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Longitudinal epigenetic variation of DNA methyltransferase genes associated with vulnerability to post-traumatic stress disorder (PTSD)

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Abstract

BACKGROUND—Epigenetic differences exist between trauma-exposed individuals with and without posttraumatic stress disorder (PTSD). It is unclear whether these epigenetic differences preexist, or arise following, trauma and PTSD onset.

METHODS—In pre- and post-trauma samples from a subset of Detroit Neighborhood Health Study participants, DNA methylation (DNAm) was measured at *DNMT1*, *DNMT3A*, *DNMT3B*, and *DNMT3L*. Pre-trauma DNAm differences and changes in DNAm from pre- to post-trauma were assessed between and within PTSD cases (n=30) and age-, gender-, and trauma exposure-matched controls (n=30). Pre-trauma DNAm was tested for association with post-trauma symptom severity (PTSS) change. Potential functional consequences of DNAm differences were explored via bioinformatic search for putative transcription factor binding sites (TFBS).

RESULTS—*DNMT1* DNAm increased following trauma in PTSD cases (p=0.001), but not controls (p=0.067). *DNMT3A* and *DNMT3B* DNAm increased following trauma in both cases (DNMT3A: p=0.009; DNMT3B: p<0.001) and controls (DNMT3A: p=0.002; DNMT3B:

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Conflict of interest

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p<0.001). In cases only, pre-trauma DNAm was lower at a *DNMT3B* CpG site that overlaps with a TFBS involved in epigenetic regulation (p=0.001); lower pre-trauma *DNMT3B* DNAm at this site was predictive of worsening of PTSS post-trauma (p=0.034). Some effects were attenuated following correction for multiple hypothesis testing.

CONCLUSIONS—DNAm among trauma-exposed individuals shows both longitudinal changes and preexisting epigenetic states that differentiate individuals who are resilient vs. susceptible to PTSD. These distinctive DNAm differences within *DNMT* loci may contribute to genome-wide epigenetic profiles of PTSD.

Keywords

posttraumatic stress disorder; epigenetics; DNA methylation; susceptibility; longitudinal; epidemiology

Introduction

PTSD is a prevalent and debilitating mental health disorder that may arise following exposure to a potentially traumatic event (Association, 2013). While the lifetime prevalence of traumatic exposure is 50–90%(Kessler *et al.*, 1995), PTSD in the general U.S. population is estimated to be only 6.8%(Kessler and Wang, 2008). Although the majority of persons exposed to trauma display resiliency (Kessler *et al.*, 1995; Breslau *et al.*, 1998; Acierno *et al.*, 2007; Kessler and Wang, 2008), the molecular underpinnings of risk remain poorly characterized. The identification of risk markers, and particularly biomarkers, that distinguish between persons at high and low risk of developing PTSD following trauma exposure has been identified as a priority research goal by the Institute of Medicine (Medicine, 2012), Department of Defense(Congressionally Directed Medical Research Programs, 2011), and the National Institute of Mental Health (NIMH, 2008). Ideally, the ability to identify persons at high risk of developing PTSD would enable providers to target evidence-based interventions to high-risk groups(Andrews and Neises, 2012). The identification of robust predictive biomarkers may also improve our understanding of the pathophysiology of PTSD and lead to more effective pharmacological interventions.

Although much work has been done to identify social and environmental factors that contribute to PTSD risk [e.g. (Kulka *et al.*, 1990; Breslau *et al.*, 1991; Brewin *et al.*, 2000; Koenen *et al.*, 2003; Breslau *et al.*, 2004; DiGrande *et al.*, 2008; Galea *et al.*, 2008; Kun *et al.*, 2009)], the biological undergirding of differential PTSD risk and resiliency remains to be more fully elucidated. Twin studies have demonstrated heritability and genetic contribution to PTSD risk(True *et al.*, 1993; Koenen *et al.*, 2002; Stein *et al.*, 2002) and targeted gene and GWAS approaches have identified both genetic risk loci(Lu *et al.*, 2008; Ressler *et al.*, 2011; Chang *et al.*, 2012; Logue *et al.*, 2013) and important gene-by-environment interactions (Binder *et al.*, 2008; Xie *et al.*, 2010; Uddin *et al.*, 2013) that contribute to risk for the disorder; nevertheless, a substantial proportion of biologically mediated variance in PTSD risk has yet to be explained.

