Genome-wide differentially methylated genes associated with posttraumatic stress disorder and longitudinal change in methylation in rape survivors

Jani Nöthling, Naeemah Abrahams, Sylvanus Toikumo, Matthew Suderman, Shibe Mhlongo, Carl Lombard, Soraya Seedat and Sian Megan Joanna Hemmings

Rape is associated with a high risk for posttraumatic stress disorder (PTSD). DNA methylation changes may confer risk or protection for PTSD following rape by regulating the expression of genes implicated in pathways affected by PTSD. We aimed to: (1) identify epigenome-wide differences in methylation profiles between rape-exposed women with and without PTSD at 3-months post-rape, in a demographically and ethnically similar group, drawn from a low-income setting; (2) validate and replicate the findings of the epigenome-wide analysis in selected genes (BRSK2 and ADCYAP1); and (3) investigate baseline and longitudinal changes in BRSK2 and ADCYAP1 methylation over six months in relation to change in PTSD symptom scores over 6 months, in the combined discovery/validation and replication samples (n = 96). Rape-exposed women (n = 852) were recruited from rape clinics in the Rape Impact Cohort Evaluation (RICE) umbrella study. Epigenome-wide differentially methylated CpG sites between rape-exposed women with (n = 24) and without (n = 24) PTSD at 3-months post-rape were investigated using the Illumina EPIC BeadChip in a discovery cohort (n = 48). Validation (n = 47) and replication (n = 49) of BRSK2 and ADCYAP1 methylation findings were investigated using EpiTYPER technology. Longitudinal change in BRSK2 and ADCYAP1 was also investigated using EpiTYPER technology in the combined sample (n = 96). In the discovery sample, after adjustment for multiple comparisons, one differentially methylated CpG site (chr10: 61385771/cg01700569, p = 0.049) and thirty-four differentially methylated regions were associated with PTSD status at 3-months post-rape. Decreased BRSK2 and ADCYAP1 methylation at 3-months and 6-months post-rape were associated with increased PTSD scores at the same time points, but these findings did not remain significant in adjusted models. In conclusion, decreased methylation of BRSK2 may result in abnormal neuronal polarization, synaptic development, vesicle formation, and disrupted neurotransmission in individuals with PTSD. PTSD symptoms may also be mediated by differential methylation of the ADCYAP1 gene which is involved in stress regulation. Replication of these findings is required to determine whether ADCYAP1 and BRSK2 are biomarkers of PTSD and potential therapeutic targets.

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INTRODUCTION

Rape and sexual assault are associated with a high risk for the development of posttraumatic stress disorder (PTSD) compared to other trauma types [1, 2]. Prospective studies have reported PTSD prevalence rates ranging between 35% and 45% at 3-months post-rape, with many survivors of sexual assault continuing to experience PTSD symptoms at 6-months and 12-months post-rape [3–6]. PTSD is a complex, multifactorial disorder and an array of environmental and genetic putative risk and protective factors mediate or contribute to the development of the disorder [3, 5, 7]. Epigenetic mechanisms, including DNA methylation, are known to respond to environmental exposures such as trauma, leading to stable changes in gene expression [8, 9]. DNA methylation responses may confer risk or protection for PTSD, as they may alter the ability to adapt to traumatic events on a molecular level [10]. Using a hypothesis-neutral, genome-wide approach to study epigenome-wide signatures (while accounting for potential environmental and biological confounding factors), and validating and replicating these findings, may bring us closer to uncovering the complexity of the disorder [10].

To date, twelve epigenome-wide association studies (EWASs) of blood DNA methylation differences in PTSD cases and controls have been published (see Table 1 for details). In sum, the majority of genes identified as differentially methylated in PTSD are linked to central nervous system functioning (e.g., neuron development, axonal outgrowth, synaptic connectivity, neurotransmitter release, neuroinflammation, and apoptosis) [11–17] and the immune response (T cell expression, cytokine and interferon release, phagocytosis) [13, 14, 18, 19].

1Department of Psychiatry, Faculty of Medicine and Health Sciences Stellenbosch University, Cape Town, South Africa. 2Gender and Health Research Unit, South African Medical Research Council, Cape Town, South Africa. 3South African Medical Research Council Unit on the Genomics of Brain Disorders, Stellenbosch University, Cape Town, South Africa.
4Division of Social and Behavioural Sciences, School of Public Health and Family Medicine, University of Cape Town, Cape Town, South Africa. 5MRC Integrative Epidemiology Unit, Population Health Sciences, Bristol Medical School, University of Bristol, Bristol, United Kingdom. 6Biostatistics Unit, South African Medical Research Council, Cape Town, South Africa. 7Division of Epidemiology and Biostatistics, Department of Global Health, Stellenbosch University, Cape Town, South Africa. ✉email: janinothling@sun.ac.za

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| Reference                  | Array and tissue type | Design and sample size | Setting and trauma type | Ethnicity                          | Gender and mean age | PTSD measure   | PTSD associated genes/networks |
|----------------------------|-----------------------|------------------------|-------------------------|------------------------------------|---------------------|----------------|--------------------------------|
| Uddin et al. [18]          | HM27; Blood           | Cross-sectional; 23 PTSD cases 77 trauma-exposed controls | Civilians from the DNHS cohort; mixture of trauma types | 79 African American, 14 Caucasian, 7 other ethnicities (not specified) | 40 Male (40%); 60 (60%) Female; 45.8 years | PCL-C         | Functional annotation clustering of differentially methylated genes implicated genes associated with the immune system in the development of PTSD. |
| Smith et al. [13]          | HM27; Blood           | Cross-sectional; 51 PTSD cases 53 trauma-exposed controls | Civilians from the GTP cohort; mixture of trauma types | 104 African American               | 64 Male (61.5%); 40 Female (38.5%); 42.7 years | CAPS          | Epigenome-wide significant differences in methylation at CpG sites in the APC5, TLR8, TPR, CLEC9A, ANK2A genes. |
| Mehta et al. [11]          | 450 K; Blood          | Cross-sectional; 32 PTSD cases without CT 29 PTSD cases with CT | Civilians; mixture of trauma types | 150 African Americans, 19 other ethnicities (not otherwise specified) | 18 Male (29.5%); 43 Female (70.5%); 41.6 years | PSS           | Pathways affected by PTSD were related to apoptosis and cellular growth rate. Pathways uniquely affected in those with PTSD and CT were related to nervous system development and tolerance induction. |
| Chen, Kobayasji and Mellman, 2016 [19] | 450 K; Blood          | Cross-sectional; 12 PTSD cases 12 trauma-exposed controls | Civilian; index traumas: 8 childhood physical or sexual abuse (33.3%); 3 sexual assault (12.5%); 9 violent crime (37.5%); 2 IPV (8.3%); 2 witnessed a violent death (8.3%) | 24 African American | 13 Male (54.2%); 11 Female (45.8%); 22 years | CAPS          | No epigenome-wide significant differences in methylation levels. Expression of genes associated with olfactory receptors, immune activation, GABA<sub>A</sub> receptor, and vitamin D synthesis was upregulated in PTSD cases. |
| Hammamieh et al. 2017 [12] | 450 K; Blood          | Cross-sectional; 79 PTSD cases 80 trauma-exposed controls | Combat exposed veterans previously deployed to Iraq or Afghanistan | 159 American ethnically matched participants (not otherwise specified) | 159 Males (100%); 33.9 years | CAPS          | Functional enrichment analysis of differentially methylated genes implicated genes related to nervous system development/ functioning, somatic complications, and endocrine signaling in the development of PTSD. |
| Kuan et al. 2017 [20]      | 450 K; Blood          | Cross-sectional; 171 current PTSD cases 100 past PTSD cases 202 trauma-exposed controls | Civilian responders to the September 11<sup>th</sup> World Trade Centre Disaster from the WTC cohort | 382 Caucasian Americans, 91 other ethnicities (not otherwise specified) | 473 Males (100%); 49.5 years | SCID          | No epigenome-wide significant differences in methylation levels. Differential methylation at CpG sites in the 2DHHC1, CSMD2, COL9A3, PDCD6IP, TBC1D24, and FAM164A genes were associated with current PTSD at a nominal level. |
| Mehta et al. [14]          | EPIC; Blood           | Cross-sectional; 8 PTSD cases 48 trauma-exposed controls | Treatment seeking Vietnam veterans with combat exposure | 96 Australian (not otherwise specified) | 96 Males (100%); 68.67 years | CAPS          | Epigenome-wide significant differences in methylation at CpG sites in the BRSK1, NGF, LCN8, DOCK2 genes and at an intergenic site (closest gene URRC33B). |
| Krygowska et al. [24]      | 450 K; Blood          | Cross-sectional; 34 PTSD cases 39 trauma-exposed controls | Police officers | 73 Dutch | 38 (52.1%) Males, 35 (47.9%) Females | CAPS          | No epigenome-wide significant differences in methylation levels. |
| Maddox et al. [17]         | 450 K; Blood          | Cross-sectional; 109 PTSD cases, 169 trauma-exposed controls | Civilians from the GTP cohort; mixture of trauma types | 278 predominately African American | 278 (100%) Females | PSS          | Genome-wide significant difference in methylation at one CpG site in HDAC4. |
| Reference | Array and tissue type | Design and sample size | Setting and trauma type | Ethnicity | Gender and mean age | PTSD measure | PTSD associated genes/networks |
|-----------|----------------------|------------------------|-------------------------|-----------|---------------------|-------------|--------------------------------|
| Rutten et al. [15] | 450 K, Blood | Discovery dataset: longitudinal; 32 high PTSD, high trauma 29 low PTSD, high trauma 32 low PTSD, low trauma | Replication dataset: longitudinal; 35 cases with PTSD 63 trauma exposure controls | Military soldiers with combat exposure, pre-deployment and post-deployment (minimum of 4 months) to Afghanistan from the PRISMO cohort. Marines with combat exposure, pre-deployment and post-deployment to Iraq from the MRS cohort | 93 Dutch Caucasian soldiers and 98 North American marines | 93 Males (100%); 27.5 years and 98 Males (100%); 22 years | SRP or CAPS | Longitudinal changes in PTSD symptoms were associated with differential methylation at CpG sites in the DUSP22, NINJ2, HOOK2, SDK1, MYT1L, PAHX, COL1A2, and HIST1H2AP52 genes in the PRISMO cohort. The finding related to HIST1H2AP52 was replicated in the MRS cohort. |
| Uddin et al. [21] | 450 K, Blood | Cross-sectional, meta-analysis; 198 with PTSD 347 trauma-exposed controls | Civilians from the DNHS, GTP, and WTC cohorts mixture of trauma types | 274 African American, 164 Caucasian American, 38 other ethnicities (not specified) | 294 Males (54%), 251 Females (46%), 46.6 years | PCL-C CAPS SCID | Epigenome-wide significant differences in methylation of CpG sites in the NRG1 and HG5 genes. |
| Logue et al. [25] | EPIC, Blood | Cross-sectional; 378 PTSD cases 135 trauma-exposed controls | Civilian Version, 164 Caucasian American, 38 other ethnicities (not specified) | 513 American veterans (not otherwise specified) | 467 (91%) Males, 46 (9%) Females, 32.7 years | CAPS | Epigenome-wide significant differences in methylation of a CpG site in the G6S2 gene. |
| Snijders et al. [16] | 450 K, Blood | Longitudinal; 123 PTSD cases 143 trauma-exposed controls | Military (marine and army) combat exposed personnel from the MRS, STARRS, and PRISMO cohorts, deployed to Iraq or Afghanistan for 4 to 7 months | 126 predominately Caucasian American marines, 78 Caucasian American army soldiers, 62 Dutch army soldiers | 266 (100%) Males; 24.3 years | CAPS, PCL, CIDI-SC, and SRP | Epigenome-wide significant differences in methylation of CpG sites in the SPRY4, SDK1, CTRC, CDH15, MAD II, HEDC genes. |
| Smith et al. [22] | 450 K, Blood | Cross-sectional, meta-analysis; 878 PTSD cases 1018 trauma-exposed controls | Three civilian samples and seven combat samples all exposed to trauma including combat and various civilian traumas from the DNHS, GTP, WTC, STARRS, MRS, INTRuST, PRISMO, VA-M-EA, VA-M-AA, and VA-NCPPTD cohorts | 986 Caucasian American, 62 Dutch, 777 African American, 57 Hispanic, 76 other ethnicities (not specified) | 1303 (68.7%) Males, 593 (31.3%) Females, 35.8 years | PCL-C, DSM-I, CAPS, MINI, SCID, CIDI-SC, SRP | Epigenome-wide significant differences in methylation of CpG sites in the AHR, RNF6, MIR3170, ATIIA, AC011899.9, FLJ43521, and LINC00899 genes. |

