DNA methylation of Nuclear Factor of Activated T Cells 1 mediates the prospective relation between exposure to different traumatic event types and post-traumatic stress disorder

James R. Occean[a,g], **Agaz H. Wani**[a], **Janelle Donglasan**[a], **Allison E. Aiello**[b], **Sandro Galea**[c], **Karestan C. Koenen**[d,e], **Annie Qu**[f], **Derek E. Wildman**[a], **Monica Uddin**[a,*]

[a]Genomics Program, College of Public Health, University of South Florida, Tampa, FL, USA

[b]Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[c]Boston University School of Public Health, Boston University, Boston, MA, USA

[d]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA, USA

[e]Psychiatric and Neurodevelopmental Genetics Unit & Department of Psychiatry, Massachusetts General Hospital, Boston, MA, USA

[f]Department of Statistics, University of California Irvine, Irvine, CA, USA

[g]Present address: Laboratory of Genetics and Genomics, National Institute on Aging, National Institutes of Health, Baltimore, MD, USA

Abstract

The mechanisms through which exposure to differing trauma types become biologically embedded to shape the risk for post-traumatic stress disorder (PTSD) is unclear. DNA methylation (5-mC), particularly in stress-relevant genes, may play a role in this relationship. Here, we conducted path analysis using generalized structural equation modeling to investigate whether blood-derived 5-mC in Nuclear Factor of Activated T Cells 1 (*NFATC1*) mediated the prospective association between each of five different trauma types ("assaultive violence", "other injury or shocking experience", "learning of trauma to loved one", "sudden, unexpected death of a close friend or

*Corresponding author: Monica Uddin, Professor, Genomics Program, College of Public Health University of South Florida, 3720 Spectrum Blvd., Ste. 304, Tampa, FL, 33612, monica43@usf.edu.

relative", and "other") and lifetime PTSD. All five trauma types were significantly associated with reduced methylation at *NFATC1* CpG site, cg17057218. Two of the five trauma types were significantly associated with increased methylation at *NFATC1* CpG site, cg22324981. Moreover, methylation at cg17057218 significantly mediated 21–32% of the total effect for four of the five trauma types, while methylation at cg22324981 mediated 27–40% of the total effect for two of the five trauma types. These CpG sites were differentially associated with transcription factor binding sites and chromatin state signatures. *NFATC1* 5-mC may be a potential mechanism in the relationship between some trauma types and prospective risk for PTSD.

Keywords

Epigenetics; methylcytosine; *NFATC1* ; glucocorticoid receptor; structural equation modeling; PTSD

1. Introduction

Post-traumatic stress disorder (PTSD) is a disabling and heterogeneous psychiatric disorder with an estimated lifetime prevalence of 6.8% among U.S. adults (Kessler et al., 2005) and 3.9% globally (Koenen et al., 2017). PTSD is unique from other psychopathologies because its diagnostic criteria necessitate exposure to a traumatic event involving significant injury, actual or threatened death (American Psychiatric Association, 2013). Exposure to trauma is thus an etiological component of PTSD. Yet, epidemiological reports indicate that the disorder will manifest in only a subset of trauma-exposed individuals (Kessler et al., 2017) —suggesting the possibility of other factors that may shape the risk of PTSD development.

The psychological susceptibility or vulnerability to PTSD is related, at least in part, to the form or type of trauma exposure. Indeed, significant differences in conditional risk of PTSD associated with exposure to different trauma types are well documented. In a nationally representative study, Kessler et al. (1995) reported an estimated conditional probability of PTSD ranging from 1.8% to 65.0% for men, and 5.4% to 48.5% for women, associated with exposure to specific traumatic events. Among the study participants with PTSD, the events nominated as "most upsetting" were combat for men and rape for women (Kessler et al., 1995). Breslau et al. (1998) later classified a list of 19 traumatic events into four composite classes and identified exposure to assaultive violence as conferring the highest conditional risk for PTSD. Similar findings were obtained from a predominantly African American sample by Goldmann et al. (2011); these authors reported experiences of assaultive violence conferred the highest risk for lifetime probable PTSD, followed by other injury or shock, sudden, unexpected death of a close relative or friend, and learning of traumas to a close relative or friend. Recent work from the World Health Organization World Mental Health surveys has also shown a differential risk of PTSD across trauma types, specifically, an increased risk of PTSD and longer duration following exposure to violence-related traumas (Kessler et al., 2017). Collectively, these findings provide evidence of a relationship between exposure to different types of traumas and PTSD development, however, the mechanisms by which these exposures confer differing conditional risk for PTSD is unclear.

Biological insights into PTSD pathophysiology point to alterations of the hypothalamic-pituitary-adrenal axis, the body's central stress response system that orchestrates the release of glucocorticoids (e.g., cortisol) following exposure to stress (Whitnall, 1993; Yehuda et al., 2004). Consequently, multiple studies have investigated epigenetic variations associated with PTSD development in stress-relevant pathways, such as the glucocorticoid receptor regulatory network (GRRN) (Labonte et al., 2014; Vukojevic et al., 2014; Yehuda et al., 2014; Yehuda et al., 2015). The GRRN is active in peripheral tissues, including blood —one of the primary tissues that transduce the HPA axis response to stress throughout the body, reviewed in (Irwin & Cole, 2011). Previous work has shown that genes belonging to the GRRN pathway undergo epigenetic modifications following exposure to glucocorticoids such as cortisol (Klengel et al., 2013). Epigenetic mechanisms influence chromatin architecture and govern DNA accessibility to transcriptional machinery, thereby playing an important role in gene regulation, reviewed in (Gibney & Nolan, 2010). As such, epigenetic modifications, particularly in GRRN genes, represent a potential mechanism wherein environmental exposures such as trauma may become biologically embedded to confer risk for PTSD. The most well-characterized epigenetic modification thus far in relation to PTSD, is DNA methylation (or 5-methylcytosine [5-mC]), which refers to the biochemical addition of a methyl ($-CH_3$) group to the 5' position of a cytosine base. Prior research has shown that 5-mC of GRRN genes, measured in blood, is associated with PTSD (Schür et al., 2017; Yehuda et al., 2015), depression (Bustamante et al., 2016; Nantharat et al., 2015), and is predictive of prospective risk for PTSD (Wani et al., 2021).

Concerning genes involved in the GRRN pathway, recent studies have implicated 5-mC in the gene encoding Nuclear Factor of Activated T Cells 1 (NFATC1) in PTSD (Kuan et al., 2017; Wani et al., 2021). The *NFATC1* gene (located on chromosome 18) encodes a Ca^{2+}/calmodulin-dependent isoform of the NFAT transcription factor family and is involved in several physiological processes, reviewed in (Kipanyula et al., 2016), including immune function regulation (Rao et al., 1997). Differential methylation of immune-related genes is extensively documented in PTSD (Daskalakis et al., 2021; Gill et al., 2009; Smith et al., 2020; Uddin et al., 2010). Moreover, recent work showed an altered transcriptional response of *NFATC1* following *in vitro* glucocorticoid stimulation (Breen et al., 2019), suggesting the gene plays a role in modulating stress response and may thus plausibly contribute to the development of stress-related psychopathology, including PTSD.

To date, however, only a few molecular studies have investigated trauma-related differences in PTSD. One study by Breen et al. (2018) employed a combined transcriptome-wide peripheral blood study that included seven trauma types; they reported differences in gene expression signatures between interpersonal- and combat-related traumas that ultimately converged on shared biological processes. Another transcriptomic study by Huckins et al. (2020) identified a gene associated with military, but not civilian, PTSD cohorts— indicating possible unique genetic etiologies in trauma-specific PTSD. An epigenome-wide association study (EWAS) of PTSD in civilians identified two genome-wide significant CpG sites in *NRG1* and *HGS*, respectively (Uddin et al., 2018), which were not observed in subsequent EWAS analyses of military-derived cohorts (Logue et al., 2020), suggesting potential mechanistic differences, possibly in addition to other factors (e.g., sex), in the loci that mediate civilian- and military-related PTSD. Nevertheless, the molecular

mechanisms underlying the relationship between exposure to different trauma types and risk for subsequent PTSD remains largely unclear, and an investigation of 5-mC in genes previously identified in stress-related psychopathology, of particular interest, *NFATC1*, may help elucidate such mechanisms.

To address this gap in knowledge, the present study sought to investigate the association between trauma types, *NFATC1* 5-mC, and lifetime PTSD. To this end, we focused our initial efforts on identifying PTSD-relevant CpG sites in *NFATC1*. We then interrogated the relation between different trauma types, methylation at the candidate CpG sites, and PTSD risk using a statistical mediation model. The different trauma types were hypothesized to confer differing conditional risk for prospective PTSD, mediated in part by potentially distinct methylation patterns of *NFATC1*.

2. Methods

2.1. Study participants

Samples included in this study were drawn from the Detroit Neighborhood Health Study (DNHS), a longitudinal population-based study composed of predominantly African American adult residents in Detroit, Michigan. The study was approved by the institutional review boards of the University of Michigan and the University of North Carolina at Chapel Hill. Details regarding the DNHS methodology have been described elsewhere (Goldmann et al., 2011; Uddin et al., 2010). Briefly, after written informed consent was obtained, the DNHS collected self-reported data on participant demographics (i.e., age, gender, and race), trauma exposures, and PTSD symptoms. Informed consent was again obtained for participants who elected to provide biospecimen.

