 2015)). Using the dexamethasone/corticotropin-releasing hormone (Dex/CRH) test, Tyrka et al. (Tyrka et al., 2016) showed a low positive correlation between GR-1_F methylation and cortisol levels ($n = 231$, $r = 0.148$, $p < 0.05$). A similar association ($r = 0.198$) was found in 114 trauma-exposed military men, half of whom had developed PTSD (Yehuda et al., 2015). By contrast, Tyrka et al. (Tyrka et al., 2012) showed a negative correlation between GR-1_F methylation and post-Dex cortisol levels in 99 healthy individuals ($r \cong -0.25$, $p < 0.05$). Such mixed results have also been reported for social stress tests. Alexander et al. (Alexander et al., 2018) found higher peak cortisol levels following the TSST in individuals with elevated methylation at the most methylated CpG. However, these

Table 2
Results of main analyses.

Outcome	AUCi (n = 204)		AUCg (n = 204)		Childhood trauma (n = 241)		Group status (n = 241)
	β	p	β	p	β	p	p
Mean methylation	-106.01	0.389	50.48	0.687	0.00088	0.536	0.446
Number of methylated loci	-2.78	0.477	2.21	0.578	0.02678	0.559	0.957
Functional methylation	-51.52	0.658	102.89	0.384	0.00109	0.479	0.750

Associations of GR-1_F methylation with the cortisol stress response (areas under the curve with respect to increase (AUCi) and ground (AUCg)) to the Trier Social Stress Test, and with childhood trauma. In addition, p -values of methylation difference by group status (patients with either schizophrenia or bipolar disorder, siblings of such patients or controls) are presented.

