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ORIGINAL ARTICLE

Longitudinal changes in glucocorticoid receptor exon 1_F methylation and psychopathology after military deployment

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Several cross-sectional studies have demonstrated the relevance of DNA methylation of the glucocorticoid receptor exon 1_F region (GR-1_F) for trauma-related psychopathology. We conducted a longitudinal study to examine GR-1_F methylation changes over time in relation to trauma exposure and the development of post-deployment psychopathology. GR-1_F methylation (52 loci) was quantified using pyrosequencing in whole blood of 92 military men 1 month before and 6 months after a 4-month deployment period to Afghanistan. GR-1_F methylation overall (mean methylation and the number of methylated loci) and functional methylation (methylation at loci associated with GR exon 1_F expression) measures were examined. We first investigated the effect of exposure to potentially traumatic events during deployment on these measures. Subsequently, changes in GR-1_F methylation were related to changes in mental health problems (total Symptom Checklist-90 score) and posttraumatic stress disorder (PTSD) symptoms (Self-Report Inventory for PTSD). Trauma exposure during deployment was associated with an increase in all methylation measures, but development of mental health problems 6 months after deployment was only significantly associated with an increased functional methylation. Emergence of post-deployment PTSD symptoms was not related to increased functional methylation over time. Pre-deployment methylation levels did not predict post-deployment psychopathology. To our knowledge, this is the first study to prospectively demonstrate trauma-related increases in GR-1_F methylation, and it shows that only increases at specific functionally relevant sites predispose for post-deployment psychopathology.

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INTRODUCTION

Exposure to traumatic stress is a major risk factor for a wide range of psychiatric disorders, including posttraumatic stress disorder (PTSD) and major depressive disorder (MDD).[1] The hypothalamus–pituitary–adrenal (HPA) axis is crucial for an adequate response to a stressful environment. The glucocorticoid receptor (GR) determines negative feedback on the HPA axis, and impaired GR functionality has been proposed as a potential pathophysiological mechanism underlying both PTSD and MDD.[2]

Over a decade ago, Weaver *et al.*[3] demonstrated that DNA cytosine methylation of the GR exon 1_7 promoter in the hippocampus of rats remains elevated after early life stress and leads to decreased GR expression and increased adult HPA axis activity. This methylation effect was particularly pronounced at a nerve growth factor-inducible protein A (NGFI-A) transcription factor binding site. McGowan *et al.*[4] translated these findings to humans, showing elevated methylation in the GR exon 1_F region (GR-1_F) in the hippocampus of suicide victims with a childhood abuse history compared with nonabused suicide victims and controls. Following these seminal reports, the number of studies examining GR-1_F methylation in relation to traumatic stress and stress-related psychiatric disorders has steadily increased (for reviews see refs 5–7). Of note, all of these studies examined peripheral tissues to assess GR-1_F methylation and significant findings were not limited to NGFI-A-binding sites. In humans, both pre- and postnatal stress have consistently been linked to increased GR-1_F methylation in most,[5] but not all studies.[8] In addition, decreased GR-1_F methylation has been found in patients with MDD,[9] whereas both increased[10] and decreased[11] GR-1_F methylation were reported in patients with PTSD compared with the controls. These studies all employed a cross-sectional design, which precludes conclusions about causality. As a result, it is still unknown whether GR-1_F methylation changes as psychopathology emerges and/or whether it represents a pre-existing vulnerability factor predicting the development of psychopathology.

We therefore quantified GR-1_F methylation levels in peripheral blood cells of Dutch military personnel before and after deployment to Afghanistan. In line with the accumulating evidence (for an overview see Daskalakis *et al.*[5]), we hypothesized that trauma exposure during deployment would increase GR-1_F methylation. In addition, we wanted to explore the direction of GR-1_F methylation change in relation to emerging psychopathology