The present investigation was limited to trauma-exposed individuals with available peripheral blood-derived genomic DNA (gDNA) samples from wave 1 (W1) or wave 2 (W2) of the DNHS. To ensure proper temporal sequencing for mediation, participants with gDNA samples from W1 were included only if they reported no trauma exposure in W2.

2.2. Measures

2.2.1. W3 lifetime PTSD—Participants were provided with a list of 19 traumatic events and asked to select the event(s) that they had experienced in their lifetime or since the last interview for post-baseline waves. From the experienced events, the respondent then nominated the "worst" event. In baseline waves, a random event was also selected for respondents who experienced multiple traumatic events. PTSD symptoms were assessed twice (once in reference to the nominated worst event and then for the randomly selected event) using the civilian version of the PTSD checklist (PCL-C) (Weathers et al., 1994) and supplemented additional questions regarding duration, timing, and impairment due to symptoms. The respondents were considered affected with lifetime PTSD in wave 3 (W3) if they met all *Diagnostic and Statistical Manual of Mental Disorders* (4th ed.; *DSM-IV*) (American Psychiatric Association, 1994) criteria in reference to either the worst or randomly chosen traumatic event in W1, W2, or in relation to a new worst trauma that may have occurred between W2 and W3. Missing data for W3 lifetime PTSD (n=173;

22% missing) were imputed using the *mice* package (Buuren & Groothuis-Oudshoorn, 2010) in R. Data inspection with density plots showed similar distribution between imputed and observed data, which indicates imputed data are indeed plausible values. The PTSD instrument described in this study was validated in the DNHS by blinded clinical interviews of a random subsample and yielded excellent internal consistency (Cronbach coefficient alpha = 0.93), a specificity of 0.97, sensitivity of 0.24, positive predicted value of 0.80, negative predictive value of 0.72, and an AUC (area under the ROC curve) of 0.76 (Uddin et al., 2011).

2.2.2.   W1/W2 5-mC—As previously mentioned, this study included participants who consented to provide a biospecimen, specifically, peripheral blood drawn via venipuncture by a phlebotomist. Detailed methods regarding sample processing in the DNHS have been described previously (Uddin et al., 2010; Weckle et al., 2015). Briefly, gDNA was isolated from peripheral blood using the Qiagen DNA Mini Kit (Qiagen, Hilden, Germany), bisulfite-converted using the Zymo EZ-96 DNA methylation kit (Zymo Research, Irvine, CA), then methylation tested using the Illumina Infinium Human Methylation 450K BeadChip platform (Illumina, San Diego, CA), as previously reported (Kim et al., 2019; Ward-Caviness et al., 2020). All steps were completed following the manufacturer's recommended protocol.

A battery of quality control (QC) steps was performed on the resulting 5-mC data. First, to account for potential sex bias associated with methylation data, samples were assessed for sex discordance using the *minfi* (Aryee et al., 2014) R package and removed if the participant's self-reported gender differed from the predicted sex. QC was next performed on the raw 5-mC beta values obtained via the Illumina GenomeStudio software and filtered for poorly performing samples, i.e., those with low mean signal intensity (<2000 arbitrary units or <50% of the overall median) (Barfield et al., 2012) or >10% missing value. Probes with detection p-value >0.01 were set to missing and those with >10% missing values were removed along with cross-reactive and polymorphic probes (Chen et al., 2013). To correct for the type II probe bias, 5-mC data was normalized using the beta-mixture quantile method (Morris et al., 2014; Teschendorff et al., 2013; Tian et al., 2017). Finally, *ComBat* adjustment was employed on logit-transformed beta-values (M-values), while controlling for trauma types, W3 lifetime PTSD, and sex, to account for potential batch effects via the empirical Bayes method (Johnson et al., 2007) used in the *sva* (Leek et al., 2012) R package. *ComBat* adjustment requires no missing data, thus, probes with missing values were imputed using the K-nearest neighbor algorithm (Peterson, 2009) then set to missing after *ComBat* adjustment. To ensure certain subjects were not overrepresented in the study, all participants contributed only one gDNA sample and had 5-mC measures at only one time-point (W1/W2).

2.2.3.   W1/W2 trauma types—The main exposure variable of interest was the W1/W2 trauma types. Study participants were asked about traumatic experiences in their lifetime (in baseline waves) and since their last interview (in post-baseline waves; W2) from a list of 19 traumatic events. These events were categorized into four traumatic event types: "assaultive violence", "other injury or shocking experience", "learning of trauma to a loved one", and

"sudden, unexpected death of a close friend or relative", as previously described (Breslau et al., 1998; Goldmann et al., 2011). An additional "other" category was included for respondents who reported experiencing "other extraordinarily stressful situation or event". Since W3 lifetime PTSD was assessed in reference to both the worst event nominated by the respondent and a randomly selected event, each trauma type variable reflects either the worst event nominated by the participant in W1/W2 or the baseline randomly selected event. Our study did not include traumatic events from W3 as these experiences would have occurred after W1/W2 methylation assessments and conflicted with the *a priori* hypothesized prospective relationship between each trauma type, 5-mC, and subsequent PTSD.

2.2.4.   Covariates—The present study included sex, age, ancestry, and cell proportions as covariates. Genomic ancestry estimates were derived from a principle component-based approach involving methylation probes near single-nucleotide polymorphisms (Barfield et al., 2014). Principal components two to four were included in this study as ancestry components to account for population stratification, as previously performed (Ratanatharathorn et al., 2017). Lastly, cell proportions were computed using the algorithm developed by Houseman (Houseman et al., 2012) implemented in the *EpiDISH* (Teschendorff et al., 2017; Teschendorff et al., 2020) R package. We included estimates of NK cells, monocytes, B cells, CD8+ T cells, and CD4+ T cells as covariates for cell proportions.

2.3.   Statistical analyses

Linear regression models were implemented using the *CpGassoc* (Barfield et al., 2012) R package to identify individual *NFATC1* CpG sites associated with W3 lifetime PTSD while controlling for other study covariates. Additionally, *post hoc* analysis was conducted using a Wilcoxon rank sum test to assess differences in methylation levels at candidate CpG sites between participants with and without W3 lifetime PTSD. Here, p-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg (Benjamini & Yekutieli, 2001) method (false discovery rate [FDR] < 0.05) and the CpG sites significantly associated with W3 lifetime PTSD were retained for downstream analyses. All analyses mentioned above were performed in R v3.6.0 (Team, 2013).

The association between trauma types, candidate *NFATC1* CpG sites, and W3 lifetime PTSD were assessed using path analysis implemented in a generalized structural equation model (GSEM). Because W3 lifetime PTSD is a binary outcome variable, the GSEM was carried out using the Bernoulli-logit link function in Stata v15.1 (StataCorp, 2017). GSEM was the method used to assess this relationship because it simultaneously tests the relationships among study variables, which reduces the chance of inflating the Type I error from multiple hypothesis testing (Li, 2011). Furthermore, to assess potential mediation effects, the average direct effect (DE), indirect effect (IE), and total effect (TE) along with mediated proportion and 95% confidence intervals (CI) were estimated with 1000 simulations for each trauma type and candidate *NFATC1* CpG sites, while adjusting for covariates, using the *medeff* command in the *mediation* (Hicks & Tingley, 2011) Stata package. The *mediation* package was used to assess these effects as it can appropriately

accommodate continuous mediator and dichotomous outcome variables (Hicks & Tingley, 2011). The associations and effects between trauma types, candidate *NFATC1* CpG sites, and W3 lifetime PTSD were considered statistically significant at a nominal *p*-value of 0.05 and when lower and upper 95% CIs did not overlap the null (i.e., zero) value.

Sensitivity analyses were conducted to assess the potential influence of chronicity effects, W3 trauma exposure, and CpG methylation outliers on the GSEM results. For the first GSEM, participants with a W2 gDNA sample and a reported experience of the same trauma type at W1 and W2 (chronicity effects) were dropped. Next, because W3 trauma could potentially inflate PTSD symptom levels reported at W3, another GSEM was conducted including W3 trauma as a predictor of W3 lifetime PTSD. Lastly, candidate *NFATC1* CpG sites were assessed for outliers using the Stata *extremes* command (StataCorp, 2017). An additional GSEM was conducted after applying 80% winsorization to CpG sites with outliers using the Stata *winsor2* command (StataCorp, 2017).

2.4.   Potential functional relevance of NFATC1 5-mC

To assess the potential functional relevance of the CpG sites that were identified as significant mediators between a trauma type and prospective risk for PTSD, the CpG sites were annotated to transcription factor binding sites (TFBS) and chromatin state signatures using the publicly available ENCODE 3 TFBS track (Moore et al., 2020) and Broad ChromHMM track (Ernst et al., 2011), respectively, from the hg19 UCSC Genome Browser (Navarro Gonzalez et al., 2021). The ENCODE 3 TFBS track was generated by the ENCODE Consortium and consists of an extensive set of human TFBS identified from ChIP-seq experiments. For the Broad Institute's ChromHMM track, a multivariate Hidden Markov Model was used to segment the genome into 15 chromatin states representing the combinatorial pattern of nine chromatin marks from ChIP-seq experiments—distinctions in the 15 defined chromatin states were also functionally validated (Ernst et al., 2011). In line with other genomic studies of whole blood (Curtis et al., 2020), we used the GM12878 B-lymphoblastoid cell line for all functional annotations as it is the most similar available cell type to whole blood. Lastly, to place our blood-based findings in the context of the brain, the tissue of interest for psychiatric disorders, we accessed publicly available data from the Human Protein Atlas (http://www.proteinatlas.org) and assessed the regional specificity and distribution of *NFATC1*'s expression levels in the brain (Uhlén et al., 2015). This database contains gene consensus normalized expression levels, summarized from the GTEx (Genotype-Tissue Expression) project and the FANTOM5 dataset, for 10 brain regions (i.e., cerebral cortex, olfactory region, hippocampal formation, amygdala, basal ganglia, thalamus, hypothalamus, midbrain, pons and medulla, and cerebellum) (Uhlén et al., 2015).