HM27 HumanMethylation27 BeadChip, PTSD posttraumatic stress disorder, DNHS Detroit Neighborhood Health Study, PCL-C PTSD Checklist–Civilian Version, GTP Grady Trauma Project, CAPS Clinician-Administered PTSD Scale, APSC acid phosphatase 5, tartrate resistant, TLX2 toll-like receptor 8, TLR translocated promotor region, CLEC9A C-type lectin domain family 9, ANXA2 annexin A2, 450K HumanMethylation 450 K BeadChip, CT childhood trauma, PSS PTSD Symptom Scale, IVT intimate partner violence, GABAA gamma-aminobutyric acid A, WTC World Trade Centre 9/11 responders study, SCID Structured Clinical Interview for DSM Disorders, ZDHHC17 zinc finger DHHC-type containing 11, ZC3H2 JUB and sushi domain-containing protein, COL9A3 collagen type IX alpha 3 chain, DTCD6P programmed cell death 6 interacting protein, TBC1D24 TBC1 domain family member 24, FAM164A family with sequence similarity 164, member A, EPIC Illumina EPIC BeadChip, BRSK1 brain-specific serine/threonine-protein kinase 1, NGF nerve growth factor, LCN8 lipocalin 8, DOCK2 dedicator of cytokinesis 2, LRRC3 leucine rich repeat containing 3B, HADC4 histone deacetylase 4, PRISMO Prospective Research in Stress-related Military Operations, MRS Marine Resiliency Study, SRP Self-Reporting Inventory for PTSD, DUSP22 dual specificity phosphatase 22, NINJ2 ninjinun 2, HOOK2 hook microtubule tethering protein 2, SDK1 sidekick cell adhesion molecule 1, MYT1L myelin transcription factor 1 like, PAHX paired box 8, COL1A2 collagen type I alpha 2 chain, HIST1H2AP52 H2A histone family, member T, pseudogene, NRGN neuregulin 1, HG5 hepatocyte growth factor-regulated tyrosine kinase substrate, TACRT Translational Research Centre for TBI and Stress Disorders, VA-RR&D Department of Veterans Affairs Rehabilitation Research and Development, TBI-VA-Boston Translomatic Brain Injury Centre of Excellence–Veteran Affairs Boston Healthcare System, G02 GOV1 switch 2, STARRS Study to Assess Risk and Resiliency in Service members, CIDI-SC Composite International Diagnostic Interview–Screening Scales, SPRY4 sprouty RTK signaling antagonist 4, SDK1 sidekick cell adhesion molecule 1, CTRC chymotrypsin C, CDH15 cadherin 15, MAD II mitotic arrest deficient 1 like 1, HEDC hexosaminidase glycosyl hydrolase family 20 catalytic domain containing, INTRuST Injury and Traumatic Stress Study, VA-M-EA Mid-Atlantic Mental Illness Research Education and Clinical Center PTSD Study, VA-NCPPTD European American cohort and VA-M-AA African American cohort, VA-NCPPTD Boston Veterans Affairs National Center for PTSD, DSM-V Diagnostic and Statistical Manual of Mental Disorders IV, MINI Mini-International Neuropsychiatric Interview, AHR human aryl hydrocarbon receptor repressor, RNF6 ring finger protein 6, MIR3170 microRNA 3170, ATIIA ATIIA phospholipid transporting 9A, FLJ43521 family with sequence similarity 75, member D1, LINC00899 long intergenic non-protein coding RNA 599. |
A meta-analysis of three North American mixed-gender civilian EWASs [13, 17, 18, 20] found that PTSD was associated with the neuregulin1 (NRG1) and hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), both of which are related to central nervous system functioning [21]. The largest EWAS meta-analysis to date included 796 participants with PTSD and 1100 healthy controls [22]. North American and European male and female participants were drawn from three civilian cohorts [13, 17, 18, 20] and seven combat-exposed cohorts [15, 16] were included. Associations with PTSD were observed at four CpG sites of the human aryl hydrocarbon receptor repressor (AHRR) gene, which has been linked to both pro-inflammatory and anti-inflammatory immune regulation [22, 23]. Ring finger protein 6 (RNF6) associated with immune function, ATPase phospholipid transporting 9A (ATP9A), associated with glucose metabolism, and family with sequence similarity 75-like protein FLJ46321 (FLJ46321), associated with cell differentiation; miRNA 3170 (MIR3170), and the long intergenic non-protein coding RNA 599 (LINC00599) genes were also associated with PTSD [22].

None of the gene-specific findings have been replicated across EWASs. Heterogeneity between and within EWASs may explain the lack of consistent findings. The majority of EWASs have been cross-sectional studies [11–15, 17–20, 22, 24, 25] and have investigated differential methylation in combat-exposed populations and first responders [12, 14–16, 20, 24, 25]. PTSD symptoms may manifest differently in combat-exposed samples (increased hypervigilance and compulsive behavior) compared to civilian samples [26, 27]. In civilians, PTSD symptom presentation, severity and recovery rates also differ depending on trauma type [26, 28, 29]. Civilian EWASs have investigated a mixture of traumas and none have investigated rape exclusively [30]. Civilian EWASs have also been predominantly conducted in mixed-gender [11, 13, 18, 19, 25, 31], North American samples [11–13, 17–20, 25].

Ethnicity-specific and sex-specific characteristics may influence methylation profiles [32–34]. Women have a two-fold increased risk of developing PTSD compared to men [34]. Increased risk for PTSD in women may be X-chromosome linked, given that PTSD may manifest differently in combat-exposed samples (increased hypervigilance and compulsive behavior) compared to civilian samples [26, 27]. In civilians, PTSD symptom presentation, severity and recovery rates also differ depending on trauma type [26, 28, 29]. Civilian EWASs have investigated a mixture of traumas and none have investigated rape exclusively [30]. Civilian EWASs have also been predominantly conducted in mixed-gender [11, 13, 18, 19, 25, 31], North American samples [11–13, 17–20, 25].

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We sought to address the design shortcomings and demographic differences in prior EWASs by conducting a cross-sectional EWAS study, complemented by validation of the results, replication, and longitudinal investigation of a demographically similar group of rape-exposed African black women in a low-income setting. Specific aims were to: (1) identify genome-wide differentially methylated CpG sites/regions associated with PTSD status at 3-months post-rape using an EWAS approach in a discovery sample; (2) validate the significant EWAS results in selected genes using an alternate methodology; (3) replicate the findings in 2 using a larger sample; (4) determine whether methylation levels of selected genes at baseline predict PTSD status change over 6-months; and (5) determine whether methylation changes in selected genes covary with PTSD symptom scores over 6 months.

**METHODS**

**Participant recruitment and setting**

Participants were recruited through the Rape Impact Cohort Evaluation (RICE) study conducted in South Africa (n = 852). A detailed description of the methods of the RICE study has been published elsewhere [40]. In short, female survivors of rape were recruited from rape clinics. Interested participants were invited to the study site to enrol in the study following informed consent procedures. Recruitment was restricted to female participants between 18 and 40 years who reported rape in the preceding 20 days of the baseline visit. In this study, we excluded women who: (1) were pregnant or lactating during the course of the study; (2) met criteria for PTSD at the baseline visit, as this would be indicative of PTSD due to a past traumatic event other than the rape; and (3) had HIV-seroconverted. Samples from 48 participants comprised the “discovery” sample, i.e., those that were included in the epigenome-wide DNA methylation analysis. These samples were subsequently utilized to technically validate the results from the EWAS study using Epityper Sequenom MassARRAY technology (Agena Bioscience, California, United States). The “replication” sample comprised 96 participants, 47 from the discovery sample and 49 additional samples.

**Clinical measures**

At the baseline visit, a research assistant supervised by a registered trauma counselor assessed each participant for PTSD (in relation to prior criterion A traumas other than the rape) on the Mini-International Neuropsychiatric Interview (MINI) version 7.0.0 [41]. An HIV rapid test, pregnancy test, blood collection for DNA analysis, and assessment of body mass index (BMI) were undertaken by a nurse at all time points (baseline, 3-months, and 6-months post-rape).

A research assistant administered a demographic questionnaire, a modified version of the Childhood Trauma Questionnaire-Short Form (CTQ-SF) [42], and a modified version of the Life Events Checklist (LEC) [43, 44] at baseline. The Davidson Trauma Scale (DTS) [45], the Alcohol Use Disorders Identification Test, alcohol consumption subscale (AUDIT-C) [46], and the Center for Epidemiologic Studies Depression Scale (CES-D) [47] was administered at all time points. The DTS was used to measure PTSD symptoms with a cut-off score of forty or more considered indicative of PTSD [45]. This cut-off was used to group participants into PTSD cases and controls at 3-months post-rape (see supplementary material for more details) [45]. All assessments were completed face-to-face and responses were recorded and electronically captured in real-time on a secure server. Item-level missing values were imputed using a multiple imputation model whilst maintaining a multivariate normal distribution.

**Demographic and clinical characteristics of the sample**

The baseline demographic and clinical characteristics of the sample were investigated using descriptive statistics. Differences in baseline demographic and clinical characteristics between the discovery/validation sample and the replication sample were investigated using non-parametric tests since most of the variables did not conform to a normal distribution. Mann-Whitney U tests were used to compare groups on continuous variables, i.e., age, body mass index (BMI), childhood trauma score, number of childhood traumas endorsed, number of lifetime traumas endorsed, alcohol use, and depression symptom scores. Chi-square statistics were used to compare groups on several categorical variables (completed secondary education, relationship status, smoking status, HIV status, medication use, childhood neglect, witnessed domestic violence in the childhood home, childhood emotional abuse, childhood physical abuse, childhood sexual abuse, imprisonment, civil unrest or war, serious injury, being close to death, murder of a family member or friend, unnatural death of a family member or friend, murder of a stranger, robbed at gun/knife point, kidnaped, hazardous alcohol use and depression status).

The same variables and methods used to investigate baseline demographic and clinical differences between the discovery/validation and replication samples were used to investigate differences those with and without PTSD at 3-months post-rape.

**Cross-sectional analyses (3 months post-rape)**

**Discovery sample.** Forty-eight participants, 24 with PTSD and 24 without PTSD at 3-months post-rape, were included in the discovery sample. We selected the 3-months post-rape time point since it was the first time point, in the parent study, at which a PTSD diagnosis could be made, based on DSM-5 criteria [48]. We implemented a cross-sectional, case-control design to identify genome-wide differentially methylated positions (DMPs) and
differentially methylated regions (DMRs) between individuals with and without PTSD. Consecutive cases of PTSD at 3-months post-rape were identified until the target number was reached. Controls were perfectly matched to cases, based on HIV status and as closely as possible (in descending hierarchical order of importance) on age, childhood trauma scores, lifetime trauma exposure, BMI, smoking, education, and income. DNA was extracted from peripheral blood samples and assessed using the Human Illumina EPIC BeadChip array (Illumina, California, United States) [49].

Raw probe intensity data (iDAT) files produced by Illumina GenomeStudio were deconvoluted and parsed into text format using the methyl R package [50] in R statistics version 3.6.2 [51]. All EWAS analyses, including quality control measures and beta normalization, were completed using the methyl R package [50].