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Figure 1. The glucocorticoid receptor exon 1_F region (GR-1_F) in *NR3C1*. (**a**) All 52 5′-C–phosphate–G-3′ sites (CpGs) where methylation was measured in the present study are numbered (red: CpGs where change in methylation was associated with GR exon 1_F expression change). Lowercase letters mark the promoter region, uppercase letters the GR-1_F exon. Black-lined boxes represent canonical (solid) and non-canonical (dashed) NGFI-A binding sites, after McGowan *et al.*[4] (**b**) *NR3C1* (Chromosome 5: 142 657 496–142 815 077) from the 5′ (left) to 3′ (right) untranslated region. Boxes on the horizontal black line represent exons (gray: non-coding, green: coding sequence). The light gray box indicates a CpG island in the proximal promoter region (from 1D to 1H). (**c**) Six common and functional haplotypes constituted by six SNPs (rs10052957, rs10482605, rs6189, rs6195, rs41423247 and rs6198) and their frequencies in the present study. Red letters represent the minor alleles. For a scaled representation of exons 2 through 9 in relation to the first exons, see Sinclair *et al.*[23]

symptoms after deployment, as previous evidence is equivocal.[5] As reported in a previous study,[11] we expected methylation changes associated with changes in GR exon 1_F expression to be most informative of change in psychopathology symptoms. Furthermore, we investigated the predictive value of pre-deployment GR-1_F methylation for the development of post-deployment psychopathology symptoms. Finally, we explored the association between GR-1_F methylation and six functional haplotypes of the GR gene (*NR3C1*), which were previously associated with GR sensitivity and might be relevant for the development of psychopathology.[12]

MATERIALS AND METHODS

Participants and assessments

The present study was part of a large longitudinal cohort of Dutch military personnel that were deployed to Afghanistan ($n = 1032$), as part of the International Security Assistance Force, between 2005 and 2008 (for details on procedures see refs 13,14). Among their duties were searching and clearing of buildings, combat patrols, transport across enemy territory and demining operations. The individuals were exposed to potentially traumatic events such as armed combat, combat casualties and enemy fire. Individuals were assessed (questionnaires were administered and blood samples were drawn) 1 month before, and 1 and 6 months after a 4-month period of deployment. This study examined methylation measures prior to and 6 months after deployment, as psychopathology levels were highest 6 months after deployment.[13] To maximize power,[15] three subgroups of approximately equal size were selected from this cohort ($N = 92$) based on exposure to potentially traumatic events during deployment (high or low) and post-deployment psychopathology (high or low, only in group with high trauma exposure).[16] The study was approved by the Institutional Review Board of the University Medical Center Utrecht (UMCU) and both oral and written informed consent was obtained from all participants.

Mental health problems were assessed with the validated Dutch version of the Symptom Checklist-90 (SCL-90), which includes the total score of eight symptom dimensions: agoraphobia, anxiety, depression, somatization, cognitive performance deficits, mistrust and interpersonal sensitivity, hostility and sleep difficulties.[17] PTSD symptoms were measured using the Self-Report Inventory for PTSD (SRIP).[18] Changes in SCL-90 and SRIP scores were significantly correlated ($\rho = 0.52$). A 19-item self-report deployment experiences checklist was used to assess traumatic stress exposure during deployment, such as direct combat stressors.[13] For childhood trauma, the Early Trauma Inventory-Self Report was used, which includes emotional, physical and sexual abuse.[19] For prediction of the development of psychopathology after deployment, individuals were divided into groups with low or high symptom levels based on previously defined cutoff scores of 124 for total SCL-90 and 38 for the SRIP.[14,17,20] This SCL-90 cutoff score delineates individuals in the general population exceeding average symptoms.[21] The SRIP cutoff score[14] is in the range with the best sensitivity and specificity for PTSD classification according to the DSM-IV (in a general population).[22]

Biological measures

Methylation was quantified in 52 CpGs in and adjacent to the 1_F region (see Figure 1, numbering in accordance with Daskalakis *et al.*[5]). The NGFI-A-binding sites in this region (CpG numbers: 17, 18, 21–26, 35–37, 42, 43) have been suggested to be relevant for GR-1_F transcription and HPA axis activity.[3]

Whole-blood EDTA samples were collected 1 month before and 6 months after deployment. Standard salting procedures were used to extract DNA. Subsequently, DNA concentration and integrity were determined using riboGreen (Thermo Fisher Scientific, Waltham, MA, USA) and BioAnalyser (Agilent Technologies, Santa Clara, CA, USA), respectively.