3.   Results

3.1.   Sample characteristics

Our study included 173 trauma-exposed participants with available gDNA samples from W1/W2 of the DNHS. Among these participants, 97 (56.1%) met the criteria for lifetime PTSD in W3. The demographic characteristics of the participants and the type of trauma exposures are described in Table 1. This study consisted of mostly female (*n*=108; 62.4%)

and Black (n=144; 83.2%) participants with a mean age of 53.7. The most frequently nominated "worst" or randomly selected trauma type was other injury or shocking experience (n=86; 49.7%). The least frequently nominated "worst" or randomly selected trauma type was other (n=40; 23.1%). Of the trauma types assessed, the participants exposed to the "other" trauma type (versus participants who did not) had the highest risk for W3 lifetime PTSD (n=28; 70.0%) followed by exposure to assaultive violence (n=45; 64.3%).

3.2. NFATC1 CpG sites associated with W3 lifetime PTSD

A total of 160 out of 166 methylation microarray probes associated with *NFATC1* survived QC assessment. The relationship between *NFATC1* 5-mC and W3 lifetime PTSD was assessed at the 160 CpG sites while controlling for study covariates. Among the 160 CpG sites, 26 were nominally associated with W3 lifetime PTSD. The top ten CpG sites are reported in Table 2, along with the annotations provided by Illumina, F-statistic, and the corresponding nominal and FDR-corrected p-value. After correction for multiple hypothesis testing, three CpG sites remained significantly associated with W3 lifetime PTSD: cg17057218 (F=16.27, p <0.001, FDR=0.014), cg22324981 (F=12.57, p <0.001, FDR=0.029), and cg04755409 (F=12.44, p <0.001, FDR=0.029). Genomic annotations showed cg17057218 was positioned within the gene body, 5' UTR, and CpG shelf, cg22324981 was positioned within the gene body and CpG shore, and cg04755409 was positioned within the gene body and CpG island. Further interrogations of cg17057218, cg22324981, and cg04755409 in *post hoc* analysis supported the finding of significant differences in 5-mC levels between participants with and without W3 lifetime PTSD (Figure 1). Specifically, those that met the criteria for lifetime PTSD in W3 had significantly lower methylation levels in W1/W2 at cg17057218 (W=4759, p=0.001, FDR=0.002) and cg04755409 (W=4782, p <0.001, FDR=0.002), but significantly higher methylation levels at cg22324981 (W=2921, p=0.019, FDR=0.019).

3.3. Association between trauma types, methylation at cg17057218, cg04755409, and cg22324981, and W3 lifetime PTSD

In path analysis using GSEM, we investigated the association between each of five trauma types, methylation at cg17057218, cg22324981, and cg04755409 and W3 lifetime PTSD. The GSEM along with unstandardized coefficients is shown in Figure 2. All five trauma types were significantly associated with cg17057218 methylation and were consistent in showing a negative direction of effect: assaultive violence (B=−0.0055, p <0.001, 95% CI=[−0.0088, −0.0022]), other injury or shocking experience (B=−0.0054, p <0.001, 95% CI=[−0.0085, −0.0022]), learning of trauma to a loved one (B=−0.0053, p <0.001, 95% CI=[−0.0084, −0.0022]), sudden, unexpected death of a close friend or relative (B=−0.0047, p=0.003, 95% CI=[−0.0078, −0.0016]), and other (B=−0.0049, p=0.004, 95% CI=[−0.0082, −0.0016]) were all associated with reduced methylation at cg17057218. In contrast, two trauma types were significantly associated with cg22324981 methylation and were consistent in showing a positive direction of effect: other injury or shocking experience (B=0.0214, p=0.002, 95% CI=[0.0079, 0.0348]), and other (B=0.0247, p <0.001, 95% CI=[0.0105, 0.0390]) were all associated with increased methylation at cg22324981. Only exposure to the "other" trauma type was significantly associated with methylation at cg04755409 (B=5.61E^{-4}, p=0.036, 95% CI=[−0.0011, −3.71E^{-5}]).

In terms of prospective risk for PTSD, we observed significant negative associations between cg17057218 methylation and W3 lifetime PTSD, and cg04755409 and W3 lifetime PTSD, but significant positive associations between cg22324981 and W3 lifetime PTSD. We also observed significant positive associations between assaultive violence and W3 lifetime PTSD (B=1.9764, p=0.003, 95% CI=[0.6552, 3.2975]); other injury or shocking experience and W3 lifetime PTSD (B=1.8101, p=0.005, 95% CI=[0.5413, 3.0789]); and learning of trauma and W3 lifetime PTSD (B=1.3738, p=0.033, 95% CI=[0.1115, 2.6361]). No significant associations were observed between sudden, unexpected death of a close friend or relative and W3 lifetime PTSD, or other and W3 lifetime PTSD in the described GSEM.

3.4. Mediational role of methylation at cg17057218, cg22324981, and cg04755409 in the relationship between trauma types and W3 lifetime PTSD

To evaluate the mediational role of cg17057218, cg22324981, and cg04755409 methylation as a potential link between different trauma types and differential risk for PTSD, we estimated the DE, IDE, and TE. Table 3 reports the effects for the trauma types that were significantly associated with methylation at cg17057218, cg22324981, and cg04755409 from the path analysis GSEM.

3.4.1. Mediation by cg17057218 methylation—A proportion of the TE for four of the five trauma types on W3 lifetime PTSD was significantly mediated by methylation at cg17057218. Specifically, methylation at cg17057218 significantly mediated 32% of the TE of other on W3 lifetime PTSD (mean$_{DE}$=0.1334, 95% CI=[−0.0438, 0.2884]; mean$_{IDE}$=0.0672, 95% CI=[0.0157, 0.1364]; mean$_{TE}$=0.2006, 95% CI=[0.0291, 0.3493], 27% of the TE of learning of trauma to a loved one on W3 lifetime PTSD (mean$_{DE}$=0.1731, 95% CI=[0.0121, 0.3053]; mean$_{IDE}$=0.0649, 95% CI=[0.0170, 0.1287]; mean$_{TE}$=0.2380, 95% CI=[0.0873, 0.3644]), 26% of the TE of other injury or shocking experience on W3 lifetime PTSD (mean$_{DE}$=0.2333, 95% CI=[0.0753, 0.3717]; mean$_{IDE}$=0.0799, 95% CI=[0.0256, 0.1505]; mean$_{TE}$=0.3131, 95% CI=[0.1667, 0.4360]), and 21% of the TE of assaultive violence on W3 lifetime PTSD (mean$_{DE}$=0.2460, 95% CI=[0.0892, 0.3773]; mean$_{IDE}$=0.0647, 95% CI=[0.0168, 0.1316]; mean$_{TE}$=0.3107, 95% CI=[0.1660, 0.4290]). Methylation at cg17057218 did not significantly mediate the TE of sudden, unexpected death of a close friend or relative on W3 lifetime PTSD.

3.4.2. Mediation by cg22324981 methylation—A proportion of the TE of two of the five trauma types on W3 lifetime PTSD was significantly mediated by methylation at cg22324981. Specifically, methylation at cg22324981 mediated 40% of the TE of other on W3 lifetime PTSD (mean$_{DE}$=0.1302, 95% CI= [−0.0424, 0.2835]; mean$_{IDE}$=0.0905, 95% CI=[0.0301, 0.1624]; mean$_{TE}$=0.2208, 95% CI=[0.0422,0.3657]) and 27% of the TE of other injury or shocking experience on W3 lifetime PTSD (mean$_{DE}$=0.2346, 95% CI=[0.0756, 0.3709]; mean$_{IDE}$=0.0876, 95% CI=[0.0303, 0.1559]; mean$_{TE}$=0.3221, 95% CI=[0.1640, 0.4568]). Methylation at cg22324981 did not significantly mediate the TE of assaultive violence, learning of trauma to a loved one, or sudden, unexpected death of a close friend or relative on W3 lifetime PTSD.

3.4.3.   Mediation by cg04755409 methylation—Methylation at cg04755409 did not significantly mediate the TE of any of the five trauma types on W3 lifetime PTSD.

3.4.4.   Sensitivity analyses—Several GSEMs were conducted to assess the influence of chronicity effects, W3 trauma exposure, and outliers on model results. The results for these sensitivity analyses are reported in Supplementary Table 1. In model 1, 44 participants with W2 gDNA sample and exposure to the same trauma type at W1 and W2 were dropped from the analysis. Exclusion of these participants did not substantially affect the GSEM results, although the DE of learning of trauma to a loved one on W3 lifetime PTSD failed to reach statistical significance. In model 2, we considered W3 trauma as an additional predictor of W3 lifetime PTSD. 91 participants reported trauma exposure at W3 and inclusion of this variable in the GSEM did not substantially affect the results. Lastly, to address the potential influence of one outlier identified for cg17057218 and nine outliers identified for cg04755409, model 3 was conducted after applying an 80% winsorization to both CpG sites. The association between the "other" trauma type and cg04755409 failed to reach statistical significance after winsorization, although the CpG site remained significantly associated with W3 lifetime PTSD. Winsorization did not substantially affect the results for cg17057218.