Epigenetic variability is considered a plausible and increasingly empirically supported contributor to the etiology of phenotypes with marked genetic and environmental

influences(Meaney, 2010), including certain psychopathologies(Toyokawa *et al.*, 2012). Indeed, recent advances have revealed that PTSD risk and resiliency is associated with differential epigenetic variation(El-Sayed *et al.*, 2012). Epigenetic mechanisms – including histone modifications, non-protein coding RNAs, and, most notably, DNA methylation (DNAm) – affect gene expression and cellular phenotype without altering the underlying DNA sequence(Feinberg, 2008; Meaney, 2010). DNAm is stably heritable across mitotic replications, but is modifiable throughout the life course in response to lived experiences and environmental exposures(Bird, 2002). In primordial mammalian germ cells, global DNAm is removed (with the exception of imprinted loci)(Reik *et al.*, 2001), with new patterns established by de novo methyltransferases DNMT3A, DNMT3B, and DNMT3L following fertilization(Bourc'his *et al.*, 2001; Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004; Kato *et al.*, 2007; Ooi *et al.*, 2007). These reprogrammed DNAm patterns are largely maintained throughout mitotic DNA replication by the action of the maintenance methyltransferase, DNMT1(Li *et al.*, 1992; Seisenberger *et al.*, 2013).

Although influenced by other variables, global DNAm patterns are largely established and maintained by the activity of the DNA methyltransferases, DNMT1, DNMT3A, DNMT3B, and DNMT3L(Feng and Fan, 2009). Gene expression evidence suggests that these DNMTs may be active throughout the life course(Robertson *et al.*, 1999; Feng *et al.*, 2005; Siegmund *et al.*, 2007), including in brain tissue(Goto *et al.*, 1994; Veldic *et al.*, 2004; Feng *et al.*, 2005) and in association with mental disorders(Veldic *et al.*, 2004; Veldic *et al.*, 2005). In addition, protein-level expression of DNMT1(Inano *et al.*, 2000; Veldic *et al.*, 2005) and DNMT3A(Feng *et al.*, 2005) has been demonstrated in the mouse and human brain. With respect to PTSD, recent work confirms that DNMT activity plays a role in mediating risk for PTSD-related phenotypes, including fear conditioning and memory consolidation (Miller and Sweatt, 2007; Feng *et al.*, 2010). Together, these findings suggest that DNAm and DNA methyltransferases represent promising targets for the identification of epigenetic underpinnings of differential PTSD risk and resiliency.

Studies of epigenetic variation have provided important insights into PTSD risk, but have been largely limited by cross-sectional analyses of post-trauma samples. Most notably, epidemiological cohorts from Detroit(Uddin *et al.*, 2010) and Atlanta(Smith *et al.*, 2011) have been the basis of research that has demonstrated cross-sectional differential DNAm that distinguishes between trauma-exposed individuals with vs. without PTSD. *DNMT3B* and *DNMT3L* were among the differentially methylated loci identified in the Detroit study(Uddin *et al.*, 2010). More recently, longitudinal DNAm data among PTSD cases and controls have been reported, including studies using samples from a cohort of U.S. military personnel deployed to Iraq and Afghanistan(Rusiecki *et al.*, 2012; Rusiecki *et al.*, 2013). To further elucidate whether differential DNAm between trauma exposed controls and PTSD cases represent pre-existing susceptibility/resiliency factors or downstream biomarkers of PTSD, additional longitudinal analyses are required. Finally, while the identification of epigenetic variation associated with mental health outcomes is important, work must begin to test the putative functionality of mental health-associated differential DNAm. For example, the identification of transcription factor binding sites (TFBS) that overlap with

differentially methylated CpG sites and to which transcription factor binding may be disrupted offer one possibility of supporting DNAm functionality(Weaver *et al.*, 2004).

Here, we analyze DNAm from individuals pre- and post-trauma to identify differences that characterize individuals who are susceptible vs. resilient to PTSD following trauma. To assess potential functional consequences of examined DNAm differences, we then perform a bioinformatic search for the presence of putative transcription factor binding sites(Weaver *et al.*, 2004). Results from this work suggest that PTSD-relevant DNAm differences in DNMT loci may exist both prior to and following trauma, with implications for future targeted interventions.