GR-1_F methylation data were available for 92 individuals. For methylation analysis (EpigenDx, Hopkinton, MA, USA[24–26]), DNA was denatured using 3 N NaOH followed by 30- min incubation at 42 °C. Bisulfite salt solution was added to the DNA and incubated for 14 h at 50 °C. Bisulfite-treated DNA was purified using Zymogen DNA columns and eluted in 20 µl $T_1E_{0.2}$ (pH 8) and 1 µl was used for each PCR. PCR was performed using 5 ng of genomic DNA and 0.2 µM of each primer. The final PCR product was purified using Sepharose beads (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and the Pyrosequencing Vacuum Prep Tool (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. Purified PCR product was sequenced using 0.2 µM pyrosequencing primer on the Pyrosequencing PSQ96 HS System (Qiagen) following the manufacturer's instructions. The percentage of methylation at each locus was determined by looking at the CpG site as an artificial C/T SNP using QCpG software (Qiagen), where *C*% equals %methylation as calculated by the equation below:

$$C\% = \mathrm{RLU(Cpeak)/RLU(Cpeak + Tpeak)}.$$

All samples resulted in good pyrosequencing signals and good quality data (for assays, sensitivity, coefficients of variance, numbers of CpG sites and chromosomal regions targeted by the primers, see Supplementary Table S1 in the online data supplement). As GR-1_F methylation levels are generally low,[27,28] two measures of overall GR-1_F methylation were examined: mean methylation across all CpGs and the number of methylated loci (the number of CpGs with >0% methylation). In addition, to optimally investigate the functional dynamics of methylation, we focused on those CpGs where we observed that longitudinal changes in methylation were significantly related to change in GR exon 1_F mRNA expression (for the latter see below), defined as functional methylation. In exploratory analyses, we examined DNA methylation at NGFI-A-binding sites.

GR exon 1_F expression data were available for 75 individuals, as RNA quality was not sufficient in 17 individuals. Pre- and post-deployment RNA from peripheral blood mononuclear cells (PBMCs) was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One µg of total RNA was converted into first-strand cDNA with oligo (dT) primers using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) as described by the manufacturer. qPCR assays were carried out in duplicate in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with specific primers (see Supplementary Table S3 in the online data supplement) and SensiMix SYBR Hi-ROX (Bioline Reagents, London, UK). For normalization of mRNA expression, RefFinder (OMICtools, Sotteville-Les-Rouen, France) was used to find the most stable reference gene among *PPIB*, *YWHAZ*, *RPL13a* and *RPL37a*. The relative abundance of mRNAs was standardized with *RPL13a* mRNA as the invariant control.

In line with the finding that lower GR-1_F methylation is associated with dampening of the cortisol response through higher GR-mediated negative feedback,[3] we hypothesized GR-1_F methylation to be negatively associated to GR sensitivity. Data on GR-binding capacity of PBMCs were available for 47 individuals, as these were acquired several years earlier from a largely overlapping cohort.[20] As previously described,[20] GR-binding capacity was quantified using a modification of the whole-cell single point-binding assay described by Yehuda *et al.*[29] Using this protocol, specific binding at 100 nM ^{3}H-dexamethasone gave a reliable Bmax, which was determined with a classical binding assay with 3-200 nM ^{3}H-dexamethasone in the absence or presence of an excess of unlabeled dexamethasone ($r^2=0.92$).[20] In short, Ficoll-Paque (Pharmacia and Upjohn, Uppsala, Sweden) was used to isolate PBMCs from heparinized whole blood, and 10^7 cells were frozen in RPMI-1640 (Gibco, Grand Island, NY, USA) in addition of 10% fetal calf serum (FCS) (Gibco) and 20% DMSO. Samples of every single individual were analyzed simultaneously. Cells were thawed, and subsequently washed twice in RPMI-1640 and incubated for 30 min at 37 °C. Again, cells were washed twice, after which they were resuspended in assay buffer (RPMI- 1640 with 10% FCS) and incubated in duplicate with 100 nM ^{3}H-dexamethasone (Amersham, Buckinghamshire, UK) in the absence or presence of excess unlabeled dexamethasone (Sigma-Aldrich, Steinheim, Germany). After 60 min incubation at 37 °C, cells were washed twice in ice cold assay buffer followed by density centrifugation over Ficoll hypaque, separating cell-bound label from free label. Liquid scintillation analysis was used to analyze cell-bound radioactivity. In two simultaneously processed samples the number of cells was counted.

To examine the effect of genetic variation in the GR gene (*NR3C1*) on GR-1_F methylation, six SNPs linked to glucocorticoid sensitivity (rs10052957, rs10482605, rs6189, rs6195, rs41423247 and rs6198)[12] were selected. SNPs were genotyped on the Illumina Human OmniExpress-24 Beadchip array, and genetic data were subjected to quality control (see Supplementary Table S4 in the online data supplement for quality control information). SNPs rs10482605, rs6189 and rs41423247 were imputed using the Haplotype Reference consortium release 1.1. R^2 values and average call rates were >0.99 for all three SNPs, whereas allele frequencies did not deviate from Hardy–Weinberg equilibrium (all *P*-values >0.05). Haplotypes were constructed using SNPHAP,[30] yielding the following *NR3C1* haplotypes: CTGACA (40%), CTGAGA (22%), TTGAGA (15%), TCGACG (13%), CTGGCA (4%) and TCAACG (4%)). *NR3C1* haplotypes and individual SNPs from 85 individuals were available.