3.5.   Potential functional and cross-tissue relevance of mediating CpG sites

Using publicly available data, we attempted to assess the functional relevance of cg17057218 and cg22324981. The ENCODE 3 TFBS track (Moore et al., 2020) showed no regional overlaps between cg17057218 and TFBS, while cg22324981 was shown to overlap several TFBS (i.e., ASH2L, BCL11A, EBF1, EP300, PAX5, and RBBP5). The Broad ChromHMM track (Ernst et al., 2011) revealed that cg17057218 overlaps a weakly transcribed genomic region, whereas cg22324981 was shown to overlap a region denoting strong enhancer activity. Finally, we attempted to assess the cross-tissue relevance of *NFATC1* expression in the tissue of interest, the brain, using the Human Protein Atlas (http://www.proteinatlas.org) (Uhlén et al., 2015). The data showed *NFATC1* was expressed in all 10 available brain anatomical regions (i.e., cerebral cortex, olfactory region, hippocampal formation, amygdala, basal ganglia, thalamus, hypothalamus, midbrain, pons and medulla, and cerebellum) and showed no brain regional specificity.

4.   Discussion

Existing evidence suggests differing conditional risk for PTSD associated with the type of trauma exposure. The mechanisms underlying this relationship remain elusive and epigenetic modifications, particularly in stress-relevant pathways, may play a role. In this study, we investigated whether blood-derived 5-mC of *NFATC1,* a gene involved in the GRRN pathway, mediated the association between five different trauma types and prospective risk for lifetime PTSD. We observed that methylation at cg17057218 mediated a significant proportion of the TE on W3 lifetime PTSD for four of the five trauma types, while methylation at cg22324981 mediated a significant proportion of the TE for two of the five trauma types. Collectively, these results highlight a complex relationship between trauma types and 5-mC and suggest *NFATC1* 5-mC as a potential mechanism that partially

mediates the relationship, between some (assaultive violence, other injury or shocking experience, learning of trauma to a loved one, and other), but not all, trauma types and prospective risk for PTSD.

This study reports several notable findings. Importantly, methylation at cg17057218 partially mediated the relations between four of five trauma types (assaultive violence, other injury or shocking experience, learning of trauma to a loved one, and other) and W3 lifetime PTSD. This finding was intriguing considering that two of the four trauma types (assaultive violence and other injury or shocking experience) can be broadly classified as directly experienced traumas. Such directly experienced trauma types have been associated with a relatively higher risk of PTSD and symptom presentation in comparison to indirectly experienced traumas (May & Wisco, 2016). Nevertheless, learning of trauma to a loved one, which could be broadly classified as an indirectly experienced trauma, was also significantly mediated by methylation at cg17057218. This suggests that qualitatively different trauma types, possibly beyond the context of direct and indirect traumatic events, may impact methylation at this locus. Furthermore, a differing pattern was observed for methylation at cg22324981. This CpG site partially mediated the relations between two trauma types (other injury or shocking experience and other) and W3 lifetime PTSD. Because the "other" trauma type can encompass several different trauma types, it is not possible to classify these two trauma types into an informative group. However, it is noteworthy that assaultive violence was not significantly associated with or mediated by cg22324981 methylation. One possible explanation is that while both assaultive violence and other injury or shocking experience can be classified as directly experienced trauma types, these trauma types may also have important and possibly differing biological relevance. Indeed, assaultive violence includes military combat exposure, whereas other injury or shocking experience includes civilian-related trauma types (e.g., natural disasters), and both have been contrasted in other biological studies of PTSD (Breen et al., 2018; Huckins et al., 2020). In sum, these findings underscore the complex nature of trauma types and their potential molecular underpinnings.

Secondly, we observed a differing, but consistent, relationship between some of the trauma types and methylation patterns at cg17057218 and cg22324981. We observed that exposure to each of the five trauma types was associated with reduced methylation at cg17057218, which concurrently, was significantly associated with lifetime PTSD in W3. In contrast, two of the five trauma types were associated with increased methylation at cg22324981, which concurrently, was also significantly associated with W3 lifetime PTSD. Although not specific to *NFATC1*, previous studies of PTSD have reported both lower (Smith et al., 2020; Uddin et al., 2018) and higher (Kang et al., 2019) DNA methylation among persons with the disorder. Interestingly, the differing methylation patterns observed here between cg17057218 and cg22324981 seem consistent with their potential biological relevance. For instance, 5-mC is known to alter the binding of transcription factors to DNA (Héberlé & Bardet, 2019), and using publicly available data of TFBS characterized from a B-lymphoblastoid cell line, we observed an overlap between several transcription factors and cg22324981, whereas no overlaps were observed for cg17057218. Similarly, publicly available chromatin states data indicate that cg17057218 was positioned in a weakly transcribed genomic region, whereas cg22324981 was shown to be positioned within a region denoting strong enhancer activity. This suggests that regional methylation changes in PTSD are not unidirectional and

future work should take the genomic context of PTSD- and trauma- relevant CpG sites into consideration.

Nevertheless, methylation at both CpG sites showed a consistent direction of relationship (negative for cg17057218 and positive for cg22324981) with their associated trauma types, and lifetime PTSD in path analysis, suggesting they may play a role in modulating stress response and related psychopathology, including PTSD. Indeed, in a secondary analysis of DNHS gene expression data (Logue et al., 2015) from a subset of individuals analyzed in this work (n=81), we observed a positive correlation between 5-mC and gene expression among those with lifetime PTSD and a negative correlation among trauma-exposed controls. Although neither correlation achieved statistical significance, these opposite directions of effect suggest that *NFATC1* may be differentially regulated among those with vs. without lifetime PTSD, as has previously been observed for other loci (Uddin et al., 2018).

Overall, we observed that 21%–40% of the TEs for four of the five trauma types (assaultive violence, other injury or shocking experience, learning of trauma to a loved one, and other) was significantly mediated by *NFATC1* methylation. This observation coupled with recent evidence of altered gene expression of *NFATC1* following *in vitro* stimulation by the synthetic glucocorticoid, dexamethasone (Breen et al., 2019), and publicly available transcriptomics data (Uhlén et al., 2015) showing expression of *NFATC1* in the brain, further implicates this locus in stress-related psychopathology. These findings could also suggest that the response to physiological triggers (e.g., synthetic glucocorticoid) may similarly reflect exposure to contextually different trauma types, at least for *NFATC1*.

Although we found significant differences in methylation levels at another *NFATC1* CpG site, cg04755409, between participants with and without W3 lifetime PTSD, cg04755409 did not significantly mediate the TE of any of the five trauma types on PTSD. This CpG site was significantly associated with the "other" trauma type in GSEM, however, sensitivity analyses suggest this association may have been influenced by outliers. Notably, methylation at this CpG site had the strongest absolute effect on W3 lifetime PTSD in the path analysis GSEM and subsequent sensitivity analyses. Using publicly available data from IMAGE-CpG (Braun et al., 2019), a web-based search tool with correlational data between blood and brain 5-mC, we also observed a significant correlation between blood and brain methylation at cg04755409 (rho=0.76, p=0.016). Thus, it remains plausible that methylation at this CpG site may play a role in PTSD-related psychopathology, despite not having a consistent significant association with the trauma types assessed in this study.

Lastly, we found that exposure to other extraordinarily stressful situation or event conferred the highest risk for lifetime PTSD, followed by assaultive violence. Although the nature of the "other" trauma type is unknown, this finding of higher risk for PTSD relating to assaultive violence exposure is consistent with previous reports from the Detroit community (Breslau et al., 1998; Goldmann et al., 2011) and other studies of events relating to assaultive violence (Kessler et al., 2017; McLaughlin et al., 2013).

This study included some limitations. Due to overlaps in participant-reported trauma exposures in different waves of the DNHS, it is possible that our relatively small sample

size did not have sufficient statistical power to detect significant differences in methylation at cg17057218, cg22324981, or cg04755409 across the trauma types. Nevertheless, we were able to detect small, but significant, differences in methylation levels at cg22324981 and/or cg17057218 associated with each of the five trauma types. Second, our assessment of 5-mC at one time-point (W1/W2), combined with an assessment of lifetime PTSD rather than PTSD onset, leaves us unable to conclude whether the observed differences in 5-mC precede PTSD or trauma exposure; however, because we prospectively assessed PTSD at wave 3, we *can* say that the 5-mC differences associated with trauma exposures reported at W1/W2 are associated with subsequent psychopathology reported at W3, enhancing the causal framework of our analyses. Third, our study did not adjust for the number of lifetime trauma types, which has been previously identified as a significant predictor of PTSD and symptom presentation (Smith et al., 2016; Wani et al., 2021). Lastly, our study focused on trauma types for which PTSD was assessed, however, this does not exclude the possibility that participants can have PTSD from other trauma types that were not selected as the worst or random event in the DNHS.