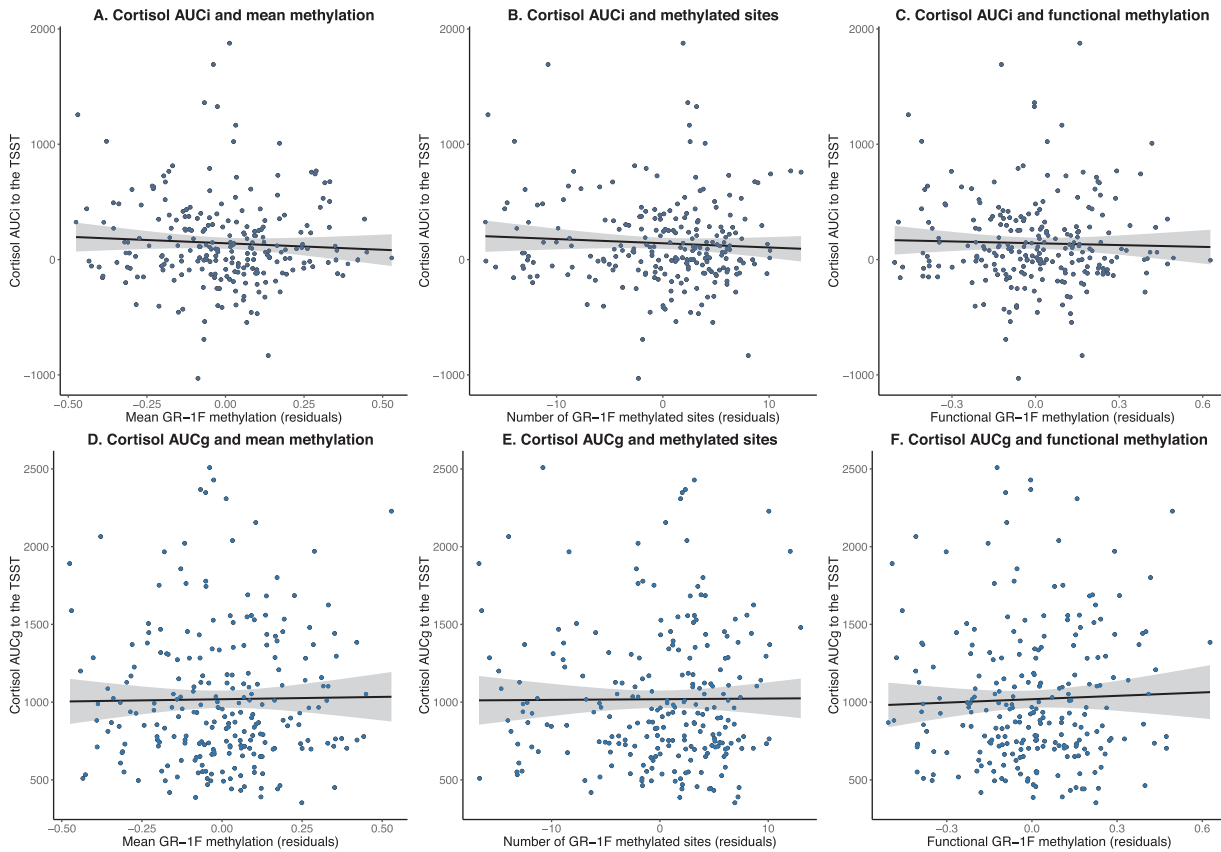


Fig. 1. Scatter plots of the cortisol stress response in relation to GR-1_F methylation. The associations of the area under the curve with respect to increase (AUCi, upper panel) and ground (AUCg, lower panel) with mean methylation (A and D), number of methylated sites (B and E) and functional methylation (C and F) are presented. The X-axis displays methylation levels adjusted for age, sex, childhood trauma and cohort.

effects only applied to trauma survivors ($n = 31$, $\beta = 0.203$, $p = 0.02$). Van der Knaap et al. (van der Knaap et al., 2015) used the Groningen Social Stress Test in 337 adolescents and found a positive association between GR-1_F methylation and cortisol levels in the recovery phase of the stress response ($\beta = 0.38$, $p < 0.001$), but not with peak cortisol levels. By contrast, Edelman et al. (Edelman et al., 2012) showed a negative association between GR-1_F methylation and total cortisol secretion during the TSST in 92 individuals ($R^2 = 0.213$, $p = 0.001$). Based on previously found positive associations between GR-1_F methylation and cortisol levels following a functional test, the power in the present study was between 68 and 88%. The null finding in the present study indicates that the functional implications of GR-1_F methylation

for HPA axis functionality are not very straightforward, possibly due to confounders (such as genetic background) that have not yet been identified.

4.2. GR-1_F methylation in relation to (childhood) trauma

The relation between childhood trauma and GR-1_F methylation has not been very consistent either. After Weaver et al. (Weaver et al., 2004) demonstrated that early life adversity resulted in elevated GR-1_F methylation in the hippocampus of rats, McGowan et al. (McGowan et al., 2009) translated this finding to humans in post-mortem research of suicide victims and controls. Most subsequent studies in peripheral