Methods and Materials

Subjects

Samples are from a subset of participants from the Detroit Neighborhood Health Study (DNHS), a longitudinal, community-representative cohort of adult residents in Detroit, MI. The current study draws on peripheral blood samples and survey data obtained at two time points were from 60 DNHS participants. Forty-six were female and fourteen male; forty-six were African-American and 12 were Caucasian, and 2 were Hispanic. The average age was 55.1 years. PTSD diagnosis was assessed via structured interview administered via telephone(Breslau *et al.*, 1998). PTSD symptoms were assessed in reference to both the traumatic event the participant regarded as their worst and one randomly selected traumatic event from the remaining traumas the participant experienced. Lifetime PTSD cases met all six DSM-IV criteria in reference to either the worst or random traumatic event. The diagnostic interview showed good validity against the Clinician Administered PTSD Scale (Blake *et al.*, 1995) as described elsewhere(Uddin *et al.*, 2010). The Institutional Review Board of the University of Michigan reviewed and approved the study protocol. Incident cases (n=30) of PTSD were identified in either waves 2, 3, or 4 of DNHS data collection among individuals for whom blood samples were available at both the wave of first PTSD diagnosis and the immediately previous, pre-incident trauma wave. Non-PTSD controls (n=30) were matched to cases on the basis of age, sex, and number of traumatic event types. DNA samples were isolated from both pre- and post-trauma time points for both cases and controls. The time between pre- and post-trauma time points was approximately 1 year. Cases and controls had no history of PTSD prior to the post-trauma wave.

Methylation quantification by targeted bisulfite pyrosequencing

DNA Isolation—DNA was isolated from whole blood acquired via venipuncture when available from DNHS participants selected for inclusion in this study. Blood spots were used as an alternate source of whole blood-derived DNA when venipuncture samples were unavailable. The exact tissue type was shared between matched case-control pairs in all instances. Venipuncture- and bloodspot-derived whole blood represent the same tissue and therefore should not differ with respect to DNAm, as confirmed by numerous studies to date (Wong *et al.*, 2008; Aberg *et al.*, 2013; Hollegaard *et al.*, 2013).

Whole blood: DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) and the QuickGene DNA Whole blood Kit S (Lifesciences, FujiFilm, Tokyo, Japan) using manufacturers' recommended protocols.

Blood spots: DNA was isolated using the QIAamp DNA Micro Kit (Qiagen) using the manufacturer's recommended protocol. For each sample, one 6mm punch was taken from dried blood spots using a disposable, sterile biopsy punch (Miltex, York, PA) within a sterile field and placed immediately into a sterile 1.7ml microcentrifuge tube. New gloves, biopsy punches, and sterile fields were utilized for each sample. Negative controls in the form of blank extractions were included with all DNA isolations.

Bisulfite conversion—For each sample, ~750ng of DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen) using the manufacturer's recommended protocol. Negative controls in the form of bisulfite conversion of water were included with each bisulfite conversion.

Pyrosequencing—Assays to assess the methylation levels of CpG sites found in the *DNMT1*, *DNMT3A*, and *DNMT3L* and *DNMT3B* (see below for assay-specific details) were custom designed using the Pyromark Q24 Assay Design Software 2.0 (Qiagen). Targeted CpG sites were selected based on prior evidence(Uddin *et al*., 2010) of involvement in epigenetic regulation of PTSD risk (*DNMT3B, DNMT3L*) and to investigate whether longitudinal, PTSD-associated DNAm differences exist across DNA methyltransferase genes more broadly (*DNMT1*, *DNMT3A*, *DNMT3B,* and *DNMT3L*). Because the *DNMT3B* target CpG is located in a CpG island, our designed assay captures DNAm at 12 CpG sites in an approximately 70 base pair region of exon 1 (see *DNMT3B* assay section below for details). Single CpG sites were assessed at *DNMT1*, *DNMT3A*, and *DNMT3L* loci (see individual assay section below for details); these CpG sites did not fall into CpG islands. *DNMT1*, *DNMT3A*, and *DNMT3L* CpG sites and 2 *DNMT3B* CpG sites assessed are also found on the HM27 and HM450K methylation bead chips from Illumina (see below for actual HG19 nucleotide location). The capacity for each assay to capture DNAm levels ranging from 0–100% was validated using commercially available demethylated and highly methylated DNA at dilutions of 1:0 (unmethylated), 3:1, 1:1, 1:3, and 0:1 (highly methylated). PCR amplification of target sequences was performed on 20ng of bisulfite-converted DNA samples using the PyroMark PCR kit (Qiagen). Bisulfite-converted, PCR-amplified DNA was pyrosequenced on the Pyromark Q24 Pyrosequencer (Qiagen) using the manufacturer's recommended protocol and default settings. All methylation analyses were conducted in triplicate with appropriate negative controls included at each of the following steps: DNA isolation, bisulfite conversion, PCR amplification, and pyrosequencing reaction.