Statistical analyses

Longitudinal changes in GR-1_F methylation around deployment were analyzed by applying mixed models for repeated measures (MMRM) using the nlme package[31] in R. The relation of changes in GR methylation with trauma exposure was investigated in a model without adjustment for symptom outcomes whereas the relation with symptom outcomes was investigated in a model with adjustment for trauma exposure during deployment (*methylation ~ methylation*$_{pre}$*+trauma during deployment × time+change in mental health problems × time, random factor: subject ID*). We only used continuous measures. PTSD symptom scores were log-transformed to improve the distribution.[6] In secondary analyses, *NR3C1* haplotypes/SNPs or childhood trauma was added to the model. To investigate the influence of GR-1_F methylation on GR-1_F expression and GR-binding, the latter two measures were used as dependent variables (for example, *expression ~ expression*$_{pre}$*+trauma during deployment × time +change in methylation × time, random factor: subject ID*).

Linear regression models were used to examine the cross-sectional relations between pre-deployment GR-1_F methylation and childhood trauma or *NR3C1* haplotypes/SNPs. Pre- and post-deployment psychopathology symptoms were compared using paired two-tailed *t*-tests. Methylation values of one individual were >8 s.ds above the mean methylation and were excluded from further analyses (resulting in $n=91$). Random effects and residuals of the main MMRM were plotted and evaluated for heteroscedasticity and did not show further aberrations.

Considering the relatedness and relatively low number of tests, the significance threshold was set at $P<0.05$ in primary analyses. However, as 12 *NR3C1* haplotypes/SNPs were investigated without prior hypotheses, the significance threshold in these analyses was set at $P<0.0042$ (0.05/12).

For prediction, receiver operation curves (ROC) were constructed to predict a high level of post-deployment mental health problems or PTSD symptoms with pre-deployment GR-1_F methylation. Individuals with a high level of pre-deployment mental health problems ($n=8$) or PTSD symptoms ($n=0$) were excluded in the respective analyses. Areas under the curve (AUCs) and corresponding *P*-values were determined using the verification package in R.[32] Sensitivity, specificity, positive and negative predictive values were calculated using the Epi package.[33]

Potential confounding was investigated by analyzing the correlations of age, body-mass index (BMI), military rank, education, previous number of deployments and white blood cell counts (including values of neutrophils, lymphocytes, monocytes, eosinophils and basophils) with methylation before and after deployment. Moreover, we analyzed the interaction of these covariates with time in our main models to investigate whether they affected the relation between our variables of interest (trauma, and change in psychopathology, GR exon 1_F expression and GR-binding) and change in GR-1_F methylation.

To investigate the possible influence of alcohol use, medication and smoking, we excluded all individuals that changed medication between baseline and follow-up ($n=18$, for types of medication see Supplementary Table S3 in the online data supplement), as well as individuals with a changed alcohol use ($n=1$) or smoking status ($n=6$) in sensitivity analyses.

Table 1. Sample characteristics (total: $n = 91$, all male)

Characteristics	*Mean*			*s.d.*			*Range*		
Age	27.5			9.3			18–54		
Childhood trauma	3.0			2.6			0–11		
Number of previous deployments	0.9			1.2			0–5		
Number of deployment-related trauma events	5.4			4.2			0–13		
BMI	24.4			2.7			18.1–35.8		
Education	Low			Moderate			High		
	42			42			6		
Rank	Private		Corporal	Non-commissioned officer			(Staff) officer		
	45		20	17			8		
	Pre-deployment			Post-deployment			*t*	df	*P*
	Mean	s.d.	Range	Mean	s.d.	Range			
	103.7	17.1	90–209	110.8	23.1	90–204	2.97	87	0.004
	26.4	4.0	22–36	31.9	9.9	22–56	4.81	87	0.000006

Abbreviation: BMI, body-mass index. Education: low: some years of high school; moderate: finished high school; high: some years of college or university. Information about rank and education was missing for one subject.