All samples passed the quality control checks (see Supplementary Material for more details). Probes not passing the quality control checks (n = 29936) were excluded from the downstream analyses. Previously identified cross-reactive probes for 43254 CpG sites were also excluded [52]. Probes targeting CpG sites on the X chromosome were retained since all participants included in the study were female. The percentage of methylated alleles for each CpG site in each sample was calculated as B = M/(M + U + 100) where M and U symbolize raw probe fluorescent intensities for methylated and unmethylated signals, respectively [53]. Technical bias and batch effects were corrected for using functional normalization (Supplementary Material, Supplementary Figs. 1–5, Supplementary Tables 1 and 2) [54]. Any residual effects were handled by including surrogate variable analysis (SVA) scores, lifetime trauma exposure, BMI, smoking, education, and income. DNA methylation (at all time points), and potential baseline confounders (age, BMI, childhood trauma score, number of PTSD endorsed, alcohol use, and depression) and categorical variables (HIV status, smoking, and medication use). Confounding variables significantly associated with PTSD or BRSK2/ADCYAP1 methylation were entered in logistic regression models as covariates, in a stepwise manner.

Replication analysis. To replicate the validation analysis, an additional 49 consecutively selected participants from the parent study were included in the DNA methylation replication analyses. These participants were not matched on PTSD status or potential methylation covariates. Samples were assayed using EpiTYPER.

Logistic regression models, including potential confounding variables, were used to determine if differential methylation of BRSK2 and ADCYAP1 at 3-months post-rape was associated with PTSD status at 3-months post-rape in the replication sample, following the same procedure applied in the validation analyses.

Comparison of previous findings from candidate gene studies and EWASs. Candidate gene studies and EWASs investigating the relationship between methylation and PTSD were identified from published literature. For EWASs, the Illumina CpG identification number for significant findings was manually recorded and cross-checked against the findings of the current EWAS. For candidate gene studies, the genomic coordinates of the sites were identified from the publications and converted to Hg19/GRCh37 positions using the BLAT function of the University of California, Santa Cruz (UCSC) genome browser (if not already indicated as Hg19/GrCh37 positions). The genomic locations were manually recorded and cross-checked with the Illumina EPIC_v1-0_B4 manifest to determine if the sites were included on the Illumina EPIC array. Significant CpG sites resulting from the current EWAS and corresponding to prior findings are reported in the results.

Agreement between the Illumina EPIC array and EpiTYPER. Spearman’s correlation coefficients were used to investigate the level of agreement between methylation levels resulting from the Illumina EPIC array at 3-months post-rape and methylation levels resulting from EpiTYPER at 3-months post-rape.

Longitudinal investigation (baseline, 3-months, and 6-months post-rape) Combined sample. The validation and replication samples were combined and methylation data from the baseline and 6-month post-rape samples were added to the dataset, for the same combined group. The group consisted of 96 participants with methylation data at all time points (baseline, 3-months, and 6-months). The samples were assayed using EpiTYPER. We investigated the same BRSK2 and ADCYAP1 CpG sites identified in the validation and replication samples but followed a longitudinal cohort design with PTSD symptom scores as the outcome, instead of a cross-sectional case-control design with PTSD status at 3-months as the outcome.

PTSD scores at each time point were compared between the discovery/validation sample and the replication sample using Mann-Whitney U tests. The relationship between PTSD, BRSK2 methylation, and ADCYAP1 methylation (at all time points), and potential baseline confounders (age, BMI, childhood trauma, lifetime trauma, alcohol use, depression, HIV status, smoking, and medication use) was investigated using Mann-Whitney U tests, Chi-square tests, and Spearman’s correlations.

Baseline ADCYAP1 and BRSK2 methylation levels were investigated as predictors of change in PTSD symptom scores over six months, in the first set of mixed regression models. In the second set of mixed regression models, we investigated change in BRSK2 and ADCYAP1 methylation levels over six months in relation to change in PTSD symptom scores over six months. Confounding variables significantly associated with PTSD or
RESULTS
Baseline demographic and clinical characteristics of the sample
Table 2 presents the baseline demographic and clinical characteristics of the discovery/validation and replication samples. The samples were similar with regard to demographic and clinical characteristics. The only variable that differed between the samples was the prevalence of lifetime exposure to the murder of a family member or friend, which was more frequently endorsed in the discovery/validation sample compared to the replication sample (25.5% vs. 8.2%, respectively; \( \chi^2 = 5.2, p = 0.022 \)).

Comparison of baseline demographic and clinical characteristics between the PTSD groups at 3-months post-rape
Table 3 presents group comparisons by PTSD status (at 3-months post-rape) in the discovery/validation sample and the replication sample, consecutively. Participants with and without PTSD had similar baseline demographic and clinical characteristics in the discovery/validation and replication samples. However, in the discovery/validation sample, those with PTSD were more likely to endorse being robbed with a gun or knife compared to those without PTSD (50% and 21.7%, respectively; \( z = 4.1, p = 0.044 \)). In the replication sample, those with PTSD endorsed less lifetime traumas (\( M = 0.5, SD = 0.7 \) compared to those without PTSD (\( M = 1.4, SD = 1.3, z = −2.5, p = 0.014 \)).

Table 2. Baseline demographic and clinical characteristics of the discovery/validation and replication samples.

| Comparison of discovery/validation sample to replication sample | Discovery/validation sample (n = 47) | Replication sample (n = 49) | \( \chi^2 \) | z | p |
|---------------------------------------------------------------|-----------------------------------|--------------------------|-----------|---|---|
| Agea | 47 (100) | 25.9 (5.4) | 49 (100) | 24.6 (5.5) | −1.3 | 0.178 |
| Secondary education completedb | 32 (68.1) | 25 (51) | 2.9 | 0.089 |
| Employedb | 13 (27.7) | 9 (18.4) | 1.2 | 0.279 |
| In a relationship/marriedb | 38 (80.9) | 38 (77.6) | 0.2 | 0.691 |
| BMIb | 47 (100) | 26.0 (6.5) | 49 (100) | 25.8 (5.7) | −0.1 | 0.956 |
| Smokerb | 5 (10.6) | 7 (14.3) | 0.3 | 0.589 |
| HIV positiveb | 27 (57.4) | 19 (38.8) | 3.4 | 0.067 |
| On ARVsb | 12 (25.5) | 14 (28.6) | 0.1 | 0.738 |
| On medications for STIb | 2 (4.3) | 2 (4.1) | 0.0 | 0.966 |
| Other medication useb,c | 1 (2.1) | 2 (4.1) | 0.3 | 0.582 |
| Childhood trauma scorea | 47 (100) | 17.2 (4.1) | 49 (100) | 16.2 (2.5) | −0.8 | 0.410 |
| Neglectb | 23 (48.9) | 18 (36.7) | 1.5 | 0.227 |
| Domestic violenceb | 10 (21.3) | 8 (16.3) | 0.4 | 0.534 |
| Emotional abuseb | 12 (25.5) | 11 (22.4) | 0.1 | 0.724 |
| Physical abuseb | 18 (38.3) | 19 (38.8) | 0.0 | 0.962 |
| Sexual abuseb | 10 (21.3) | 11 (22.4) | 0.0 | 0.890 |
| Number of childhood traumasa | 47 (100) | 1.6 (1.6) | 49 (100) | 1.4 (1.5) | −0.6 | 0.530 |
| Number of lifetime traumasa,d | 47 (100) | 1.6 (1.5) | 49 (100) | 1.13 (1.2) | −1.7 | 0.092 |
| Imprisonmentb | 2 (4.3) | 1 (2.0) | 0.4 | 0.533 |
| Civil unrest or warb | 3 (6.4) | 1 (2.0) | 1.1 | 0.287 |
| Serious injuryb | 8 (17.0) | 3 (6.1) | 2.8 | 0.094 |
| Being close to deathb | 13 (27.7) | 14 (28.6) | 0.0 | 0.921 |
| Murder of family/friendb | 12 (25.5) | 4 (8.2) | 5.2 | 0.022* |
| Unnatural death of family/friendb | 9 (19.1) | 5 (10.2) | 1.5 | 0.214 |
| Murder of strangerb | 10 (21.3) | 5 (10.2) | 2.2 | 0.135 |
| Robbed with gun/knife usedb | 17 (36.2) | 18 (36.7) | 0.0 | 0.954 |
| Kidnappedb | 3 (6.4) | 4 (8.2) | 0.1 | 0.737 |
| PTSD symptom scorea | 47 (100) | 67.1 (21.7) | 49 (100) | 65.7 (18.6) | −0.8 | 0.431 |
| Alcohol use severity scorea | 47 (100) | 1.4 (2.2) | 49 (100) | 1.9 (2.5) | −1.2 | 0.242 |
| Hazardous alcohol useb | 12 (25.5) | 15 (30.6) | 0.3 | 0.580 |
| Depression symptom scorea | 47 (100) | 32.4 (13.9) | 49(100) | 31.7 (12.1) | −0.2 | 0.854 |
| Depression statusb | 41 (87.2) | 45 (91.8) | 0.5 | 0.461 |

PTSD Posttraumatic stress disorder, \( M \) mean, \( SD \) standard deviation, \( BMI \) body mass index, \( ARV \) antiretrovirals, \( STI \) sexually transmitted infection.

aContinuous variables.
bCategorical variables.
cMedication prescribed for chronic sinusitis (\( n = 1 \)) and hypertension (\( n = 2 \)).
dLifetime traumas refer to directly experiencing the trauma; *\( p < 0.05 \).
Table 3. Baseline demographic and clinical characteristics of rape-exposed participants with and without posttraumatic stress disorder at 3-months post-rape in the discovery/validation and replication samples.