Details of each custom assay are listed below.

DNMT1: PCR forward primer: TTTTTTTAGGTGTGATGGGGATAAAG

PCR reverse primer (biotinylated): CAAAAACTCTCACAAACCCTTAAA

PCR program (50 cycles):

Initial	15 minutes at 95°C
Denaturation	30 seconds at 94°C
Annealing	30 seconds at 58°C
Extension	30 seconds at 72°C
Final	10 minutes at 72°C
Hold	4°C

Sequencing primer: GTGATGGGGATAAAGT

Target sequence: AG**CG**AGAAGCCCCCAAGGGTTTGTGAGA (**CpG target in bold**; hg19: chr19:10,305,909–10,305,936)

DNMT3A: PCR forward primer: GGTGGGAGGTTGAATGAAATGA

PCR reverse primer (biotinylated): AATACCCAACCCCAAATCCTAC

PCR program (50 cycles):

Initial	15 minutes at 95°C
Denaturation	30 seconds at 94°C
Annealing	30 seconds at 58°C
Extension	30 seconds at 72°C
Final	10 minutes at 72°C
Hold	4°C

Sequencing primer: AGTTGGAAGATTTTGTG

Target sequence:

TGTGCCTACACAC**CG**CCCTCACCCCTTCACYGTGGGGGCTGTTCTCCTTCCCCAT GGAGYGCTCAGGGCTCTAGGTTCCTGACTTGGGGCACCTCTGTCTAATTCCACC AGCACAGCCACTCACTATGTGCTCATCTCACTCCTCCAGCAGCYGCTGTAGGAC TTG GGGCTGGGCACC (**CpG target in bold**; hg19: chr2:25,565,782–25,565,959)

DNMT3B: PCR forward primer: GGGGTTAAGTGGTTTAAGTAAAT

PCR reverse primer (biotinylated): CCTCCAAAAATCCCTAAAAAAAATCTCTCC

PCR program (45 cycles):

Initial	15 minutes at 95°C
Denaturation	30 seconds at 94°C
Annealing	30 seconds at 52°C
Extension	30 seconds at 72°C
Final	10 minutes at 72°C
Hold	4°C

Sequencing primer: GTTAAGTGGTTTAAGTAAATTTAG

Target sequence:

CT**CG**G**CG**AT**CG**G**CG**C**CG**GAGATT**CGCG**AGCCCAG**CG**CCCTGCA**CG**GC**CG**CCA GC**CG**GCCTCC**CG**CCAGCCAGCCCCGACCCGCGGCTCCGCCGCCCAGCCGCGCCC CAGCCAGCCCTGCGGCAGGTGAGCGCCCCGGGGCCC (**CpG targets in bold**; hg19: chr20:31,350,382–31,350,523)

DNMT3L: PCR forward primer: AGTTTTTTTTATTGGGGTAGTTAGG

PCR reverse primer (biotinylated): CTTAAAACCAAAAAACCACATTTTATTCA

PCR program (45 cycles):

Initial	15 minutes at 95°C
Denaturation	30 seconds at 94°C
Annealing	30 seconds at 50°C
Extension	30 seconds at 72°C
Final	10 minutes at 72°C
Hold	4°C

Sequencing primer: GATTTAGGGATAGAGAGGG

Target sequence: G**CG**GTAGGGAGTGGGAAATCTGAATAA (**CpG target in bold**; hg19: chr21:45,683,527–45,683,553)

To demonstrate the ability of our assays to resolve DNAm differences as small as reported, we computed intraclass correlation coefficients (ICC) between triplicate replicates for each assay. Average within-sample coefficient of variation was computed using a two-way mixed model, using an absolute agreement definition (Shrout and Fleiss, 1979), as implemented in SPSS. ICCs for the 15 total CpG sites assayed ranged from 0.703 to 0.937, with a mean ICC of 0.855 (standard deviation: 0.066). This strongly supports the conclusion that these assays are capable of consistently resolving small DNAm differences.