Despite these limitations, our study is strengthened by its analytical methods, which consisted of identifying PTSD-relevant CpG sites, that were then interrogated using path analysis GSEM, followed by a mediational assessment method for binary outcomes (W3 lifetime PTSD) that enabled us to assess the DE, IDE, and TE for various trauma types. Whereas other methylation studies have primarily focused on early childhood traumas or individual traumatic events, we assessed the relative importance of different trauma types on *NFATC1* 5-mC and differential risk of prospective PTSD. Furthermore, we attempted to identify some preliminary and potential biological relevance of the mediating CpG sites as well as placing *NFATC1*'s expression in the context of brain tissue. In sum, our results indicate a relationship between some trauma types (assaultive violence, other injury or shocking experience, learning of trauma to a loved one, and other extraordinarily stressful situation or event), *NFATC1* 5-mC, and prospective risk of PTSD. Future studies are needed to assess and corroborate the importance of *NFATC1* 5-mC in the overall context of trauma exposure and PTSD psychopathology, as well as its relation to other genes, ideally in larger cohorts. In addition, given the bidirectional nature of regional *NFATC1* methylation changes in PTSD, our findings also echo the importance of developing strategies to alter methylation at specific CpG sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

American Psychiatric Association. (1994). Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). American Psychiatric Publishing, Incorporated.

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (DSM-5®). American Psychiatric Publishing, Incorporated.

Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, & Irizarry RA (2014). Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics, 30(10), 1363–1369. 10.1093/bioinformatics/btu049 [PubMed: 24478339]

Barfield RT, Almli LM, Kilaru V, Smith AK, Mercer KB, Duncan R, Klengel T, Mehta D, Binder EB, & Epstein MP (2014). Accounting for population stratification in DNA methylation studies. Genetic epidemiology, 38(3), 231–241. 10.1002/gepi.21789 [PubMed: 24478250]

Barfield RT, Kilaru V, Smith AK, & Conneely KN (2012). CpGassoc: an R function for analysis of DNA methylation microarray data. Bioinformatics, 28(9), 1280–1281. 10.1093/bioinformatics/bts124 [PubMed: 22451269]

Benjamini Y, & Yekutieli D (2001). The control of the false discovery rate in multiple testing under dependency. Annals of statistics, 1165–1188. 10.1214/aos/1013699998

Braun P, Han S, Nagahama Y, Gaul L, Heinzman J, Hing B, Grossbach A, Dlouhy B, Howard M, Kawasaki H, Potash J, & Shinozaki G (2019). 28 - IMAGE-CpG: DEVELOPMENT OF A WEB-BASED SEARCH TOOL FOR GENOME-WIDE DNA METHYLATION CORRELATION BETWEEN LIVE HUMAN BRAIN AND PERIPHERAL TISSUES WITHIN INDIVIDUALS. European Neuropsychopharmacology, 29, S796. 10.1016/j.euroneuro.2017.08.029

Breen MS, Bierer LM, Daskalakis NP, Bader HN, Makotkine I, Chattopadhyay M, Xu C, Buxbaum Grice A, Tocheva AS, Flory JD, Buxbaum JD, Meaney MJ, Brennand K, & Yehuda R (2019). Differential transcriptional response following glucocorticoid activation in cultured blood immune cells: a novel approach to PTSD biomarker development. Translational Psychiatry, 9(1), 201. 10.1038/s41398-019-0539-x [PubMed: 31434874]

Breen MS, Tylee DS, Maihofer AX, Neylan TC, Mehta D, Binder EB, Chandler SD, Hess JL, Kremen WS, Risbrough VB, Woelk CH, Baker DG, Nievergelt CM, Tsuang MT, Buxbaum JD, & Glatt SJ (2018). PTSD Blood Transcriptome Mega-Analysis: Shared Inflammatory Pathways across Biological Sex and Modes of Trauma. Neuropsychopharmacology, 43(3), 469–481. 10.1038/npp.2017.220 [PubMed: 28925389]

Breslau N, Kessler RC, Chilcoat HD, Schultz LR, Davis GC, & Andreski P (1998). Trauma and Posttraumatic Stress Disorder in the Community: The 1996 Detroit Area Survey of Trauma. Archives of General Psychiatry, 55(7), 626–632. 10.1001/archpsyc.55.7.626 [PubMed: 9672053]

Bustamante AC, Aiello AE, Galea S, Ratanatharathorn A, Noronha C, Wildman DE, & Uddin M (2016). Glucocorticoid receptor DNA methylation, childhood maltreatment and major depression. Journal of Affective Disorders, 206, 181–188. 10.1016/j.jad.2016.07.038 [PubMed: 27475889]

Buuren S. v., & Groothuis-Oudshoorn K (2010). mice: Multivariate imputation by chained equations in R. Journal of statistical software, 1–68. 10.18637/jss.v045.i03

Chen YA, Lemire M, Choufani S, Butcher DT, Grafodatskaya D, Zanke BW, Gallinger S, Hudson TJ, & Weksberg R (2013). Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics, 8(2), 203–209. 10.4161/epi.23470 [PubMed: 23314698]

Curtis SW, Gerkowicz SA, Cobb DO, Kilaru V, Terrell ML, Marder ME, Barr DB, Marsit CJ, Marcus M, Conneely KN, & Smith AK (2020). Sex-specific DNA methylation differences in people exposed to polybrominated biphenyl. Epigenomics, 12(9), 757–770. 10.2217/epi-2019-0179 [PubMed: 32496131]

Daskalakis NP, Xu C, Bader HN, Chatzinakos C, Weber P, Makotkine I, Lehrner A, Bierer LM, Binder EB, & Yehuda R (2021). Intergenerational trauma is associated with expression alterations in glucocorticoid- and immune-related genes. Neuropsychopharmacology, 46(4), 763–773. 10.1038/s41386-020-00900-8 [PubMed: 33173192]

Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, Epstein CB, Zhang X, Wang L, Issner R, & Coyne M (2011). Mapping and analysis of chromatin state dynamics in nine human cell types. Nature, 473(7345), 43–49. 10.1038/nature09906 [PubMed: 21441907]

Gibney ER, & Nolan CM (2010). Epigenetics and gene expression. Heredity, 105(1), 4–13. 10.1038/hdy.2010.54 [PubMed: 20461105]

Gill JM, Saligan L, Woods S, & Page G (2009). PTSD is associated with an excess of inflammatory immune activities. Perspectives in psychiatric care, 45(4), 262–277. 10.1111/j.1744-6163.2009.00229.x [PubMed: 19780999]

Goldmann E, Aiello A, Uddin M, Delva J, Koenen K, Gant LM, & Galea S (2011). Pervasive exposure to violence and posttraumatic stress disorder in a predominantly African American Urban Community: the Detroit Neighborhood Health Study. Journal of traumatic stress, 24(6), 747–751. 10.1002/jts.20705 [PubMed: 22144187]

Héberlé É, & Bardet AF (2019). Sensitivity of transcription factors to DNA methylation. Essays in biochemistry, 63(6), 727–741. 10.1042/EBC20190033 [PubMed: 31755929]

Hicks R, & Tingley D (2011). Causal mediation analysis. The Stata Journal, 11(4), 605–619. 10.1177/1536867X1201100407

Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, Wiencke JK, & Kelsey KT (2012). DNA methylation arrays as surrogate measures of cell mixture distribution. BMC bioinformatics, 13(1), 1–16. 10.1186/1471-2105-13-86 [PubMed: 22214541]

Huckins LM, Chatzinakos C, Breen MS, Hartmann J, Klengel T, da Silva Almeida AC, Dobbyn A, Girdhar K, Hoffman GE, & Klengel C (2020). Analysis of Genetically Regulated Gene Expression Identifies a Prefrontal PTSD Gene, SNRNP35, Specific to Military Cohorts. Cell Reports, 31(9), 107716. 10.1016/j.celrep.2020.107716 [PubMed: 32492425]

Irwin MR, & Cole SW (2011). Reciprocal regulation of the neural and innate immune systems. Nature reviews. Immunology, 11(9), 625–632. 10.1038/nri3042

Johnson WE, Li C, & Rabinovic A (2007). Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics, 8(1), 118–127. 10.1093/biostatistics/kxj037 [PubMed: 16632515]

Kang JI, Kim TY, Choi JH, So HS, & Kim SJ (2019). Allele-specific DNA methylation level of FKBP5 is associated with post-traumatic stress disorder. Psychoneuroendocrinology, 103, 1–7. 10.1016/j.psyneuen.2018.12.226 [PubMed: 30605803]

Kessler RC, Aguilar-Gaxiola S, Alonso J, Benjet C, Bromet EJ, Cardoso G, Degenhardt L, de Girolamo G, Dinolova RV, Ferry F, Florescu S, Gureje O, Haro JM, Huang Y, Karam EG, Kawakami N, Lee S, Lepine J-P, Levinson D, Navarro-Mateu F, Pennell B-E, Piazza M, Posada-Villa J, Scott KM, Stein DJ, Ten Have M, Torres Y, Viana MC, Petukhova MV, Sampson NA, Zaslavsky AM, & Koenen KC (2017). Trauma and PTSD in the WHO World Mental Health Surveys. European Journal of Psychotraumatology, 8(sup5). 10.1080/20008198.2017.1353383

Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, & Walters EE (2005). Lifetime Prevalence and Age-of-Onset Distributions of DSM-IV Disorders in the National Comorbidity Survey Replication. Archives of General Psychiatry, 62(6), 593–602. 10.1001/archpsyc.62.6.593 [PubMed: 15939837]

Kessler RC, Sonnega A, Bromet E, Hughes M, & Nelson CB (1995). Posttraumatic Stress Disorder in the National Comorbidity Survey. Archives of General Psychiatry, 52(12), 1048–1060. 10.1001/archpsyc.1995.03950240066012 [PubMed: 7492257]