|                                      | Discovery/validation sample (n = 47) | Replication sample (n = 49) |
|-------------------------------------|-------------------------------------|-----------------------------|
|                                     | Group difference (n (%) M (SD) n (%) M (SD) χ² z p) | Group difference (n (%) M (SD) n (%) M (SD) χ² z p) |
| Ageb                               | 24 (100) 25.1 (5.3) 23 (100) 26.7 (5.5) −1.0 0.296 15 (100) 24.7 (4.7) 34 (100) 24.5 (5.9) −0.5 0.616 |
| Secondary education completedc     | 16 (66.7) 16 (69.6) 0.1 0.831 10 (66.7) 15 (44.1) 2.1 0.146 |
| Employedc                          | 4 (16.7) 9 (39.1) 3.0 0.085 1 (6.7) 8 (23.5) 2.0 0.160 |
| In a relationship/marriedc         | 19 (79.2) 19 (82.6) 0.1 0.764 11 (77.3) 27 (79.4) 0.2 0.638 |
| BMIb                               | 24 (100) 24.8 (5.4) 23 (100) 27.2 (7.4) −1.1 0.268 15 (100) 25.3 (4.8) 34 (100) 26.0 (6.1) −0.3 0.745 |
| Smokerc                            | 3 (12.5) 2 (8.6) 0.2 0.672 1 (6.7) 8 (23.5) 2.0 0.160 |
| HIV positivec                      | 14 (58.3) 13 (56.5) 0.0 0.900 7 (46.7) 12 (35.3) 0.6 0.451 |
| On ARVsc                           | 6 (25.0) 6 (26.1) 0.0 0.932 4 (26.7) 10 (29.4) 0.0 0.845 |
| On medications for STIc            | 1 (4.2) 1 (4.3) 0.0 0.975 2 (5.9) 2 (5.9) 0.9 0.338 |
| Other medication usec,d            | 0 (0.0) 1 (4.3) 1.1 0.302 2 (5.9) 2 (5.9) 0.9 0.338 |
| Childhood trauma scoreb           | 24 (100) 18.2 (4.6) 23 (100) 16.2 (3.3) −1.7 0.098 15 (100) 15.7 (2.5) 34 (100) 16.4 (2.6) −1.0 0.299 |
| Neglectc                           | 13 (54.2) 10 (43.5) 0.5 0.464 5 (33.3) 13 (38.2) 0.1 0.743 |
| Domestic violencec                 | 7 (29.2) 3 (13.0) 1.8 0.177 3 (20.0) 5 (14.7) 0.2 0.644 |
| Emotional abusec                   | 9 (37.5) 3 (13.0) 3.7 0.055 3 (20.0) 8 (23.5) 0.1 0.785 |
| Physical abusec                    | 10 (41.7) 8 (34.8) 0.2 0.627 5 (33.3) 14 (41.2) 0.3 0.604 |
| Sexual abusec                      | 7 (29.2) 3 (13.0) 1.8 0.177 2 (13.3) 9 (26.5) 1.0 0.310 |
| Number of childhood traumasb      | 24 (100) 1.9 (1.7) 23 (100) 1.2 (1.3) −1.6 0.120 15 (100) 1.2 (1.7) 34 (100) 1.4 (1.5) −0.8 0.430 |
| Number of lifetime traumasb,sa     | 24 (100) 2.0 (1.6) 23 (100) 1.2 (1.2) −1.9 0.063 15 (100) 0.5 (0.7) 34 (100) 1.4 (1.3) −2.5 0.014 |
| Imprisonmentc                      | 2 (8.3) 0 (0.0) 2.0 0.157 1 (6.7) 1 (2.9) 0.5 0.502 |
| Civil unrest or warc               | 2 (8.3) 1 (4.3) 0.3 0.576 0 (0.0) 1 (2.9) 0.5 0.502 |
| Serious injuryc                    | 6 (25.0) 2 (8.6) 2.2 0.137 1 (6.7) 2 (5.9) 0.0 0.916 |
| Being close to deathc              | 7 (29.2) 6 (26.1) 0.1 0.813 3 (20.0) 11 (32.4) 0.8 0.378 |
| Murder of family/friendc           | 5 (20.8) 7 (30.4) 0.6 0.450 0 (0.0) 4 (11.8) 1.9 0.166 |
| Unnatural death of family/friendc  | 5 (20.8) 4 (17.4) 0.2 0.764 0 (0.0) 5 (14.7) 2.5 0.117 |
| Murder of strangerc                | 7 (29.2) 3 (13.0) 1.8 0.177 0 (0.0) 5 (14.7) 2.5 0.117 |
| Robbed with a gun/knife usedc      | 12 (50.0) 5 (21.7) 4.1 0.044 3 (20.0) 15 (44.1) 2.6 0.107 |
| Kidnappedc                         | 3 (12.5) 0 (0.0) 3.1 0.080 1 (6.7) 3 (8.8) 0.1 0.799 |
| PTSD symptom scoreb                | 24 (100) 75.7 (17.9) 23 (100) 58.1 (22.0) −2.9 0.004 15 (100) 63.4 (20.4) 34 (100) 66.7 (18.0) −0.1 0.914 |
| Alcohol use severity scoreb        | 24 (100) 1.7 (2.4) 23 (100) 1.2 (1.9) −0.9 0.394 15 (100) 1.6 (2.6) 34 (100) 2.1 (2.5) −1.0 0.299 |
| Hazardous alcohol usec             | 7 (29.2) 5 (21.7) 0.3 0.559 4 (26.7) 11 (32.4) 0.2 0.691 |
| Depression symptom scoreb          | 24 (100) 35.1 (12.9) 23 (100) 29.5 (14.7) −1.4 0.173 15 (100) 28.4 (13.5) 34 (100) 33.1 (11.4) −1.4 0.149 |
| Depression statusc                 | 22 (91.7) 19 (82.6) 0.9 0.352 13 (86.7) 32 (94.1) 0.8 0.380 |

PTSD Posttraumatic stress disorder, M mean, SD standard deviation, BMI body mass index, ARV antiretrovirals, STI sexually transmitted infection.

aThe 3-month post-rape time point was used in the analysis since it is the first time point in the parent study at which a PTSD diagnosis can be made. PTSD status at 3-months post-rape was used as the outcome to address the first three aims of the study. PTSD symptom score rather than PTSD status was used as the outcome in the longitudinal analysis to address aim four and aim five of the study. One participant included in the discovery sample (n = 48) was not included in the validation sample (n = 47).

bContinuous variables.

cCategorical variables.

dMedication prescribed for chronic sinusitis (n = 1) and hypertension (n = 2).

*Lifetime traumas refer to directly experiencing the trauma.
Discovery sample: genome-wide differentially methylated genes associated with PTSD status at 3-months post-rape

Table 4 presents selected findings from the top twenty DMPs that were associated with PTSD before correction for multiple comparisons (p < 0.05) (see Supplementary Table 3 and Supplementary Figs. 7–9 for more details). Only one DMP, cg01700569, remained significant after correcting for multiple testing (adjusted p < 0.05). This intergenic site (cg01700569) is located 24694 bases downstream of solute carrier family 16 member 9 (SLC16A9). Other genes previously linked to mood, anxiety, or trauma-related disorders included protein zeta-1 (FEZ1), ADCYAP1, BRSK2, catenin alpha 3 (CTNNA3), and par-3 family cell polarity regulator (PARD3).

Thirty-four DMRs were identified from the regional analysis after Bonferroni correction for multiple testing. The regions previously linked to mood, anxiety, or trauma-related disorders included coiled-coil and C2 domain-containing protein 2 A (CC2D2A), BRSK2, and ADCYAP1. The findings related to these genes are also presented in Table 4.

Validation and replication sample: differential methylation of BRSK2 in relation to PTSD status at 3-months post-rape

The BRSK2 region (chr11:1463541–1463670; adjusted p < 0.05) identified from the EWAS included five CpG sites (CpG1-cg121186219, CpG2-cg14064268, CpG3-cg10599025, CpG4-cg17429870, CpG5-cg18651858) that showed decreased methylation in participants with PTSD (see Fig. 1). Based on prior findings, DNA methylation of these CpG sites in blood was highly correlated with DNA methylation in the prefrontal cortex, superior temporal gyrus, and the cerebellum (see Supplementary Fig. 10a–d) [59]. Three of the five CpG sites (CpG3, CpG4, and CpG5) were investigated in the validation and replication sample. We could not investigate CpG1 or CpG2, as the mass of CpG1 was too low to be measured by the Epityper mass spectrometer, and CpG2 contained a silent peak that overlapped with the non-methylated peak for this site (see Supplementary Table 4 for the genomic coordinates and sequence for CpG3, CpG4, and CpG5).

Baseline age, HIV status, BMI, smoking status, childhood trauma score, lifetime trauma, alcohol use, depression, and medication use were not associated with BRSK2 methylation at 3-months post-rape in either the validation or replication samples. PTSD status at 3-months post-rape was associated with lifetime trauma (β = −2.47, p = 0.014) in the replication sample only (see Supplementary Tables 5 and 6). In the validation analysis, methylation levels of BRSK2 CpG3 (β = −0.04, p = 0.050, OR 0.96) and CpG4 (β = −0.04, p = 0.052, OR 0.96) at 3-months post-rape were not significantly associated with PTSD status at 3-months post-rape. Decreased methylation of BRSK2 CpG5 (β = −0.04, p = 0.048, OR 0.96) at 3-months post-rape was significantly associated with PTSD status at 3-months post-rape, but the association was no longer significant when lifetime trauma was added as a covariate to the model (see Supplementary Tables 7). In the replication analysis, methylation levels of BRSK2 CpG3 (β = −0.00, p = 0.889, OR 1.00), CpG4 (β = −0.01, p = 0.667, OR 0.99) and CpG5 (β = 0.00, p = 0.866, OR 1.00) were not significantly associated with PTSD status at 3-months post-rape (see Supplementary Table 8).

Validation and replication samples: differential methylation of ADCYAP1 in relation to PTSD status at 3-months post-rape

The ADCYAP1 region (chr18:905177–905180) identified from the EWAS included only two differentially methylated CpG sites (CpG1 – cg22388954, CpG2 – cg11773720) which both showed increased methylation in participants with PTSD (see Fig. 2). Based on prior findings, DNA methylation of these CpG sites in blood was not correlated with DNA methylation in brain tissue (Supplementary Fig. 11a, b) [59]. Epityper signals for ADCYAP1 CpG1 and CpG2 were combined for analysis, due to their proximity to each other (see supplementary Table 9 for the genomic coordinates and sequence of CpG1 and CpG2).

Baseline age, HIV status, BMI, smoking status, childhood trauma score, lifetime trauma, alcohol use, depression, and medication use were not associated with ADCYAP1 methylation at 3-months post-rape in the validation or replication samples (see Supplementary Tables 5 and 6). In the validation analysis, methylation levels of ADCYAP1 CpG1&2 (β = −0.09, p = 0.382, OR 0.92) were not significantly associated with PTSD status at 3-months post-rape (see supplementary Tables 7). In the replication sample, methylation levels of ADCYAP1 CpG1&2 (β = −0.06, p = 0.639, OR 0.94) were also not significantly associated with PTSD status at 3-months post-rape (see supplementary Table 8).

Agreement between the Illumina EPIC array and Epityper

Large positive correlations were found when comparing the Illumina EPIC array and Epityper methylation levels for BRSK2 CpG3 (r = 0.881, p < 0.000), CpG4 (r = 0.900, p < 0.000), and CpG5 (r = 0.831, p = 0.831) at 3-months post-rape (see Supplementary Table 10). Small, non-significant correlations were found when comparing the Illumina EPIC array and Epityper methylation levels for ADCYAP1 CpG1&2 (r = 0.254, p > 0.05; see Supplementary Table 11).

Replication of previous candidate gene and EWAS findings

Differential methylation of five CpG sites previously investigated was replicated in this EWAS study, prior to correction for multiple testing (see Supplementary Table 12 and 13). These sites were located in the HTR3A (chr11:113846004, cg20621129, p = 0.028) [67], AHRR (two CpG sites: chr5:3733738, cg05575921, p = 0.033; chr5:377358, cg26703534, p = 0.031) [22], DUSP22 (chr6:291882, cg21548813, p = 0.032) [15] and TPR (chr1:186344558, cg24577137, p = 0.0008) genes [13]. Since decreased methylation of AHRR is strongly linked to smoking, [22] we investigated the link between smoking and AHRR methylation (based on the values obtained from our EWAS) and found decreased AHRR methylation levels in smokers (M = 78.91, SD = 14.95, n = 5) compared to non-smokers (M = 93.88, SD = 1.45, n = 42) at cg05575921 (z = −2.92, p = 0.001).

Combined sample: longitudinal relationship between BRSK2, ADCYAP1, PTSD scores, and confounding variables

Baseline childhood trauma, alcohol use, and depression were associated with PTSD scores at one or more time points. Baseline childhood and lifetime trauma scores were associated with BRSK2 methylation at one or more time points. Baseline HIV status was associated with ADCYAP1 methylation at 3-months post-rape (see Supplementary Table 14).

Combined sample: longitudinal change in PTSD symptom scores

The mean PTSD scores at baseline, 3-months, and 6-months, stratified by sample (discovery/validation, replication, combined), are presented in Fig. 3. There were no significant differences between the discovery/validation samples and the replication sample for either baseline (z = −0.79, p = 0.431), 3-month (z = −1.37, p = 0.172), or 6-month (z = −0.15, p = 0.883) PTSD scores. There was a significant decline in PTSD scores from baseline to 3-months (p < 0.000) and from 3-months to 6-months (p = 0.021), in the combined sample.