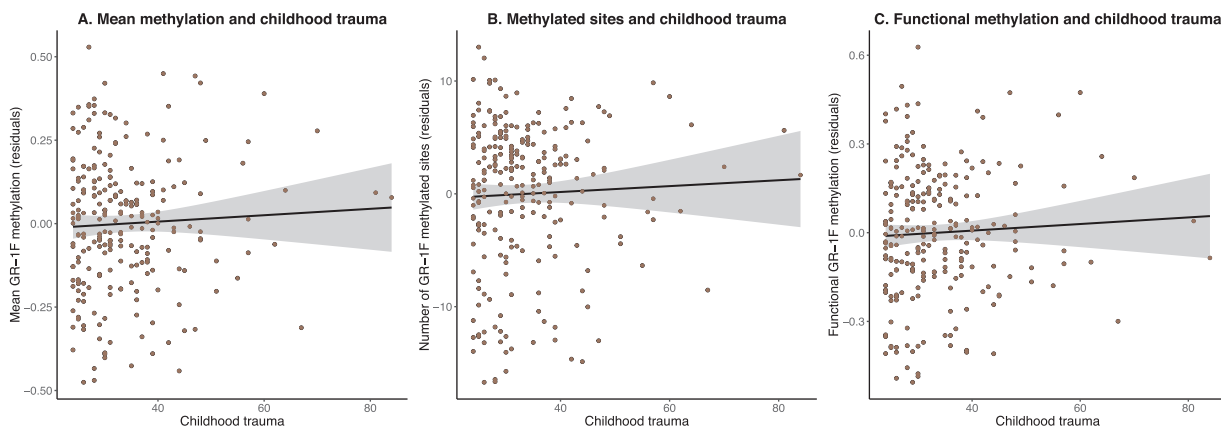


Fig. 2. Scatter plots of GR-1_F methylation in relation to childhood trauma. The association of childhood trauma with A. mean methylation, B. number of methylated sites and C. functional methylation are presented. The Y-axis displays methylation levels adjusted for age, sex and cohort.

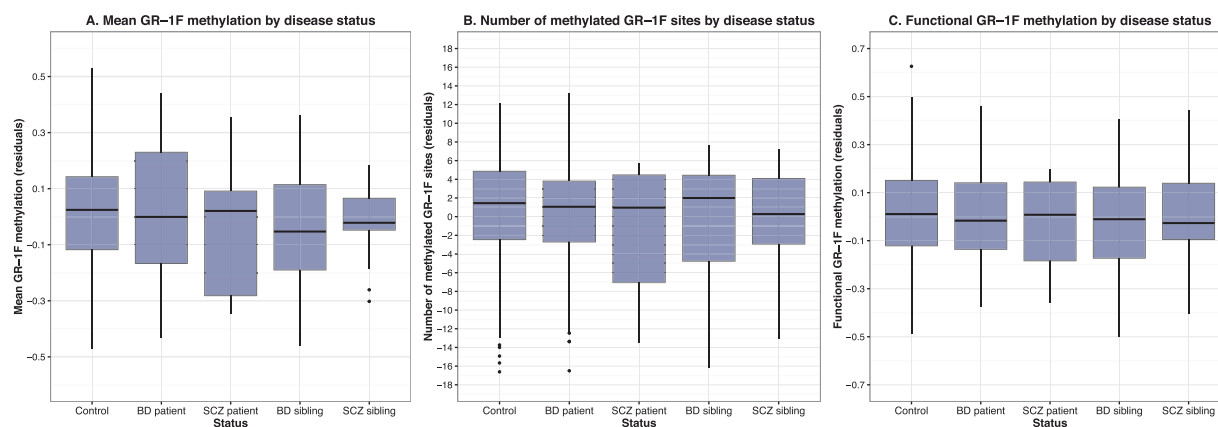


Fig. 3. Boxplot of GR-1_F methylation in healthy controls without siblings with bipolar disorder or schizophrenia ($n = 131$), patients with bipolar disorder ($n = 43$), schizophrenia patients ($n = 13$), siblings of patients with bipolar disorder ($n = 24$) and siblings of schizophrenia patients ($n = 30$). The Y-axis displays methylation levels adjusted for age, sex, childhood trauma and cohort.

tissues also demonstrated increased GR-1_F methylation following in utero or postnatal adverse events (Daskalakis and Yehuda, 2014). In addition, our recent longitudinal study showed a positive association between trauma exposure during military deployment and GR-1_F methylation change around deployment (Schür et al., 2017). However, a large study published in 2016 even reported a negative association between early life adversity and GR-1_F methylation (Tyrka et al., 2016). Moreover, there are several studies reporting no significant association (Vukojevic et al., 2014; Yehuda et al., 2015). This includes our recent study where we found no association between childhood trauma and either basal or longitudinal changes in GR-1_F methylation (Schür et al., 2017). In summary, the current study does not corroborate a positive association between the total level or any specific subtype of childhood trauma and GR-1_F methylation and weakens assertions of its biomarker properties.