Kim GS, Smith AK, Xue F, Michopoulos V, Lori A, Armstrong DL, Aiello AE, Koenen KC, Galea S, Wildman DE, & Uddin M (2019). Methylomic profiles reveal sex-specific differences in leukocyte composition associated with post-traumatic stress disorder. Brain, Behavior, and Immunity, 81, 280–291. 10.1016/j.bbi.2019.06.025

Kipanyula MJ, Kimaro WH, & Seke Etet PF (2016). The Emerging Roles of the Calcineurin-Nuclear Factor of Activated T-Lymphocytes Pathway in Nervous System Functions and Diseases. Journal of aging research, 2016, 5081021–5081021. 10.1155/2016/5081021 [PubMed: 27597899]

Klengel T, Mehta D, Anacker C, Rex-Haffner M, Pruessner JC, Pariante CM, Pace TW, Mercer KB, Mayberg HS, Bradley B, Nemeroff CB, Holsboer F, Heim CM, Ressler KJ, Rein T, & Binder EB

(2013). Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. Nat Neurosci, 16(1), 33–41. 10.1038/nn.3275 [PubMed: 23201972]

Koenen KC, Ratanatharathorn A, Ng L, McLaughlin KA, Bromet EJ, Stein DJ, Karam EG, Meron Ruscio A, Benjet C, Scott K, Atwoli L, Petukhova M, Lim CCW, Aguilar-Gaxiola S, Al-Hamzawi A, Alonso J, Bunting B, Ciutan M, de Girolamo G, Degenhardt L, Gureje O, Haro JM, Huang Y, Kawakami N, Lee S, Navarro-Mateu F, Pennell BE, Piazza M, Sampson N, ten Have M, Torres Y, Viana MC, Williams D, Xavier M, & Kessler RC (2017). Posttraumatic stress disorder in the World Mental Health Surveys. Psychological Medicine, 47(13), 2260–2274. 10.1017/S0033291717000708 [PubMed: 28385165]

Kuan P, Waszczuk M, Kotov R, Marsit C, Guffanti G, Gonzalez A, Yang X, Koenen K, Bromet E, & Luft B (2017). An epigenome-wide DNA methylation study of PTSD and depression in World Trade Center responders. Translational Psychiatry, 7(6), e1158–e1158. 10.1038/tp.2017.130 [PubMed: 28654093]

Labonte B, Azoulay N, Yerko V, Turecki G, & Brunet A (2014). Epigenetic modulation of glucocorticoid receptors in posttraumatic stress disorder. Translational Psychiatry, 4, e368. 10.1038/tp.2014.3 [PubMed: 24594779]

Leek JT, Johnson WE, Parker HS, Jaffe AE, & Storey JD (2012). The sva package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics, 28(6), 882–883. 10.1093/bioinformatics/bts034 [PubMed: 22257669]

Li SD (2011). Testing Mediation Using Multiple Regression and Structural Equation Modeling Analyses in Secondary Data. Evaluation Review, 35(3), 240–268. 10.1177/0193841×11412069 [PubMed: 21917711]

Logue MW, Miller MW, Wolf EJ, Huber BR, Morrison FG, Zhou Z, Zheng Y, Smith AK, Daskalakis NP, Ratanatharathorn A, Uddin M, Nievergelt CM, Ashley-Koch AE, Baker DG, Beckham JC, Garrett ME, Boks MP, Geuze E, Grant GA, Hauser MA, Kessler RC, Kimbrel NA, Maihofer AX, Marx CE, Qin XJ, Risbrough VB, Rutten BPF, Stein MB, Ursano RJ, Vermetten E, Vinkers CH, Ware EB, Stone A, Schichman SA, McGlinchey RE, Milberg WP, Hayes JP, & Verfaellie M (2020). An epigenome-wide association study of posttraumatic stress disorder in US veterans implicates several new DNA methylation loci. Clinical Epigenetics, 12(1), 46. 10.1186/s13148-020-0820-0 [PubMed: 32171335]

Logue MW, Smith AK, Baldwin C, Wolf EJ, Guffanti G, Ratanatharathorn A, Stone A, Schichman SA, Humphries D, Binder EB, Arloth J, Menke A, Uddin M, Wildman D, Galea S, Aiello AE, Koenen KC, & Miller MW (2015). An analysis of gene expression in PTSD implicates genes involved in the glucocorticoid receptor pathway and neural responses to stress. Psychoneuroendocrinology, 57, 1–13. 10.1016/j.psyneuen.2015.03.016 [PubMed: 25867994]

May CL, & Wisco BE (2016). Defining trauma: How level of exposure and proximity affect risk for posttraumatic stress disorder. Psychological Trauma: Theory, Research, Practice and Policy, 8(2), 233–240. 10.1037/tra0000077

McLaughlin KA, Koenen KC, Hill ED, Petukhova M, Sampson NA, Zaslavsky AM, & Kessler RC (2013). Trauma Exposure and Posttraumatic Stress Disorder in a National Sample of Adolescents. Journal of the American Academy of Child & Adolescent Psychiatry, 52(8), 815–830.e814. 10.1016/j.jaac.2013.05.011 [PubMed: 23880492]

Moore JE, Purcaro MJ, Pratt HE, Epstein CB, Shoresh N, Adrian J, Kawli T, Davis CA, Dobin A, Kaul R, Halow J, Van Nostrand EL, Freese P, Gorkin DU, Shen Y, He Y, Mackiewicz M, Pauli-Behn F, Williams BA, Mortazavi A, Keller CA, Zhang X-O, Elhajjajy SI, Huey J, Dickel DE, Snetkova V, Wei X, Wang X, Rivera-Mulia JC, Rozowsky J, Zhang J, Chhetri SB, Zhang J, Victorsen A, White KP, Visel A, Yeo GW, Burge CB, Lécuyer E, Gilbert DM, Dekker J, Rinn J, Mendenhall EM, Ecker JR, Kellis M, Klein RJ, Noble WS, Kundaje A, Guigó R, Farnham PJ, Cherry JM, Myers RM, Ren B, Graveley BR, Gerstein MB, Pennacchio LA, Snyder MP, Bernstein BE, Wold B, Hardison RC, Gingeras TR, Stamatoyannopoulos JA, Weng Z, & The EPC (2020). Expanded encyclopaedias of DNA elements in the human and mouse genomes. Nature, 583(7818), 699–710. 10.1038/s41586-020-2493-4 [PubMed: 32728249]

Morris TJ, Butcher LM, Feber A, Teschendorff AE, Chakravarthy AR, Wojdacz TK, & Beck S (2014). ChAMP: 450k chip analysis methylation pipeline. Bioinformatics, 30(3), 428–430. 10.1093/bioinformatics/btt684 [PubMed: 24336642]

Nantharat M, Wanitchanon T, Amesbutr M, Tammachote R, & Praphanphoj V (2015). Glucocorticoid receptor gene (NR3C1) promoter is hypermethylated in Thai females with major depressive disorder. Genetics and Molecular Research, 14(4), 19071.10.4238/2015 [PubMed: 26782558]

Navarro Gonzalez J, Zweig AS, Speir ML, Schmelter D, Rosenbloom KR, Raney BJ, Powell CC, Nassar LR, Maulding ND, Lee CM, Lee BT, Hinrichs AS, Fyfe AC, Fernandes JD, Diekhans M, Clawson H, Casper J, Benet-Pagès A, Barber GP, Haussler D, Kuhn RM, Haeussler M, & Kent WJ (2021). The UCSC Genome Browser database: 2021 update. Nucleic Acids Res, 49(D1), D1046–d1057. 10.1093/nar/gkaa1070 [PubMed: 33221922]

Peterson LE (2009). K-nearest neighbor. Scholarpedia, 4(2), 1883. 10.4249/scholarpedia.1883

Rao A, Luo C, & Hogan PG (1997). Transcription factors of the NFAT family: regulation and function. Annual review of immunology, 15(1), 707–747. 10.1146/annurev.immunol.15.1.707

Ratanatharathorn A, Boks MP, Maihofer AX, Aiello AE, Amstadter AB, Ashley-Koch AE, Baker DG, Beckham JC, Bromet E, Dennis M, Garrett ME, Geuze E, Guffanti G, Hauser MA, Kilaru V, Kimbrel NA, Koenen KC, Kuan P-F, Logue MW, Luft BJ, Miller MW, Mitchell C, Nugent NR, Ressler KJ, Rutten BPF, Stein MB, Vermetten E, Vinkers CH, Youssef NA, Workgroup VM-AM, Workgroup PPE, Uddin M, Nievergelt CM, & Smith AK (2017). Epigenome-wide association of PTSD from heterogeneous cohorts with a common multi-site analysis pipeline. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 174(6), 619–630. 10.1002/ajmg.b.32568

Schür RR, Boks MP, Rutten BPF, Daskalakis NP, de Nijs L, van Zuiden M, Kavelaars A, Heijnen CJ, Joëls M, Kahn RS, Geuze E, Vermetten E, & Vinkers CH (2017). Longitudinal changes in glucocorticoid receptor exon 1F methylation and psychopathology after military deployment. Translational Psychiatry, 7(7), e1181–e1181. 10.1038/tp.2017.150 [PubMed: 28742078]

Smith AK, Ratanatharathorn A, Maihofer AX, Naviaux RK, Aiello AE, Amstadter AB, Ashley-Koch AE, Baker DG, Beckham JC, & Boks MP (2020). Epigenome-wide meta-analysis of PTSD across 10 military and civilian cohorts identifies methylation changes in AHRR. Nature communications, 11(1), 1–9. 10.1038/s41467-020-19615-x

Smith HL, Summers BJ, Dillon KH, & Cougle JR (2016). Is worst-event trauma type related to PTSD symptom presentation and associated features? Journal of Anxiety Disorders, 38, 55–61. 10.1016/j.janxdis.2016.01.007 [PubMed: 26826984]

StataCorp. (2017). Stata Statistical Software: Release 15. In StataCorp LLC.