Combined sample: baseline BRSK2 and ADCYAP1 methylation levels and longitudinal change in PTSD scores

Table 5 presents the results of the mixed regression models investigating baseline BRSK2 and ADCYAP1 methylation as
Table 4. Genome-wide differentially methylated positions (DMPs) and regions (DMRs) associated with posttraumatic stress disorder in the discovery sample.

| Gene Name* | Positionb | Probe | Location in Gene,c,d | β    | SE   | t/z   | p       | Adj. p | Other exposures/phenotypes associated with the CpG site* | Mood, anxiety or trauma-related disorders previously associated with the site in EWAS or GWAS studies*n,f | Reference of prior candidate gene study linking the gene to PTSDg |
|------------|-----------|-------|-----------------------|------|------|-------|---------|--------|------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|
| **Differentially methylated positions (DMPs)** | | | | | | | | | | | |
| NA, SLC16A9a | Chr10:61385771 | cg01700569 | Intergenic | 0.031 | 0.004 | 7.119 | 6.187e−08 | 0.049233 | None | None | NA | NA |
| FEZ1 | Chr11:125365803 | cg06309855 5’UTR | 0.022 | 0.004 | 5.727 | 2.930e−06 | 0.777284 | Gestational age | Depression, Bipolar | None | Ressler et al. [64] |
| ADCYAP1 | Chr18:905177 | cg22388954 5’UTR; TSS200 | −0.025 | 0.005 | −5.181 | 1.371e−05 | 0.999998 | B acute lymphoblastic leukemia | None | Ressler et al. [64] |
| BRSK2 | Chr11:1431833 | cg09450823 Body | 0.036 | 0.007 | 5.115 | 1.653e−05 | 0.999998 | None | PTSD (paralog BRSK1) | None |
| CTNNA3 | Chr10:68940214 | cg23307744 Body | −0.012 | 0.002 | −5.012 | 2.208e−05 | 0.999998 | None | Depression | None |
| PARD3 | Chr10:35016204 | cg18026072 Body | −0.009 | 0.002 | −4.961 | 2.534e−05 | 0.999998 | None | Depression | None |
| **Differentially methylated regions (DMRs)** | | | | | | | | | | | |
| CC2D2A | Chr4:15471214-15471399 | cg21329975 TSS1500 | 0.015 | 0.002 | 7.856 | 3.964e−15 | 3.285e−09 | Depression | None |
| | | cg16509355 TSS1500 | | | | | | | | | |
| | | cg21123203 Intergenic | | | | | | | | | |
| | | cg02964094 TSS200 | | | | | | | | | |
| | | cg18470593 TSS200 | | | | | | | | | |
| | | cg20184469 TSS200 | | | | | | | | | |
| BRSK2 | Chr11:1463541-1463670 | cg12186219 Body | 0.112 | 0.016 | 7.131 | 9.935e−13 | 8e−07 | PTSD (paralog BRSK1) | None |
| | | cg14064268 Body | | | | | | | | |
| | | cg10590925 Body | | | | | | | | |
| | | cg17429870 Body | | | | | | | | |
| Gene Namea | Positionb | Probe | Location in Gene,c,d | β    | SE  | t/z   | p      | Adj. p | Other exposures/phenotypes associated with the CpG sitee | Mood, anxiety or trauma-related disorders previously associated with the site in EWAS or GWAS studiesf | Reference of prior candidate gene study linking the gene to PTSDg |
|------------|-----------|-------|----------------------|------|-----|-------|--------|--------|----------------------------------------------------------|-----------------------------------------------------------------|---------------------------------------------------------------|
| ADCYAP1    | Chr18:905177-905180 | cg18651858 | Body                | −0.022 | 0.003 | −6.761 | 1.370e−11 | 1.13e−05 | None                                                    | B Acute lymphoblastic leukemia                                   | Ressler et al. [64]                                           |
| cg22388954 | 5'UTR; TSS200     | 5'UTR; TSS200     | None                |        |      |        |        |        |                                                         |                                                                 |                                                               |
| cg11773720 | 5'UTR; TSS200     | 5'UTR; TSS200     | None                |        |      |        |        |        |                                                         |                                                                 |                                                               |

SE Standard error, Adj adjusted, EWAS epigenome-wide association study, GWAS genome-wide association study, PTSD posttraumatic stress disorder, SLC16A9 solute carrier family 16 member 9, Chr chromosome, NA not applicable, FEZ1 fasciculation and elongation protein zeta 1, 5'UTR 5' untranslated region, ADCYAP1 adenylate cyclase activating polypeptide 1, TSS200 transcription start site 200, BRSK2 brain-specific serine/threonine-protein kinase 2, BRSK1 brain-specific serine/threonine-protein kinase 1, MCEE methylmalonyl-CoA epimerase, CTNNA3 catenin alpha 3, PARD3 PAR-3 family cell polarity regulator, CC2D2A coiled-coil and C2 domain-containing protein 2A, TSS1500 transcription start site 1500.

aIdentified using the GENECODE database.
bIdentified using the Human Genome 19 (HG19) build from the Genome Reference Consortium.
cIdentified using the University of California Santa Cruz (UCSC) Genomic Institute/Genome Browser.
dMultiple listings indicate splice variants.
eIdentified using the Medical Research Council’s Integrative Epidemiology Unit (MRC-IEU) catalog of epigenome-wide association studies (EWAS) [60] and the China National Center for Bioinformation National Genomics Data Center EWAS atlas [61].
fIdentified using the European Molecular Biology Laboratory-European Bioinformatic Institute (EMBL-EBI) genome-wide association studies (GWAS) Catalog for GWAS studies [62].
gIdentified through a literature search in PubMed [63].
hCpG sites located in a region not attributed to a gene, the gene closest to the CpG site is provided.
predictors of change in PTSD symptom scores over time. Decreased baseline BRSK2 CpG3, CpG4, and CpG5 methylation levels were significant predictors of increased PTSD symptom scores at 3-months (CpG3 $\beta = -0.39, p < 0.001$, CpG4 $\beta = -0.33, p = 0.005$, CpG5 $\beta = -0.27, p = 0.009$) and 6-months (CpG3 $\beta = -0.49, p < 0.001$, CpG4 $\beta = -0.44, p < 0.001$, CpG5 $\beta = -0.38, p < 0.001$) post-rape. However, the relationships between BRSK2 CpG3, CpG4, and CpG5 methylation levels and PTSD scores at 3- and 6-months post-rape were no longer significant when childhood trauma, alcohol consumption, depression, and lifetime trauma were added to the models as covariates.

Increased baseline ADCYAP1 CpG1&2 methylation was a significant predictor of increased PTSD scores at baseline ($\beta = 5.34, p < 0.001$) and decreased PTSD scores at 6-months ($\beta = -3.52, p = 0.004$) post-rape, but the associations were no longer significant when covariates were added to the model.

Combined sample: longitudinal change in ADCYAP1 and BRSK2 methylation levels in relation to longitudinal change in PTSD scores

Table 6 presents the results of the mixed regression models investigating change in BRSK2 and ADCYAP1 methylation over time as predictors of change in PTSD symptom scores over time. Decreased BRSK2 CpG3 ($\beta = -0.39, p < 0.001$), CpG4 ($\beta = -0.36, p = 0.001$), and CpG5 ($\beta = -0.32, p = 0.001$) methylation at 3-months post-rape was associated with increased PTSD scores at 3-months post-rape. Decreased BRSK2 CpG3 ($\beta = -0.49, p < 0.001$), CpG4 ($\beta = -0.46, p < 0.001$), and CpG5 ($\beta = -0.43, p < 0.001$) methylation at 6-months post-rape was also associated with increased PTSD scores at 6-months post-rape. The relationship between PTSD score at 3-month post-rape and methylation of BRSK2 CpG3 ($\beta = -0.30, p = 0.049$) was the only association that remained significant after the addition of covariates to the models.
Increased baseline ADcyAP1 CpG1&2 methylation was associated with increased PTSD scores at baseline ($\beta = 4.67, p < 0.001$), while decreased ADcyAP1 CpG1&2 methylation at 3-months ($\beta = -2.61, p = 0.001$) and 6-months ($\beta = -5.01, p < 0.001$) was associated with increased PTSD scores at 3-months and 6-months post-rape. The associations were no longer significant when covariates were added to the model.

**DISCUSSION**

In this study, we identified one DMP (cg01700569) and thirty-four DMRs associated with PTSD at 3-months post-rape on an epigenome-wide level. The gene closest to the aforementioned DMP is SLC16A9. Although investigating this DMP further may have been of value, little is known about it in the context of mental health. The site (cg01700569) is located in an intergenic region, which further complicates the interpretation of the clinical significance of the finding.

We investigated two DMRs in the BRSK2 and ADcyAP1 genes further. We were able to validate, but not replicate, the BRSK2 CpG5 finding, confirming decreased BRSK2 methylation in rape-exposed participants with PTSD at 3-months post-rape, compared to those without PTSD. We also found that decreased baseline BRSK2 CpG3, CpG4, and CpG5 methylation was associated with increased PTSD scores at 3-months and 6-months post-rape. Decreased BRSK2 methylation at 3-months and 6-months post-rape was associated with increased PTSD scores at the same time points. However, the associations between decreased BRSK2 CpG3 methylation at 3-months post-rape and increased PTSD scores at 3-months post-rape were the only ones that remained significant after childhood trauma, alcohol consumption, depression, and lifetime trauma were added as covariates to the models.

We were unable to validate or replicate our ADcyAP1 CpG1&2 findings. We found that decreased baseline ADcyAP1 CpG1&2 methylation was associated with increased PTSD scores at 6-months post-rape. Decreased ADcyAP1 methylation at 3- and 6-months post-rape was also associated with increased PTSD scores at the same time points, while decreased baseline ADcyAP1 CpG1&2 methylation was associated with decreased PTSD scores at baseline. The findings did not remain significant after PTSD covariates were added to the models.

Decreased methylation of the BRSK2 paralog, BRSK1 [68], has been associated with a PTSD diagnosis in a prior EWAS [14]. BRSK1 and BRSK2 share a 68% overlap in genetic sequence, both are highly expressed in the brain, and decreased expression of both has been linked to disorganized presynaptic vesicle formation, uncoordinated release and reuptake of neurotransmitters, altered axonal development, and abnormal neuronal polarization in animal studies [68–73]. In human studies, a BRSK2 polymorphism (rs1881509) has been associated with heroin dependence [69], and functional variants of BRSK2 have been associated with autism spectrum disorder, cognitive impairment, intellectual disability, and speech delays [74, 75].

BRSK1 and BRSK2 are expressed most strongly in the cerebellum and the hippocampus [69]. The hippocampus is closely linked to PTSD since it is involved in memory consolidation [76]. When memories are not consolidated into autobiographical memory networks, they may involuntarily resurface (e.g., flashbacks, intrusions, nightmares, and dissociation) and activate the limbic system, which induces the fight-or-flight response [77]. Differential methylation and expression of BRSK2 may also alter the expression of neurotransmitters previously found to be associated with PTSD (norepinephrine, epinephrine, dopamine, and serotonin) through altered presynaptic vesicle and synaptic cleft development [78, 79].

In addition to their functions in the brain, BRSK1 and BRSK2 have been linked to metabolic processes and glucose homeostasis [80, 81]. Animal studies have found increased expression of BRSK1 and BRSK2 in pancreatic cells and knockdown of BRSK2 resulted in a significant increase in serum insulin levels [80, 81]. In a human study, BRSK2 was found to be highly expressed in human pancreatic insulin-producing B cells, and activation of BRSK2 was linked to reduced insulin secretion [81]. Moreover an EWAS found that participants with type 1 diabetes and neuropathy showed decreased methylation at four CpG sites in the BRSK2 gene compared to participants with type 1 diabetes without neuropathy [82].