Table 2. Summaries of the main analyses: associations of change in methylation (mean, number of methylated sites and functional) with trauma exposure and change in mental health problems, PTSD symptoms, GR exon 1_F expression and GR-binding

Methylation	*Trauma exposure* (n = *91*)			*Mental health problems* (n = *88*)			*PTSD symptoms* (n = *88*)			*GR exon 1_F expression* (n = *75*)			*GR-binding* (n = *47*)		
	β	t	P	β	t	P	β	t	P	β	t	P	β	t	P
Mean	0.040	3.02	0.003	0.004	1.65	0.10	0.233	1.05	0.30	−0.543	−2.63	0.011	−463	−2.56	0.014
Number of methylated loci	0.745	3.24	0.002	0.089	1.98	0.050	4.356	1.14	0.26	−0.039	−3.41	0.001	−29.9	−2.68	0.010
Functional	0.056	3.16	0.002	0.010	2.89	0.005	0.450	1.53	0.13	−0.526	−4.02	< 0.001	−317	−2.51	0.016

Abbreviations: GR, glucocorticoid receptor; PTSD, posttraumatic stress disorder.

RESULTS

Sample characteristics, including information on age, childhood trauma, number of previous deployments, number of deployment-related trauma events, BMI, education, rank, mental health problems and PTSD symptoms are listed in Table 1.

Mean methylation per locus ranged from 0 to 2.60% (see Supplementary Table S2 in the online data supplement). Methylation changes per locus were highly correlated, especially for CpGs 31 to 52 (see Supplementary Figure S1 in the online data supplement).

Change in GR-1_F methylation was associated with change in GR exon 1_F expression at a nominal significance level at the following 17 CpGs: 1, 9, 11, 12, 23, 24, 28, 34, 39, 40, 45–51 (all *P*-values < 0.05, see Figure 1, as well as Supplementary Table S2 and Supplementary Figure S2 in the online data supplement). The mean methylation at these sites constituted our measure of functional methylation. Changes in our three main methylation measures (mean methylation, number of methylated sites and functional methylation) over time were significantly correlated with a minimal ρ of 0.89. Increases in all three methylation measures were associated with decreases in GR exon 1_F expression (mean methylation: $\beta = -0.54$, $P = 0.011$; number of methylated loci: $\beta = -0.039$, $P = 0.001$; functional methylation: $\beta = -0.526$, $P < 0.001$, see Table 2 and Figures 2a and b) and GR-binding (mean methylation: $\beta = -463$, $P = 0.014$; number of methylated loci: $\beta = -29.9$, $P = 0.010$; functional methylation: $\beta = -317$, $P = 0.016$). In exploratory analyses, methylation change at NGFI-A binding sites was not associated with a change in GR exon 1_F expression ($\beta = -0.22$, $P = 0.36$) or GR-binding ($\beta = -413$, $P = 0.06$) and was therefore not further analyzed.

GR-1_F methylation and exposure to potentially traumatic events

Trauma exposure during deployment was significantly associated with an increase in all three methylation measures (mean methylation: $\beta = 0.040$, $P = 0.003$; number of methylated loci: $\beta = 0.75$, $P = 0.002$; functional methylation: $\beta = 0.56$, $P = 0.002$, see Table 2) and with an increase in methylation at 23 individual CpGs: 2, 4, 8, 9, 17, 19, 20, 22, 28, 31, 32, 34, 35, 39, 40, 45–52 (see Supplementary Table S2 in the online data supplement).

No significant associations were observed between childhood trauma and our main methylation measures (change in methylation over time: all *P*-values > 0.54; pre-deployment methylation: all *P*-values > 0.40).

GR-1_F methylation and the development of psychopathology

Although the development of mental health problems was only at trend level significance associated with an increase in the number of methylated loci ($\beta = 0.089$, $P = 0.050$) (Figure 2c) and not with mean methylation ($\beta = 0.004$, $P = 0.10$), it was significantly associated with an increase in functional methylation ($\beta = 0.010$, $P = 0.005$) (Table 2 and Figure 2d). Significant associations between change in mental health problems and methylation change existed at 14 CpGs (22, 31–35, 39–41, 44, 47, 48, 51, 52).