4.3. GR-1_F methylation in relation to psychopathology

As the functional implications of GR-1_F methylation for HPA axis functionality are equivocal, the same can be expected for its link with psychopathology. Indeed, effects in opposite directions have been described for MDD (Na et al., 2014; Nantharat et al., 2015), PTSD (Perroud et al., 2014; Yehuda et al., 2015) and general psychopathology as measured using the Symptom Checklist 90 (Schür et al., 2017; Yehuda et al., 2015). To our knowledge GR-1_F methylation had not yet been investigated in patients with bipolar disorder or schizophrenia (or siblings of such patients) versus healthy controls. Although the present study did not show altered GR-1_F methylation in relation to these disorders, our group sizes were rather small (13 schizophrenia patients and 30 siblings; 43 patients with bipolar disorder and 24 siblings) and we could not adjust for possible confounding effects of medication as all subjects used medication.

4.4. Strengths and limitations

The main strength of this study is its large sample size with data on the cortisol stress response and GR-1_F methylation in the complete GR-1_F region for 204 individuals. Moreover, it is one of the largest studies investigating the relationship between childhood trauma and GR-1_F methylation ($n = 241$, three studies had larger sample sizes (Martín-Blanco et al., 2014; Tyrka et al., 2016; Van der Knaap et al., 2014)). Importantly, in contrast to the vast majority of previous studies on GR-1_F methylation, we investigated all 52 CpGs in this region. Finally, we included both healthy controls and patients (as well as unaffected siblings) with bipolar disorder or schizophrenia.

A limitation of this study is that only the GR-1_F region was

investigated, whereas this constitutes only a minor part of the whole *NR3C1* gene (Sinclair et al., 2012). Therefore, it could be argued that only limited explained variance to the cortisol stress response was to be expected. We chose to focus particularly on the GR-1_F region, as there is a large body of evidence showing relevance of this specific region in relation to childhood trauma, HPA axis functionality and psychopathology, despite generally very low methylation levels (see for example (Daskalakis and Yehuda, 2014)). As in almost all previous studies (Daskalakis and Yehuda, 2014; Tyrka et al., 2016), GR-1_F methylation was determined in peripheral tissues (blood and saliva), with unknown relevance to the structures where negative feedback on the HPA axis actually takes place (e.g. the hippocampus). However, cortisol reaches many different cell types and may alter their epigenomes equally (Turecki and Meaney, 2014). Moreover, a substantial part of the negative feedback takes place at the level of the pituitary gland, which is accessible to systemic corticosteroids. Nevertheless, important methylation differences across cell types and tissues exist (Davies et al., 2012; Hannon et al., 2015). Of note, we investigated the influence of white blood cell types on our main analyses in a subset of participants and found no changes in results. Other limitations include possible recall and social desirability bias inherent to the use of the Childhood Trauma Questionnaire. Furthermore, the number of schizophrenia patients was low ($n = 13$) and none of these patients or the patients with bipolar disorder were medication-naïve, making it impossible to exclude confounding effects of medication on the relation between psychopathology and GR-1_F methylation.

4.5. Conclusion and future directions

Our findings do not support a role for peripheral GR-1_F methylation as a proxy for cortisol levels in response to stress, nor a link between GR-1_F methylation and childhood trauma or psychopathology. Although a wider dispersion of GR-1_F methylation may yield more convincing associations, our results are in line with several recent studies indicating that the application of GR-1_F methylation to predict the cortisol stress response or vulnerability to psychopathology is complex. Therefore, our results support a cautious use of single biological measures and imply a broader and more integrative approach to explain variance in the cortisol stress response (Houtepen et al., 2016), as well as the relation between stress and psychopathology. Possibly, future longitudinal studies may reveal GR-1_F methylation to be a useful parameter at the level of individuals.

Conflict of interest

Funders had no role in design and reporting of the study. All authors

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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.2018.07.018>.

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