The BRSK2 CpG sites investigated in this study were located in intron 4 of the gene. The function of methylation in gene bodies is not well established, but methylation is abundant in these regions and is generally positively correlated with expression [83]. Assuming the latter, we can hypothesize that decreased methylation of BRSK2 may contribute to adverse neuronal development, neuronal maintenance, and dysregulated blood glucose levels which may explain the increased risk for diabetes and cardiovascular disease observed in prior PTSD studies [84, 85]. The relationship between BRSK2 methylation and adverse neuronal development and maintenance is further supported by prior findings of a high correlation between BRSK2 blood methylation and methylation in brain tissue [59].
| Model | $\beta$ | Std error | t | p    | 95% CI | Lower | Upper |
|-------|--------|-----------|---|------|--------|-------|-------|
| **Baseline BRSK2 CpG3 methylation** | | | | | | | |
| 1A Baseline × CpG3 (baseline) | 0.07 | 0.10 | 0.71 | 0.482 | −0.13 | 0.27 |
| 3-months × CpG3 (baseline) | −0.39 | 0.10 | −3.81 | 0.0002* | −0.60 | −0.19 |
| 6-months × CpG3 (baseline) | −0.49 | 0.10 | −4.76 | 0.000004* | −0.70 | −0.29 |
| 1B Baseline × CpG3 (baseline) | −0.12 | 0.10 | −1.17 | 0.247 | −0.33 | 0.08 |
| 3-months × CpG3 (baseline) | −0.16 | 0.15 | −1.09 | 0.276 | −0.45 | 0.13 |
| 6-months × CpG3 (baseline) | −0.12 | 0.15 | −0.81 | 0.418 | −0.41 | 0.17 |
| Baseline × childhood trauma | 1.54 | 0.49 | 3.13 | 0.002* | 0.57 | 2.51 |
| 3-months × childhood trauma | 1.21 | 0.66 | 1.84 | 0.069 | −0.09 | 2.52 |
| 6-months × childhood trauma | −0.035 | 0.66 | −0.53 | 0.598 | −1.65 | 0.96 |
| Baseline × alcohol consumption | −1.36 | 0.76 | −1.79 | 0.077 | −2.87 | 0.15 |
| 3-months × alcohol consumption | −0.96 | 1.23 | −0.78 | 0.438 | −3.40 | 1.48 |
| 6-months × alcohol consumption | −1.65 | 1.23 | −1.35 | 0.181 | −4.09 | 0.78 |
| Baseline × depression | 0.73 | 0.14 | 5.29 | 0.0000008* | 0.46 | 1.01 |
| 3-months × depression | 0.00 | 0.22 | 0.00 | 1.00 | −0.45 | 0.45 |
| 6-months × depression | 0.49 | 0.22 | 1.49 | 0.175 | −0.09 | 1.07 |
| **Baseline BRSK2 CpG4 methylation** | | | | | | | |
| 2A Baseline × CpG4 (baseline) | 0.07 | 0.11 | 0.59 | 0.558 | −0.16 | 0.29 |
| 3-months × CpG4 (baseline) | −0.33 | 0.12 | −2.85 | 0.005* | −0.56 | −0.10 |
| 6-months × CpG4 (baseline) | −0.44 | 0.12 | −3.83 | 0.0002* | −0.67 | −0.21 |
| 2B Baseline × CpG4 (baseline) | −0.08 | 0.12 | −0.70 | 0.486 | −0.31 | 0.15 |
| 3-months × CpG4 (baseline) | −0.14 | 0.15 | −0.93 | 0.357 | −0.44 | 0.16 |
| 6-months × CpG4 (baseline) | −0.19 | 0.15 | −1.29 | 0.201 | −0.49 | 0.10 |
| Baseline × childhood trauma | 1.44 | 0.51 | 2.84 | 0.005* | 0.43 | 2.44 |
| 3-months × childhood trauma | 1.20 | 0.70 | 1.73 | 0.086 | −0.17 | 2.58 |
| 6-months × childhood trauma | −0.09 | 0.69 | −0.12 | 0.902 | −1.45 | 1.28 |
| Baseline × alcohol consumption | −1.41 | 0.77 | −1.84 | 0.069 | −2.93 | 0.11 |
| 3-months × alcohol consumption | −1.00 | 1.23 | −0.81 | 0.421 | −3.44 | 1.45 |
| 6-months × alcohol consumption | −1.60 | 1.22 | −1.32 | 0.191 | −4.02 | 0.82 |
| Baseline × depression | 0.71 | 0.14 | 5.08 | 0.000002* | 0.43 | 0.98 |
| 3-months × depression | −0.01 | 0.23 | −0.05 | 0.963 | −0.46 | 0.44 |
| 6-months × depression | 0.53 | 0.22 | 2.40 | 0.018* | 0.09 | 0.98 |
| **Baseline BRSK2 CpG4 methylation** | | | | | | | |
| 2C Baseline × CpG4 (baseline) | −0.04 | 0.12 | −0.36 | 0.720 | −0.27 | 0.19 |
| 3-months × CpG4 (baseline) | −0.12 | 0.16 | −0.75 | 0.458 | −0.42 | 0.19 |
Table 5 continued

| Model | \( \beta \) | Std error | \( t \) | \( p \) | 95% CI Lower | 95% CI Upper |
|-------|-------------|-----------|--------|--------|-------------|-------------|
| 6-months × CpG4 (baseline) | −0.16 | 0.15 | −1.01 | 0.314 | −0.46 | 0.15 |
| Baseline × childhood trauma | 1.20 | 0.53 | 2.24 | 0.027* | 0.14 | 2.25 |
| 3-months × childhood trauma | 1.17 | 0.77 | 1.53 | 0.130 | −0.35 | 2.69 |
| 6-months × childhood trauma | −0.34 | 0.76 | −0.45 | 0.654 | −1.84 | 1.16 |
| Baseline × alcohol consumption | −1.47 | 0.76 | −1.93 | 0.056 | −2.99 | 0.04 |
| 3-months × alcohol consumption | −1.03 | 1.24 | −0.83 | 0.407 | −3.49 | 1.43 |
| 6-months × alcohol consumption | −1.67 | 1.22 | −1.37 | 0.175 | −4.09 | 0.75 |
| Baseline × depression | 0.74 | 0.14 | 5.28 | 0.0000008* | 0.46 | 1.02 |
| 3-months × depression | 0.00 | 0.23 | 0.01 | 0.996 | −0.45 | 0.46 |
| 6-months × depression | 0.57 | 0.22 | 2.52 | 0.013* | 0.12 | 1.01 |
| Baseline × lifetime trauma | 2.11 | 1.39 | 1.52 | 0.132 | −0.65 | 4.88 |
| 3-months × lifetime trauma | 0.71 | 2.27 | 0.32 | 0.753 | −3.79 | 5.22 |
| 6-months × lifetime trauma | 2.19 | 2.23 | 0.98 | 0.329 | −2.24 | 6.62 |
| Baseline BRSK2 CpG5 methylation | 3A Baseline × CpG5 (baseline) | 0.16 | 0.10 | 1.60 | 0.112 | −0.04 | 0.35 |
| 3-months × CpG5 (baseline) | −0.27 | 0.10 | −2.66 | 0.009* | −0.47 | −0.07 |
| 6-months × CpG5 (baseline) | −0.38 | 0.10 | −3.73 | 0.0003* | −0.58 | −0.18 |
| 3B Baseline × CpG5 (baseline) | −0.06 | 0.10 | −0.57 | 0.573 | −0.25 | 0.14 |
| 3-months × CpG5 (baseline) | −0.12 | 0.14 | −0.84 | 0.405 | −0.39 | 0.16 |
| 6-months × CpG5 (baseline) | −0.06 | 0.14 | −0.44 | 0.657 | −0.33 | 0.21 |
| Baseline × childhood trauma | 1.60 | 0.50 | 3.21 | 0.002* | 0.61 | 2.58 |
| 3-months × childhood trauma | 1.33 | 0.66 | 2.01 | 0.047* | 0.02 | 2.65 |
| 6-months × childhood trauma | −0.27 | 0.66 | −0.41 | 0.683 | −1.58 | 1.04 |
| Baseline × alcohol consumption | −1.44 | 0.77 | −1.88 | 0.064 | −2.96 | 0.08 |
| 3-months × alcohol consumption | −1.01 | 1.23 | −0.82 | 0.412 | −3.46 | 1.43 |
| 6-months × alcohol consumption | −1.72 | 1.23 | −1.41 | 0.163 | −4.16 | 0.71 |
| Baseline × depression | 0.72 | 0.14 | 5.13 | 0.000002* | 0.44 | 1.00 |
| 3-months × depression | 0.00 | 0.23 | 0.01 | 0.991 | −0.45 | 0.45 |
| 6-months × depression | 0.48 | 0.22 | 2.14 | 0.035* | 0.03 | 0.93 |
| 3C Baseline × CpG5 (baseline) | −0.03 | 0.10 | −0.29 | 0.774 | −0.23 | 0.17 |
| 3-months × CpG5 (baseline) | −0.10 | 0.14 | −0.71 | 0.477 | −0.38 | 0.18 |
| 6-months × CpG5 (baseline) | −0.03 | 0.14 | −0.22 | 0.827 | −0.31 | 0.25 |
| Baseline × childhood trauma | 1.34 | 0.53 | 2.56 | 0.012* | 0.30 | 2.39 |
| 3-months × childhood trauma | 1.28 | 0.73 | 1.74 | 0.085 | −0.18 | 2.73 |
| 6-months × childhood trauma | −0.58 | 0.73 | −0.80 | 0.425 | −2.03 | 0.86 |
| Baseline × alcohol consumption | −1.49 | 0.76 | −1.96 | 0.054 | −3.01 | 0.02 |
| 3-months × alcohol consumption | −1.04 | 1.24 | −0.84 | 0.402 | −3.50 | 1.41 |
| 6-months × alcohol consumption | −1.78 | 1.22 | −1.46 | 0.148 | −4.21 | 0.65 |
| Baseline × depression | 0.75 | 0.14 | 5.33 | 0.0000007* | 0.47 | 1.03 |
| 3-months × depression | 0.01 | 0.23 | 0.06 | 0.950 | −0.44 | 0.47 |
| 6-months × depression | 0.52 | 0.23 | 2.29 | 0.024* | 0.07 | 0.97 |
| Baseline × lifetime trauma | 2.12 | 1.39 | 1.53 | 0.129 | −0.63 | 4.88 |
| 3-months × lifetime trauma | 0.74 | 2.26 | 0.33 | 0.75 | −3.74 | 5.22 |
| 6-months × lifetime trauma | 2.52 | 2.23 | 1.13 | 0.262 | −1.92 | 6.96 |
| Baseline ADCYAP1 CpG1&2 methylation | 4A Baseline × CpG1&2 (baseline) | 5.34 | 1.02 | 5.26 | 0.0000009* | 3.33 | 7.36 |
| 3-months × CpG1&2 (baseline) | −1.03 | 1.12 | −0.92 | 0.360 | −3.25 | 1.19 |
| 6-months × CpG1&2 (baseline) | −3.52 | 1.18 | −2.97 | 0.004* | −5.86 | −1.17 |
| 4B Baseline × CpG1&2 (baseline) | −0.73 | 0.88 | −0.83 | 0.407 | −2.47 | 1.01 |
| 3-months × CpG1&2 (baseline) | 2.75 | 1.39 | 1.98 | 0.050 | −0.00 | 5.50 |
We investigated ADCYAP1 further, since its protein product, PACAP, has been identified as a master regulator of the HPA-axis and the stress response [86]. The highest concentration of PACAP in the brain is found in the hypothalamus [87]. PACAP binding in the hypothalamus triggers the release of corticotrophin-releasing hormone (CRH) and signals the activation of the stress response [86]. In the adrenal medulla, PACAP binding to PAC1R (product of ADCYAP1R1) stimulates the release of catecholamines as part of the sympathetic nervous system (SNS) [88]. PACAP binding to PACR1 in preganglionic neurons triggers the release of phenylethanolamine-N-methyltransferase (PNMT) and tyrosine hydroxylase (TH) in effector organs of the SNS. PNMT and TH are catecholamine-synthesizing enzymes and sustain the release of catecholamines in the effector organs during the stress response [88].

Researchers investigating PACAP/ADCYAP1 and PACR1/ADCYAP1R1 in relation to PTSD in a predominantly African-American sample with a mixture of trauma types found that, in women more than men, increased PACAP blood levels were associated with increased PTSD symptom severity and an increased acoustic startle reflex response [64, 89]. They also found that women carrying the ADCYAP1R1 rs2267735 CC genotype showed decreased ADCYAP1R1 mRNA expression, increased PTSD symptom severity, increased dark-enhanced startle response, and increased amygdala and hippocampal activity in response to viewing threatening face stimuli [64–66, 89]. In both men and women, increased methylation of ADCYAP1R1 was associated with decreased cortical mRNA expression and increased PTSD symptom severity [64, 90]. However, the functional effects of ADCYAP1 and ADCYAP1R1 seem to be more pronounced in women compared to men [64–66], due to the presence of several estrogen response elements (EREs) in the ADCYAP1R1 promoter. The CC genotype of rs2267735 has been associated with decreased binding of estrogen receptor alpha to the EREs and decreased expression of ADCYAP1R1 [91]. The role of estrogen in ADCYAP1R1 and HPA-axis activity may in part explain why women have an increased risk of PTSD compared to men [35, 92].