The development of PTSD symptoms was not associated with change in any methylation measure (all *P*-values > 0.12, see Table 2). At a nominal significance level, change in PTSD

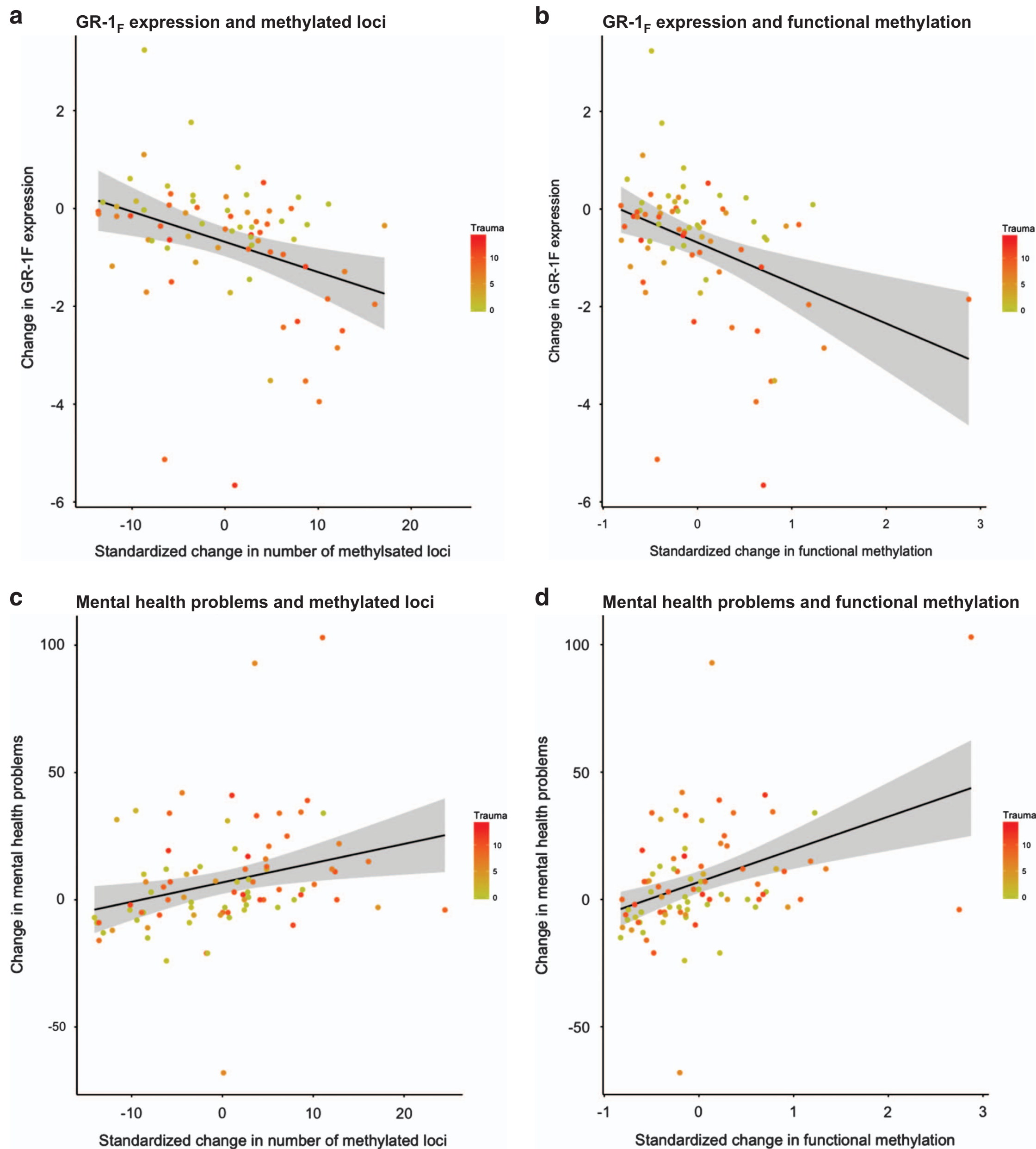

Figure 2. Change in mental health problems in relation to (**a**) the number of methylated loci ($n=88$, $\beta=0.089$, $P=0.050$) and (**b**) functional methylation ($n=88$, $\beta=0.010$, $P=0.005$), and change in GR exon 1_F expression in relation to (**c**) the number of methylated loci ($n=75$, $\beta=-0.039$, $P=0.001$) and (**d**) functional methylation ($n=75$, $\beta=-0.53$, $P<0.001$). Standardized change indicates that methylation levels after deployment were adjusted for pre-deployment values.

symptoms was associated with methylation change at 9 individual CpGs (10, 20, 25, 31, 35–37, 44, 51, see Supplementary Table S2 in the online data supplement).