Team, R. C. (2013). R: A language and environment for statistical computing. In Vienna, Austria.

Teschendorff AE, Breeze CE, Zheng SC, & Beck S (2017). A comparison of reference-based algorithms for correcting cell-type heterogeneity in Epigenome-Wide Association Studies. BMC bioinformatics, 18(1), 1–14. 10.1186/s12859-017-1511-5 [PubMed: 28049414]

Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, & Beck S (2013). A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics, 29(2), 189–196. 10.1093/bioinformatics/bts680 [PubMed: 23175756]

Teschendorff AE, Shijie C, Zheng MSC, Imports, M., biocViews DNAMethylation, M., & DifferentialMethylation, I. (2020). Package 'EpiDISH'.

Tian Y, Morris TJ, Webster AP, Yang Z, Beck S, Feber A, & Teschendorff AE (2017). ChAMP: updated methylation analysis pipeline for Illumina BeadChips. Bioinformatics, 33(24), 3982–3984. 10.1093/bioinformatics/btx513 [PubMed: 28961746]

Uddin M, Aiello AE, Wildman DE, Koenen KC, Pawelec G, de Los Santos R, Goldmann E, & Galea S (2010). Epigenetic and immune function profiles associated with posttraumatic stress disorder. Proceedings of the National Academy of Sciences of the United States of America, 107(20), 9470–9475. 10.1073/pnas.0910794107 [PubMed: 20439746]

Uddin M, Galea S, Chang S-C, Aiello AE, Wildman DE, de los Santos R, & Koenen KC (2011). Gene Expression and Methylation Signatures of MAN2C1 are Associated with PTSD. Disease Markers, 30, 513659. 10.3233/DMA-2011-0750

Uddin M, Ratanatharathorn A, Armstrong D, Kuan P-F, Aiello AE, Bromet EJ, Galea S, Koenen KC, Luft B, Ressler KJ, Wildman DE, Nievergelt CM, & Smith A (2018). Epigenetic meta-analysis across three civilian cohorts identifies NRG1 and HGS as blood-based biomarkers for post-

traumatic stress disorder. Epigenomics, 10(12), 1585–1601. 10.2217/epi-2018-0049 [PubMed: 30456986]

Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson Å, Kampf C, Sjöstedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigyarto CA-K, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist P-H, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, & Pontén F (2015). Tissue-based map of the human proteome. Science, 347(6220), 1260419. 10.1126/science.1260419 [PubMed: 25613900]

Vukojevic V, Kolassa I-T, Fastenrath M, Gschwind L, Spalek K, Milnik A, Heck A, Vogler C, Wilker S, & Demougin P (2014). Epigenetic modification of the glucocorticoid receptor gene is linked to traumatic memory and post-traumatic stress disorder risk in genocide survivors. Journal of Neuroscience, 34(31), 10274–10284. 10.1523/JNEUROSCI.1526-14.2014 [PubMed: 25080589]

Wani AH, Aiello AE, Kim GS, Xue F, Martin CL, Ratanatharathorn A, Qu A, Koenen K, Galea S, Wildman DE, & Uddin M (2021). The impact of psychopathology, social adversity and stress-relevant DNA methylation on prospective risk for post-traumatic stress: A machine learning approach. Journal of Affective Disorders, 282, 894–905. 10.1016/j.jad.2020.12.076 [PubMed: 33601733]

Ward-Caviness CK, Pu S, Martin CL, Galea S, Uddin M, Wildman DE, Koenen K, & Aiello AE (2020). Epigenetic predictors of all-cause mortality are associated with objective measures of neighborhood disadvantage in an urban population. Clinical Epigenetics, 12(1), 44. 10.1186/s13148-020-00830-8 [PubMed: 32160902]

Weathers FW, Litz BT, Herman D, Huska J, & Keane T (1994). The PTSD checklistcivilian version (PCL-C). Boston, MA: National Center for PTSD, 10.

Weckle A, Aiello AE, Uddin M, Galea S, Coulborn RM, Soliven R, Meier H, & Wildman DE (2015). Rapid Fractionation and Isolation of Whole Blood Components in Samples Obtained from a Community-based Setting. Journal of Visualized Experiments(105). 10.3791/52227

Whitnall MH (1993). Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. Progress in Neurobiology, 40(5), 573–629. 10.1016/0301-0082(93)90035-Q [PubMed: 8484004]

Yehuda R, Daskalakis NP, Lehrner A, Desarnaud F, Bader HN, Makotkine I, Flory JD, Bierer LM, & Meaney MJ (2014). Influences of maternal and paternal PTSD on epigenetic regulation of the glucocorticoid receptor gene in Holocaust survivor offspring. American Journal of Psychiatry, 171(8), 872–880. 10.1176/appi.ajp.2014.13121571

Yehuda R, Flory JD, Bierer LM, Henn-Haase C, Lehrner A, Desarnaud F, Makotkine I, Daskalakis NP, Marmar CR, & Meaney MJ (2015). Lower methylation of glucocorticoid receptor gene promoter 1F in peripheral blood of veterans with posttraumatic stress disorder. Biological Psychiatry, 77(4), 356–364. 10.1016/j.biopsych.2014.02.006 [PubMed: 24661442]

Yehuda R, Halligan SL, Golier JA, Grossman R, & Bierer LM (2004). Effects of trauma exposure on the cortisol response to dexamethasone administration in PTSD and major depressive disorder. Psychoneuroendocrinology, 29(3), 389–404. 10.1016/s0306-4530(03)00052-0 [PubMed: 14644068]

HIGHLIGHTS

- Exposure to different trauma types is associated with differences in conditional risk for post-traumatic stress disorder (PTSD).

- DNA methylation at three Nuclear Factor of Activated T Cells 1 (*NFATC1*) CpG sites were significantly associated with PTSD.

- DNA methylation at *NFATC1* CpG sites, cg17057218 and cg22324981, mediated 21–40% of the total effect for four of the five trauma types (assaultive violence, other injury or shocking experience, learning of trauma to a loved one, and other) on PTSD.

- Potential functional relevance: cg17057218 overlaps a weakly transcribed genomic region, while cg22324981 overlaps several transcription factor binding sites and a region denoting strong enhancer activity, suggesting that PTSD- and trauma-relevant methylation differences may be genomic context-specific.

Figure 1.
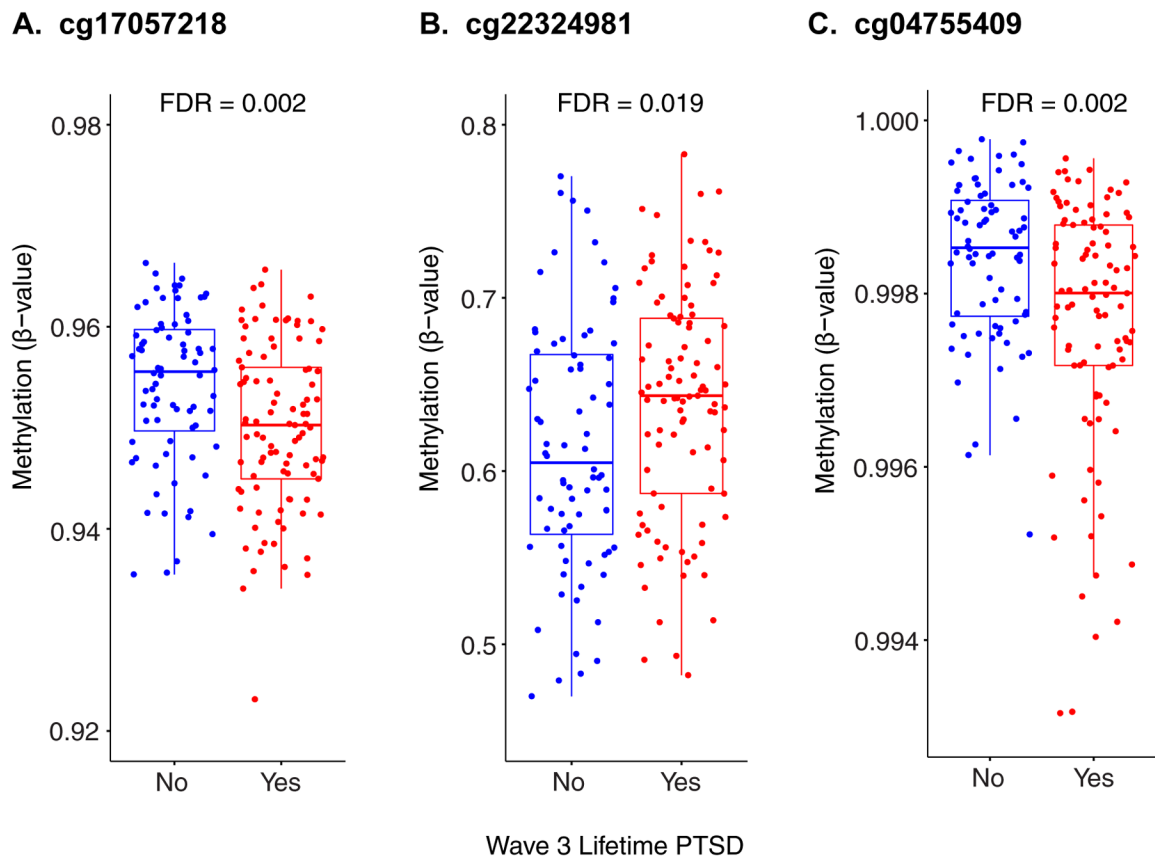Methylation at cg17057218, cg22324981, and cg04755409 between participants with and without W3 lifetime PTSD. Participants with W3 lifetime PTSD had (A) lower W1/W2 methylation at cg17057218 (W=4759, p=0.001, FDR=0.002); (B) higher methylation at cg22324981 (W=2921, p=0.019, FDR=0.019); and (C) lower methylation at cg04755409 (W=4782, p <0.001, FDR=0.002) based on Wilcoxon rank sum test.