The two ADCYAP1 CpG sites investigated in this study are located in a CpG island spanning the 1st intron of the gene. Methylation in CpG islands and in the 1st intron of a gene is generally associated with decreased expression of the gene [93–95]. Our longitudinal findings, therefore, correspond with prior findings since decreased methylation of ADCYAP1 is likely to result in increased expression of PACAP and increased PTSD symptom severity [65, 66, 91, 96]. Decreased PACAP is also likely to result in decreased binding to PAC1 and reduced activation of the HPA-axis [86, 88]. Based on prior findings, ADCYAP1 CpG1&2 DNA methylation in blood was not significantly correlated with DNA methylation at the same sites in brain tissue [59]. However, the brain regions investigated did not specifically focus on the region where PACAP is most abundantly expressed i.e., the paraventricular nucleus of the hypothalamus, and investigating blood-brain methylation in this region may show different results [37]. It is also likely that the expression of PACAP in the endocrine system has a more profound effect on the regulation of the HPA-axis compared to PACAP expression in the brain [37].

We found that, before correction for multiple testing, CpG sites in HTR3A [67], AHRR [22], DUSP22 [15], and TPR [13] were

| Table 5 continued | Model | β (95% CI) | Std error | t | p | 95% CI (Lower, Upper) |
| --- | --- | --- | --- | --- | --- | --- |
| 6-months × CpG1&2 (baseline) | 1.66 (−1.10, 4.41) | 1.39 | 1.20 | 0.235 | −1.00 | 2.26 |
| Baseline × childhood trauma | 1.84 (0.92, 2.76) | 0.47 | 3.95 | 0.0001* | 0.05 | 0.05 |
| 3-months × childhood trauma | 1.11 (0.05, 2.22) | 0.59 | 1.88 | 0.062 | −0.05 | 0.87 |
| 6-months × childhood trauma | −0.29 (−1.45, 0.87) | 0.59 | −0.49 | 0.625 | −1.45 | 0.87 |
| Baseline × alcohol consumption | −1.48 (−3.01, 0.05) | 0.77 | −1.93 | 0.057 | −3.01 | 0.05 |
| 3-months × alcohol consumption | −1.43 (−3.85, 0.98) | 1.22 | −1.18 | 0.242 | −3.85 | 0.98 |
| 6-months × alcohol consumption | −1.91 (−4.32, 0.51) | 1.21 | −1.57 | 0.120 | −4.32 | 0.51 |
| Baseline × depression | 0.73 (0.46, 1.01) | 0.14 | 5.29 | 0.0000008* | 0.46 | 1.01 |
| 3-months × depression | −0.13 (−0.56, 0.30) | 0.22 | −0.59 | 0.554 | −0.56 | 0.30 |
| 6-months × depression | 0.42 (0.00, 0.85) | 0.22 | 1.96 | 0.052 | −0.00 | 0.85 |
| 4C Baseline × CpG1&2 (baseline) | −0.54 (−2.30, 1.22) | 0.89 | −0.61 | 0.541 | −2.30 | 1.22 |
| 3-months × CpG1&2 (baseline) | 2.76 (0.05, 5.57) | 1.42 | 1.95 | 0.055 | −0.05 | 5.57 |
| 6-months × CpG1&2 (baseline) | 1.59 (−1.22, 4.40) | 1.41 | 1.13 | 0.263 | −1.22 | 4.40 |
| Baseline × childhood trauma | 1.87 (0.95, 2.80) | 0.47 | 4.01 | 0.0001* | 0.95 | 2.80 |
| 3-months × childhood trauma | 1.06 (−0.11, 2.24) | 0.59 | 1.79 | 0.076 | −0.11 | 2.24 |
| 6-months × childhood trauma | −0.36 (−1.53, 0.82) | 0.59 | −0.60 | 0.550 | −1.53 | 0.82 |
| Baseline × alcohol consumption | −1.44 (−2.96, 0.08) | 0.76 | −1.88 | 0.063 | −2.96 | 0.08 |
| 3-months × alcohol consumption | −1.43 (−3.86, 1.01) | 1.22 | −1.17 | 0.247 | −3.86 | 1.01 |
| 6-months × alcohol consumption | −1.91 (−4.34, 0.52) | 1.22 | −1.56 | 0.121 | −4.34 | 0.52 |
| Baseline × depression | 0.74 (0.46, 1.01) | 0.14 | 5.33 | 0.0000006* | 0.46 | 1.01 |
| 3-months × depression | −0.13 (−0.56, 0.30) | 0.22 | −0.61 | 0.546 | −0.56 | 0.30 |
| 6-months × depression | 0.42 (−0.01, 0.85) | 0.22 | 1.92 | 0.058 | −0.01 | 0.85 |
| Baseline × HIV status | −4.51 (−11.55, 2.53) | 3.54 | −1.27 | 0.206 | −11.55 | 2.53 |
| 3-months × HIV status | −0.39 (−11.61, 10.84) | 5.65 | −0.07 | 0.945 | −11.61 | 10.84 |
| 6-months × HIV status | 1.24 (−9.99, 12.46) | 5.65 | 0.22 | 0.827 | −9.99 | 12.46 |

CI: confidence interval. BRSK2 brain-specific serine/threonine-protein kinase 2, ADCYAP1 adenylate cyclase activating polypeptide 1.
Table 6. Summary statistics of the mixed regression models investigating change in BRSK2 and ADCYAP1 methylation over time as predictors of change in posttraumatic stress symptoms scores over time.

| Model | $\beta$ | Std error | t   | p       | 95% CI   | Lower  | Upper  |
|-------|---------|-----------|-----|---------|----------|--------|--------|
|       |         |           |     |         |          |        |        |
| Baseline BRSK2 CpG3 methylation | | | | | | | |
| 1A    | 0.07    | 0.10      | 0.71| 0.482   | −0.13 0.27 |
|       | −0.39   | 0.10      | −3.81| 0.0002*| −0.60 −0.19 |
|       | −0.49   | 0.10      | −4.76| 0.000004*| −0.70 −0.29 |
| 1B    | −0.16   | 0.10      | −1.60| 0.111   | −0.37 0.04 |
|       | −0.31   | 0.15      | −2.06| 0.041*  | −0.60 −0.01 |
|       | −0.15   | 0.14      | −1.02| 0.308   | −0.44 0.14 |
|       | 1.43    | 0.49      | 2.93| 0.004*  | 0.47 2.40 |
|       | 1.39    | 0.66      | 2.11| 0.037*  | 0.08 2.70 |
|       | −0.51   | 0.66      | −0.78| 0.436   | −1.81 0.78 |
|       | −1.31   | 0.76      | −1.72| 0.088   | −2.81 0.20 |
|       | −0.86   | 1.22      | −0.71| 0.481   | −3.28 1.56 |
|       | −1.60   | 1.24      | −1.29| 0.199   | −4.06 0.86 |
|       | 0.74    | 0.14      | 5.34| 0.000006*| 0.46 1.01 |
|       | 0.06    | 0.22      | 0.26| 0.793   | −0.38 0.50 |
|       | 0.50    | 0.23      | 2.16| 0.034*  | 0.04 0.97 |
| 1C    | −0.15   | 0.10      | −1.42| 0.157   | −0.35 0.06 |
|       | −0.30   | 0.15      | −1.99| 0.049*  | −0.60 −0.00 |
|       | −0.12   | 0.15      | −0.80| 0.423   | −0.41 0.17 |
|       | 1.18    | 0.52      | 2.89| 0.024*  | 0.16 2.21 |
|       | 1.34    | 0.73      | 1.84| 0.068   | −0.10 2.78 |
|       | −0.82   | 0.73      | −1.12| 0.263   | −2.25 0.62 |
|       | −1.35   | 0.75      | −1.79| 0.077   | −2.84 0.15 |
|       | −0.88   | 1.23      | −0.71| 0.477   | −3.31 1.56 |
|       | −1.66   | 1.24      | −1.34| 0.184   | −4.11 0.80 |
|       | 0.77    | 0.14      | 5.55| 0.000002*| 0.49 1.05 |
|       | 0.07    | 0.23      | 0.31| 0.761   | −0.38 0.52 |
|       | 0.53    | 0.23      | 2.27| 0.026*  | 0.07 1.00 |
|       | 2.02    | 1.36      | 1.48| 0.141   | −0.68 4.72 |
|       | 0.57    | 2.22      | 0.26| 0.797   | −3.84 4.98 |
|       | 2.28    | 2.27      | 1.01| 0.317   | −2.23 6.79 |
| Baseline BRSK2 CpG4 methylation | | | | | | | |
| 2A    | 0.03    | 0.11      | 0.32| 0.749   | −0.17 0.24 |
|       | −0.36   | 0.11      | −3.40| 0.001*  | −0.57 −0.15 |
|       | −0.46   | 0.11      | −4.92| 0.0003*| −0.68 −0.25 |
| 2B    | −0.13   | 0.11      | −1.21| 0.230   | −0.35 0.84 |
|       | −0.30   | 0.15      | −2.04| 0.043*  | −0.59 −0.01 |
|       | −0.22   | 0.15      | −1.49| 0.138   | −0.51 0.07 |
|       | 1.36    | 0.49      | 2.75| 0.007*  | 0.38 2.34 |
|       | 1.48    | 0.69      | 2.14| 0.034*  | 0.11 2.84 |
|       | −0.28   | 0.69      | −0.40| 0.689   | −1.64 1.09 |
|       | −1.35   | 0.76      | −1.77| 0.080   | −2.87 0.16 |
|       | −0.85   | 1.22      | −0.70| 0.488   | −3.27 1.57 |
|       | −1.59   | 1.22      | −1.30| 0.197   | −4.02 0.84 |
|       | 0.72    | 0.14      | 5.15| 0.000001*| 0.44 0.99 |
|       | 0.07    | 0.22      | 0.32| 0.751   | −0.37 0.52 |
|       | 0.55    | 0.23      | 2.38| 0.020*  | 0.098 1.01 |
| 2C    | −0.10   | 0.11      | −0.88| 0.379   | −0.32 0.12 |
|       | −0.28   | 0.15      | −1.88| 0.062   | −0.58 0.14 |

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| Model | $\beta$ | Std error | $t$ | $p$ | 95% CI | Lower | Upper |
|-------|--------|-----------|-----|-----|--------|--------|-------|
| 6-months × CpG4 (6-months) | $-0.19$ | 0.15 | $-1.25$ | 0.215 | $-0.48$ | 0.11 |
| Baseline × childhood trauma | 1.12 | 0.52 | 2.14 | 0.035* | 0.08 | 2.15 |
| 3-months × childhood trauma | 1.46 | 0.76 | 1.93 | 0.056 | $-0.04$ | 2.96 |
| 6-months × childhood trauma | $-0.53$ | 0.76 | $-0.70$ | 0.488 | $-2.03$ | 0.97 |
| Baseline × alcohol consumption | $-1.41$ | 0.76 | $-1.85$ | 0.067 | $-2.92$ | 0.10 |
| 3-months × alcohol consumption | $-0.87$ | 1.23 | $-0.71$ | 0.478 | $-3.31$ | 1.56 |
| 6-months × alcohol consumption | $-1.64$ | 1.22 | $-1.34$ | 0.183 | $-4.08$ | 0.79 |
| Baseline × depression | 0.75 | 0.14 | 5.34 | 0.0000006* | 0.47 | 1.02 |
| 3-months × depression | 0.79 | 0.23 | 3.53 | 0.035 | 0.35 | 0.73 |
| 6-months × depression | 0.53 | 0.76 | $-0.70$ | 0.488 | $-2.03$ | 0.97 |
| Baseline × lifetime trauma | 2.00 | 1.39 | 1.44 | 0.154 | $-0.76$ | 4.75 |
| 3-months × lifetime trauma | 0.49 | 2.22 | 0.22 | 0.826 | $-3.93$ | 4.91 |
| 6-months × lifetime trauma | 2.12 | 2.25 | 0.95 | 0.345 | $-2.33$ | 6.59 |