GR-1_F methylation and genetic variation in NR3C1

None of the *NR3C1* haplotypes or individual SNPs was significantly associated with either pre-deployment or prospective changes in GR-1_F methylation measures after correcting for multiple testing (data not shown). Nevertheless, rs10052957 showed an association with prospective methylation changes at a nominal significance level (TC carriers with CC as reference; mean methylation: $\beta=-0.25$, $P=0.046$; number of methylated loci: $\beta=-4.21$, $P=0.045$; functional methylation: $\beta=-0.38$, $P=0.022$).

Confounder and sensitivity analyses

Confounding by age, BMI, military rank, education, previous number of deployments and white blood cell counts (including values of neutrophils, lymphocytes, monocytes, eosinophils and basophils) was highly unlikely as these measures were not correlated with methylation before or after deployment (*P*-values >0.05). In addition, cell-type composition was similar before and after deployment.[16] Furthermore, including the interactions of these covariates with time did not affect the associations between change in GR-1_F methylation with trauma exposure during deployment, change in mental health problems or PTSD symptoms, change in GR-1_F expression or change in GR-binding.

No individuals used antidepressants at any time point (see Supplementary Table S5 in the online data supplement). Exclusion

of individuals with changed medication, smoking status or alcohol use status after deployment (total $n = 23$) strengthened most associations of GR-1_F methylation in the longitudinal analyses (as indicated by the βs) except for the associations with GR-binding, which did not remain significant (see Supplementary Table S6 in the online data supplement).

Prediction of post-deployment psychopathology by pre-deployment GR-1_F methylation

Pre-deployment methylation did not significantly predict a high level of post-deployment mental health problems (mean methylation: AUC = 0.51, $P = 0.47$; number of methylated loci: AUC = 0.54, $P = 0.32$; functional methylation: AUC = 0.51, $P = 0.47$) or PTSD symptoms (mean methylation: AUC = 0.58, $P = 0.11$; number of methylated loci: AUC = 0.56, $P = 0.18$; functional methylation: AUC = 0.56, $P = 0.17$).

DISCUSSION

We examined longitudinal changes in whole-blood GR-1_F methylation from 1 month prior to until 6 months after a 4-month period of military deployment in relation to deployment-related trauma and the development of mental health problems and PTSD symptoms. We found that deployment-related trauma was associated with an increase in all GR-1_F methylation measures. However, only an increase in GR-1_F methylation at loci associated with GR exon 1_F expression (functional methylation) was significantly related to the development of post-deployment mental health problems. In contrast, the emergence of PTSD symptoms was not associated with an increase in functional methylation over time. Pre-deployment GR-1_F methylation did not predict a high level of psychopathology symptoms after deployment. Moreover, six functional *NR3C1* haplotypes and the six SNPs constituting these haplotypes were not associated with changes in GR-1_F methylation.

We believe this study provides the first longitudinal evidence in humans that methylation differences between trauma- and non-trauma-exposed individuals previously reported in the majority of cross-sectional studies (see for review Daskalakis *et al.*[5]) reflect a response to traumatic stress exposure. An increase in GR-1_F methylation at functionally relevant sites could result in impaired negative feedback on the HPA axis,[34] leading to increased vulnerability to psychopathology. The here reported association between functional GR-1_F methylation increase and emerging mental health problems fits this hypothesis. In contrast, we did not find any associations between childhood trauma and GR-1_F methylation, whereas most, but not all,[8] previous studies found a positive association (see for review Daskalakis *et al.*[5]). Of note, the fact that elevated GR-1_F methylation levels could constitute a vulnerability factor for the development of psychopathology was not supported by our prediction models and has not been investigated in prior studies. Moreover, the only two previous longitudinal studies on GR-1_F methylation do not indicate that psychotherapeutic interventions influence this hypothesized pathophysiological mechanism. Perroud *et al.*[35] reported no change in mean GR-1_F methylation in 61 patients with borderline personality disorder after intensive dialectical behavior therapy. Yehuda *et al.*[28] showed that pre-treatment GR-1_F methylation levels (sum and number of methylated loci) significantly differed between responders ($n = 8$) and non-responders ($n = 8$) receiving psychotherapy for PTSD, but did not find a significant methylation change over time.