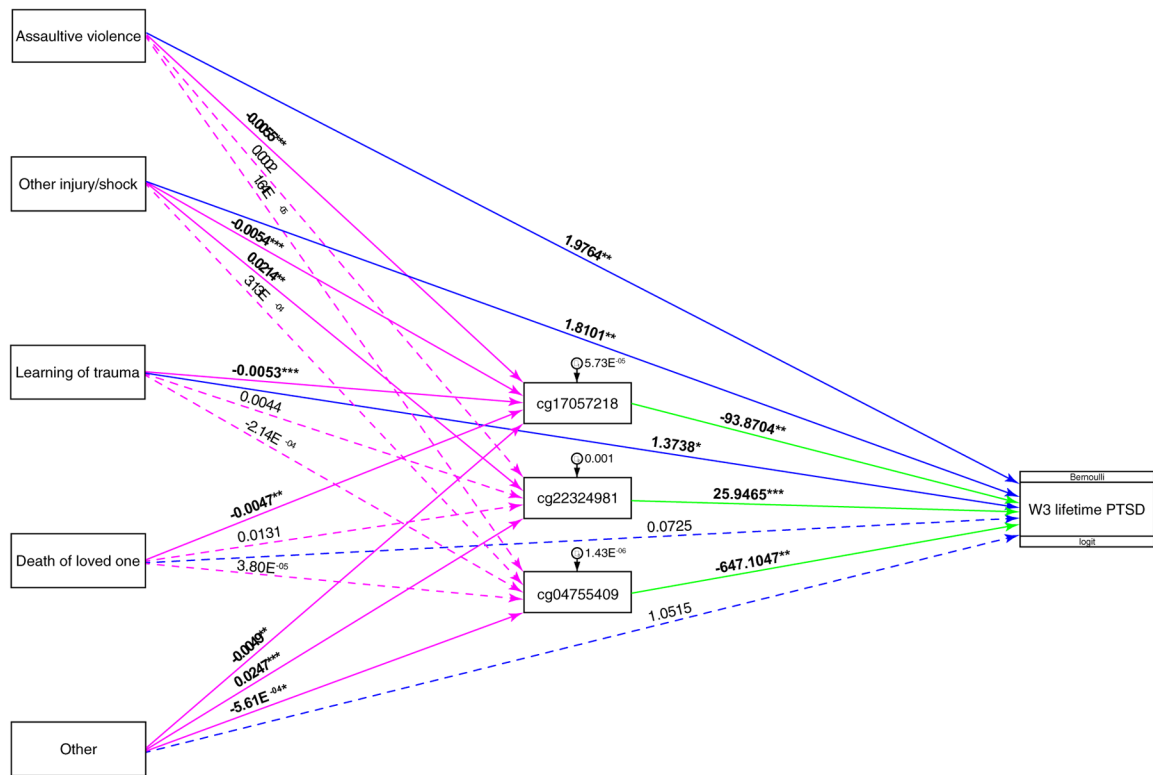**Figure 2.**
Path analysis using generalized structural equation modeling. Shown are unstandardized coefficients of exposures to mediators (magenta), mediators to outcome (green), and exposures to outcome (blue). Trauma types are abbreviated, and model is adjusted for sex, age, ancestry, and cell proportions (paths are not shown). Non-significant paths are dashed; *p <0.05; **p <0.01; ***p <0.001.

Table 1.

Sample characteristics, n=173[§]

	All participants	W3 lifetime PTSD	
		No	**Yes**
Demographics			
Age	53.7±13.7	55.0±15.1	52.7±12.6
Sex			
Female	108 (62.4%)	43 (39.8%)	65 (60.2%)
Male	65 (37.6%)	33 (50.7%)	32 (49.2%)
Race			
Black	144 (83.2%)	63 (43.8%)	81 (56.3%)
White	24 (13.9%)	10 (41.7%)	14 (58.3%)
Other	5 (2.9%)	3 (60.0%)	2 (40.0%)
W1/W2 trauma types			
Assaultive violence			
No	103 (59.5%)	51 (49.5%)	52 (50.5%)
Yes	70 (40.5%)	25 (35.7%)	45 (64.3%)
Other injury or shocking experience			
No	87 (50.3%)	44 (50.6%)	43 (49.4%)
Yes	86 (49.7%)	32 (37.2%)	54 (62.8%)
Learning about traumas to a loved one			
No	109 (63.0%)	51 (46.8%)	58 (53.2%)
Yes	64 (37.0%)	25 (39.1%)	39 (60.9%)
Sudden, unexpected death of a close friend or relative			
No	90 (52.0%)	32 (35.6%)	58 (64.4%)
Yes	83 (48.0%)	44 (53.0%)	39 (47.0%)
Other			
No	133 (76.9%)	64 (48.1%)	69 (51.9%)
Yes	40 (23.1%)	12 (30.0%)	28 (70.0%)

[§]All statistics are presented in n (percentage) and mean±standard deviation for age.

Table 2.

Characteristics of the top 10 *NFATC1* CpG sites associated with W3 lifetime PTSD[§]

Illumina ID	Genomic coordinates	Genomic annotation	CpG annotationn	F-statistic	*p*-value	FDR
cg17057218	Chr18:77,183,423	Body;5'UTR	Shelf	16.27	$8.47E^{-05}$	**0.014**
cg22324981	Chr18:77,283,493	Body	Shore	12.57	$5.15E^{-04}$	**0.029**
cg04755409	Chr18:77,233,327	Body	Island	12.44	$5.48E^{-04}$	**0.029**
cg05718035	Chr18:77,240,530	Body	Island	9.08	$3.00E^{-03}$	0.116
cg15784814	Chr18:77,171,832	Body;5'UTR	Island	8.33	$4.43E^{-03}$	0.116
cg07952877	Chr18:77,171,705	Body;5'UTR	Island	8.29	$4.54E^{-03}$	0.116
cg00431336	Chr18:77,223,422	Body	Shore	8.07	$5.08E^{-03}$	0.116
cg26550337	Chr18:77,203,542	Body;5'UTR	Island	6.46	$1.20E^{-02}$	0.240
cg05753993	Chr18:77,199,432	Body	Shore	6.01	$1.53E^{-02}$	0.246
cg05165553	Chr18:77,171,010	Body;5'UTR	Island	6.00	$1.54E^{-02}$	0.246

[§]Genomic annotation and CpG annotation (relation to UCSC CpG island) were determined from the annotation provided for each probe by Illumina. Coordinates and annotations are provided based on GRCh37/hg19 genome assembly.

Table 3.

Effects of W1/W2 trauma types on W3 lifetime PTSD, as mediated by methylation at cg17057218, cg22324981, or cg04755409

	Direct effect	Indirect effect	Total effect	Proportion mediated
	Mean [95% CI]	Mean [95% CI]	Mean [95% CI]	Mean [95% CI]
	cg17057218			
Assaultive violence	**0.2460** [0.0892, 0.3773]	**0.0647** [0.0168, 0.1316]	**0.3107** [0.1660, 0.4290]	**0.2060** [0.1509, 0.3899]
Other injury/shock	**0.2333** [0.0753, 0.3717]	**0.0799** [0.0256, 0.1505]	**0.3131** [0.1667, 0.4360]	**0.2525** [0.1832, 0.4791]
Learning of trauma	**0.1731** [0.0121, 0.3053]	**0.0649** [0.0170, 0.1287]	**0.2380** [0.0873, 0.3644]	**0.2669** [0.1767, 0.7253]
Death of loved one	0.0029 [−0.1590, 0.1518]	**0.0665** [0.0171, 0.1297]	0.0694 [−0.0852, 0.2076]	0.6121 [−7.3505, 8.7927]
Other	0.1334 [−0.0438, 0.2884]	**0.0672** [0.0157, 0.1364]	**0.2106** [0.0291, 0.3493]	**0.3224** [0.1833, 1.5197]
	cg22324981			
Other injury/shock	**0.2346** [0.0756, 0.3709]	**0.0876** [0.0303, 0.1559]	**0.3221** [0.1640, 0.4568]	**0.2689** [0.1917, 0.5339]
Other	0.1302 [−0.0424, 0.2835]	**0.0905** [0.0301, 0.1624]	**0.2208** [0.0422, 0.3657]	**0.4010** [0.2417, 1.4903]
	cg04755409			
Other	0.1323 [−0.0428, 0.2826]	0.0300 [−0.0179, 0.0931]	0.1622 [−0.0148, 0.3167]	0.1699 [22120.7443, 1.1455]

Note: Trauma types are abbreviated. Significant effects (when lower and upper CIs do not overlap zero) are bolded.