**Baseline BRSK2 CpG5 methylation**

| 3A | Baseline × CpG5 (baseline) | 0.10 | 0.09 | 1.07 | 0.285 | $-0.08$ | 0.28 |
| 3-months × CpG5 (3-months) | $-0.32$ | 0.09 | $-3.40$ | 0.001* | $-0.51$ | $-0.14$ |
| 6-months × CpG5 (6-months) | $-0.43$ | 0.10 | $-4.42$ | 0.00002* | $-0.62$ | $-0.24$ |
| 3B | Baseline × CpG5 (baseline) | $-0.11$ | 0.10 | $-1.10$ | 0.275 | $-0.30$ | 0.09 |
| 3-months × CpG5 (3-months) | $-0.25$ | 0.14 | $-1.81$ | 0.073 | $-0.53$ | 0.02 |
| 6-months × CpG5 (6-months) | $-0.15$ | 0.13 | $-1.11$ | 0.269 | $-0.42$ | 0.12 |
| Baseline × childhood trauma | 1.43 | 0.49 | 2.91 | 0.004* | 0.46 | 2.40 |
| 3-months × childhood trauma | 1.46 | 0.67 | 2.17 | 0.032* | 0.13 | 2.78 |
| 6-months × childhood trauma | $-0.35$ | 0.66 | $-0.54$ | 0.593 | $-1.65$ | 0.95 |
| Baseline × alcohol consumption | $-1.37$ | 0.76 | $-1.79$ | 0.076 | $-2.88$ | 0.15 |
| 3-months × alcohol consumption | $-0.98$ | 1.23 | $-0.80$ | 0.429 | $-3.41$ | 1.46 |
| 6-months × alcohol consumption | $-1.62$ | 1.23 | $-1.32$ | 0.190 | $-4.06$ | 0.82 |
| Baseline × depression | 0.72 | 0.14 | 5.18 | 0.0000001* | 0.45 | 1.00 |
| 3-months × depression | 0.05 | 0.22 | 0.21 | 0.834 | $-0.40$ | 0.49 |
| 6-months × depression | 0.52 | 0.23 | 2.26 | 0.027* | 0.06 | 0.98 |
| 3C | Baseline × CpG5 (baseline) | $-0.08$ | 0.10 | $-0.81$ | 0.417 | $-0.27$ | 0.11 |
| 3-months × CpG5 (3-months) | $-0.24$ | 0.14 | $-1.68$ | 0.095 | $-0.51$ | 0.04 |
| 6-months × CpG5 (6-months) | $-0.12$ | 0.14 | $-0.86$ | 0.390 | $-0.39$ | 0.15 |
| Baseline × childhood trauma | 1.19 | 0.52 | 2.28 | 0.024* | 0.16 | 2.22 |
| 3-months × childhood trauma | 1.41 | 0.74 | 1.91 | 0.058 | $-0.05$ | 2.87 |
| 6-months × childhood trauma | $-0.62$ | 0.73 | $-0.86$ | 0.391 | $-2.06$ | 0.81 |
| Baseline × alcohol consumption | $-1.42$ | 0.76 | $-1.87$ | 0.065 | $-2.93$ | 0.88 |
| 3-months × alcohol consumption | $-0.99$ | 1.23 | $-0.81$ | 0.423 | $-3.45$ | 1.46 |
| 6-months × alcohol consumption | $-1.68$ | 1.23 | $-1.37$ | 0.175 | $-4.12$ | 0.76 |
| Baseline × depression | 0.75 | 0.14 | 5.37 | 0.0000005* | 0.48 | 1.03 |
| 3-months × depression | 0.06 | 0.23 | 0.26 | 0.800 | $-0.40$ | 0.51 |
| 6-months × depression | 0.55 | 0.23 | 2.36 | 0.020* | 0.09 | 1.01 |
| Baseline × lifetime trauma | 2.03 | 1.38 | 1.47 | 0.145 | $-0.71$ | 4.77 |
| 3-months × lifetime trauma | 0.62 | 2.24 | 0.28 | 0.781 | $-3.82$ | 5.06 |
| 6-months × lifetime trauma | 2.23 | 2.26 | 0.98 | 0.328 | $-2.27$ | 6.72 |

**Baseline ADCYAP1 CpG1&2 methylation**

| 4A | Baseline × CpG5 (baseline) | 4.67 | 0.92 | 5.10 | 0.0000001* | 2.86 | 6.49 |
| 3-months × CpG5 (3-months) | $-2.61$ | 0.80 | $-3.26$ | 0.001* | $-4.20$ | $-1.02$ |
| 6-months × CpG5 (6-months) | $-5.01$ | 1.12 | $-4.48$ | 0.00002* | $-7.23$ | $-2.80$ |
| 4B | Baseline × CpG5 (baseline) | $-1.32$ | 0.83 | $-1.16$ | 0.113 | $-2.97$ | 0.32 |
| 3-months × CpG5 (3-months) | $-1.46$ | 0.92 | $-1.59$ | 0.116 | $-3.29$ | 0.37 |
associated with PTSD. The results from our study are in line with recent results from the largest EWAS meta-analysis of PTSD published to date [22], where AHRR cg05575921 and cg26703534 were found to exhibit reduced DNA methylation in individuals with PTSD. Decreased AHRR methylation at these CpG sites was also associated with decreased kynurenine and kynurenine acid in the same study [22]. Kynurenine ligand binding to aryl hydrocarbon receptors has been associated with the expression of anti-inflammatory genes which may be disrupted by decreased methylation of AHRR [22, 25]. This may result in increased levels of proinflammatory cytokines and the low-grade inflammatory state often observed in PTSD [97, 98]. Uuprearrangement in kynurenine to restore the imbalance between pro-inflammatory and anti-inflammatory cytokines may also result in reduced levels of serotonin since both kynurenine and serotonin are synthesized from tryptophan [99]. A strong link between decreased AHRR methylation and smoking has also been reported in previous studies although some studies have reported a significant relationship between AHRR methylation and PTSD independent of the effect of smoking [22, 100–102].

Our findings should be interpreted in light of a number of limitations. First, the EWAS was conducted in a small sample of participants. However, the study was well designed to limit variation between groups. Second, we used DNA extracted from whole blood to measure methylation levels while differential methylation in brain tissue is a more direct approximation of PTSD pathophysiology. However, based on prior findings, we observed that blood-brain methylation was highly correlated at the BRSK2 CpG sites investigated in this study, but not at the ADCYAP1 CpG sites. Blood is easily accessible and blood biomarkers of PTSD risk may be a more pragmatic approach for personalized treatment of individuals at high risk of developing PTSD following trauma exposure [103]. Third, we may have overcorrected for confounding variables in the EWAS given that SVA was used along with the inclusion of cell-type composition as a covariate in the final models. Fourth, we did not investigate methylation quantitative trait loci (meQTL) located in the BRSK2 and ADCYAP1 genes. SNPs located in these genes may predict or mediate the methylation profiles observed in relation to PTSD status and symptom scores. Finally, DNA methylation in relation to gene expression and/or protein levels was not objectively measured and conclusions related to the functional effects of methylation are speculative.

The study has many strengths. First, all participants were rape-exposed women from similar sociodemographic backgrounds and from the same ethnicity group thus making the sample relatively homogenous. Second, the analyses were robust with a variety of confounding factors controlled for i.e., participants who were pregnant/lactating were excluded, none of the participants were on psychotropic medication and participants were of similar age. Baseline measures of age, HIV status, BMI, smoking, childhood trauma, lifetime trauma, alcohol use, and depression were controlled for by matching participants on these variables in the cross-sectional EWAS and including these factors as covariates/confounders in the longitudinal analyses. Third, we attempted to expand the findings of the EWAS by including longitudinal data which allowed us to investigate changes in methylation in relation to change in PTSD symptom scores over time. Fourth, investigating the agreement between

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**Table 6 continued**

| Model                        | $\beta$ | Std error | $t$ | $p$  | 95% CI Lower | 95% CI Upper |
|------------------------------|---------|-----------|-----|-----|--------------|--------------|
| 6-months $\times$ CpG5 (6-months) | $-0.44$ | 1.28      | $-0.34$ | 0.734 | $-2.97$ | 2.10 |
| Baseline $\times$ childhood trauma | $1.77$ | 0.47      | 3.76 | 0.0003* | 0.84 | 2.70 |
| 3-months $\times$ childhood trauma | $1.44$ | 0.61      | 2.37 | 0.019* | 0.24 | 2.63 |
| 6-months $\times$ childhood trauma | $-0.19$ | 0.60      | $-0.31$ | 0.757 | $-1.37$ | 1.00 |
| Baseline $\times$ alcohol consumption | $-1.41$ | 0.77      | $-1.84$ | 0.068 | $-2.93$ | 0.11 |
| 3-months $\times$ alcohol consumption | $-1.27$ | 1.24      | $-1.02$ | 0.309 | $-3.73$ | 1.19 |
| 6-months $\times$ alcohol consumption | $-1.74$ | 1.22      | $-1.42$ | 0.158 | $-4.17$ | 0.69 |
| Baseline $\times$ depression | 0.74     | 0.14      | 5.35 | 0.0000006* | 0.46 | 1.01 |
| 3-months $\times$ depression | $-0.03$ | 0.22      | $-0.16$ | 0.876 | $-0.46$ | 0.40 |
| 6-months $\times$ depression | 0.46     | 0.21      | 2.14 | 0.035* | 0.03 | 0.88 |
| 4C Baseline $\times$ CpG5 (baseline) | $-1.12$ | 0.83      | $-1.35$ | 0.182 | $-2.78$ | 0.54 |
| 3-months $\times$ CpG5 (3-months) | $-1.54$ | 0.93      | $-1.66$ | 0.100 | $-3.38$ | 0.30 |
| 6-months $\times$ CpG5 (6-months) | $-0.48$ | 1.29      | $-0.37$ | 0.712 | $-3.03$ | 2.08 |
| Baseline $\times$ childhood trauma | 1.80     | 0.47      | 3.84 | 0.0002* | 0.87 | 2.73 |
| 3-months $\times$ childhood trauma | 1.37     | 0.61      | 2.23 | 0.027* | 0.16 | 2.58 |
| 6-months $\times$ childhood trauma | $-0.26$ | 0.61      | $-0.42$ | 0.675 | $-1.46$ | 0.95 |
| Baseline $\times$ alcohol consumption | $-1.38$ | 0.76      | $-1.81$ | 0.074 | $-2.90$ | 0.17 |
| 3-months $\times$ alcohol consumption | $-1.29$ | 1.25      | $-1.04$ | 0.302 | $-3.77$ | 1.18 |
| 6-months $\times$ alcohol consumption | $-1.76$ | 1.23      | $-1.43$ | 0.157 | $-4.21$ | 0.69 |
| Baseline $\times$ depression | 0.74     | 0.14      | 5.40 | 0.0000005* | 0.47 | 1.01 |
| 3-months $\times$ depression | $-0.04$ | 0.22      | $-0.20$ | 0.844 | $-0.48$ | 0.39 |
| 6-months $\times$ depression | 0.45     | 0.22      | 2.08 | 0.040* | 0.02 | 0.88 |
| Baseline $\times$ HIV status | $-4.28$ | 3.53      | $-1.21$ | 0.229 | $-11.30$ | 2.74 |
| 3-months $\times$ HIV status | 2.16     | 5.78      | 0.37 | 0.709 | $-9.32$ | 13.65 |
| 6-months $\times$ HIV status | 1.86     | 5.60      | 0.33 | 0.740 | $-9.27$ | 12.99 |

CI = confidence interval, BRSK2 = brain-specific serine/threonine-protein kinase 2, ADCYAP1 = adenylyl cyclase activating polypeptide 1.
the results obtained from the two different laboratory methods used (illumina EPIC array and Epityper) also allowed identification of potential bias/variation introduced by the different procedures involved in each method.

In summary, this study provides evidence that differential methylation of genes related to neurogenesis/development, glucose homeostasis, and HPA-axis regulation may be involved in PTSD development following rape. Our findings are supported by previous research implicating ADCYAP1/ADCYAPI-R1 (especially in women) and BRSK1/BRSK2 in the development of PTSD. However, replication of these findings is required to determine whether the differentially methylated regions identified in this study are consistently linked to the development of PTSD.

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AUTHOR CONTRIBUTIONS
JN, NA, CL, SS, and SMJH contributed to the conception and design of the study. JN, ST, MS, SM, CL, and SMJH analyzed the data. All authors contributed to the interpretation of the data. NA was involved in the acquisition of the primary data. MS developed the statistical package used in the epigenome-wide analysis. JN drafted the paper and all other authors revised it. All authors read and approved the paper.

COMPETING INTERESTS
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Jani Nöthling.

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