In contrast to our study, it was previously observed that individuals with a high level of post-deployment PTSD symptoms had increased GR sensitivity[36] and GR-binding[14,37] prior to deployment (whereas depressive symptom development was associated with decreased GR sensitivity[36]). Moreover, Yehuda *et al.*[11] found lower GR-1_F methylation and evidence for increased GR sensitivity in PTSD patients compared with trauma-exposed controls. These opposing directionalities of effects cannot be ascribed to a difference in pathophysiology between PTSD symptoms and mental health problems (measured with the SCL-90), as Yehuda *et al.*[11] found negative associations of GR-1_F methylation with both outcomes, whereas we found positive associations (not significant for PTSD symptoms). Of note, both increased and decreased functionality of the GR have been linked to psychopathology, and Raison and Miller[2] previously suggested that these mechanisms are specific to PTSD and MDD, respectively. However, two recent studies contradict this hypothesis, showing decreased GR-1_F methylation in MDD[9] and increased GR-1_F methylation in PTSD.[10] Moreover, PTSD and MDD often co-occur which complicates the interpretation of findings. This is illustrated by the significant correlation ($\rho = 0.50$) between change in depressive symptoms (subscale SCL-90) and change in PTSD symptoms in the present study.

The main strength of the current study is its longitudinal design, with GR-1_F methylation, expression, GR-binding and psychiatric outcomes assessed before and after military deployment. Evidence from longitudinal studies is paramount as it is much less influenced by genetic predispositions and other confounding factors that may influence both methylation levels and psychopathology than cross-sectional studies. In addition, the present study investigated the entire GR exon 1_F region, allowing examination of all CpGs in relation to GR exon 1_F expression. This approach highlights that associations between change in GR-1_F methylation and expression are site-specific and present at 17 out of 52 CpGs. Thirteen of these 17 CpGs have previously been associated with trauma and/or psychopathology (CpG numbers 1, 9, 23, 28, 34, 40 and 45–51, see this review[5]). Our findings do not support an important role for NGFI-A binding sites in GR-1_F transcription. This is in line with recent evidence suggesting that several other transcription factors are important in the GR exon 1_F region.[38] Other strengths are the generally healthy cohort at baseline, the absence of antidepressant use at any time point and the sensitivity analyses that exclude a confounding effect of change in any medication, smoking or alcohol use status. Finally, we explored the associations between *NR3C1* haplotypes and SNPs with GR-1_F methylation and did not find any significant associations with baseline GR-1_F methylation, which is in line with a recent study,[39] or with methylation change. This finding highlights the fact that GR-1_F methylation is just one biological mechanism influencing GR sensitivity, whereas *NR3C1* haplotypes may be independently associated with GR functioning and psychopathology.[12]

A possible limitation is the relevance of peripheral blood methylation to the brain. Several studies suggest that methylation differences across tissues are substantial,[40,41] even though consistent effects of various methylation quantitative trait loci are found across tissues.[42] However, the effect of trauma, mediated through stress hormones or cytokines, appears to affect the epigenome in a wide range of cell and tissue types.[7] This is in line with findings of Tyrka *et al.*,[34] who demonstrated a cross-sectional relation between trauma-related whole-blood GR-1_F methylation with decreased negative feedback of the HPA axis. Also, increased GR-1_F methylation in peripheral blood cells has consistently been linked to (early life) adversity.[5] The question of generalizability of methylation patterns across tissues also pertains to our measurement of GR exon 1_F expression and GR-binding in PBMCs, instead of whole blood.[43] Another limitation is our focus on GR-1_F, whereas methylation in other parts of *NR3C1* may hold additional information about the relation between trauma and GR function.[44] In addition, only Dutch Caucasian males were investigated that limits the generalizability. This may be relevant as there is evidence for ethnic and sex-specific differences in cortisol stress responsivity in relation to DNA methylation.[45]

Furthermore, in addition to baseline GR exon 1_F expression data examined here, GR exon 1_F expression data following stress may provide additional information of its relation with GR-1_F methylation. Finally, we used self-report questionnaires to assess psychiatric outcomes, constituting a possible source of social desirability bias, and our questionnaire for exposure to potentially traumatic events does not take severity and impact into account.

In conclusion, this is the first longitudinal evidence-linking changes in GR-1_F methylation to trauma exposure and the development of psychopathology symptoms. Our data indicate that trauma exposure increases GR-1_F methylation and that only increases at specific functionally relevant sites may predispose for post-deployment psychopathology. These results suggest an important role for epigenetic regulation of GR functioning after trauma exposure in the development of psychopathology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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