Transcription factor binding site prediction

Putative TFBS were identified that overlap target CpG sites using the MatInspector(Cartharius *et al.*, 2005) tool from Genomatix, with default parameters. Input sequence included 200bp up and downstream of the CpG site. Only putative TFBS that directly overlapped CpG sites of interest were retained.

Statistical analyses

Statistical testing was performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY). DNAm at *DNMT3B* CpG sites was treated on a regional and an individual CpG site basis, similar to previous work (Rusiecki *et al.*, 2013). Regional values were calculated as the mean of 12 CpG sites. Paired-sample t-tests were used to test for differences in pre-trauma DNAm between cases and controls and to test for differences between pre- and post-trauma time points within cases and controls. Linear regression was used to test whether pre-trauma DNAm levels are predictive of post-trauma symptom severity (PTSS) changes. PTSS change was calculated as the difference between post-trauma PTSS and pre-trauma PTSS. Analyses included severity scores of individual

symptom criteria (hyperarousal, avoidance, or intrusion symptoms) as well as a total severity score that is inclusive of each symptom subdomain. Regression models were adjusted for age, gender, and pre-trauma symptom severity. The contribution of pre-trauma DNAm to post-trauma PTSS change was tested via the change in R square values comparing full to reduced models. We present primary results uncorrected for multiple testing as is consistent with the current state of the science of DNAm variation in association with psychiatric endpoints(Perroud *et al.*, 2011; Unternaehrer *et al.*, 2012; Perroud *et al.*, 2013; Rusiecki *et al.*, 2013). In addition, to assess the extent to which our results may be attenuated by multiple hypothesis testing correction, we calculated stringent Bonferroni-corrected significance values(Dunn, 1961) as well as false discovery rate (FDR) Q values(Benjamini, 1995). FDR has recently been utilized to correct multiple hypothesis testing in studies utilizing DNAm data, with user-defined Q-values ranging from 0.05 to 0.2(Provencal *et al.*, 2013; Zhao *et al.*, 2013).

Results

PTSD cases and controls do not differ in age, gender, ethnicity, or pre-trauma symptom severity, including individual symptoms of intrusion, avoidance, and hyperarousal (Table 1).

Pre-trauma DNAm variation is associated with PTSD

PTSD-associated DNAm variation may both pre-exist trauma and be associated with post-trauma PTSD outcome. To test for pre-existing protective/risk factors, pre-trauma DNAm at *DNMT1*, *DNMT3A*, *DNMT3B*, and *DNMT3L* loci was compared between trauma exposed individuals with vs. without PTSD. Pre-trauma DNAm was higher in cases compared with controls at a single *DNMT3B* CpG site (CpG 9) (Figure 1; t=2.250, 29 df, p=0.032); no difference in pre-trauma *DNMT3B* regional DNAm mean was observed (t=1.538, 29 df, p=0.135). We observed no pre-trauma differences between cases and controls at *DNMT1* (t=0.582, 29 df, p=0.565), *DNMT3A* (t=0.579, 29 df, p=0.567), and *DNMT3L* (t=1.386, 29df, p=0.176) loci.

Pre-trauma DNAm variation predicts post-trauma changes in trauma symptom severity

To explore whether this PTSD-associated pre-trauma DNAm is predictive of trauma response, we performed linear regression analyses with pre-trauma DNAm of *DNMT3B* at CpG 9 and PTSS change as predictor and outcome variables, respectively. Controlling for age, gender, and pre-trauma symptom severity, pre-trauma DNAm of CpG 9 (Figure 2; unstandardized B=−2.318, SE=1.25 p=0.034) predicted post-trauma symptom severity change. In this model, only pre-trauma symptom severity and pre- trauma DNAm were significant predictor variables. *DNMT3B* CpG 9 DNAm explained approximately 6.8% of the variance in PTS severity change, as revealed by a comparison of the full and reduced models. The full model that included *DNMT3B* CpG 9 DNAm, age, gender, and pre-trauma symptom severity explained approximately 24% of the variance in post-trauma PTSS change (Adjusted R Square=0.242, p=0.005).

Because the relationship between pre-trauma DNAm and post-trauma changes in PTS symptom severity may be driven by distinct symptom subdomains (hyperarousal, avoidance,

and intrusion), we regressed separately each subdomain symptom severity change onto pre-trauma DNAm, controlling for age, gender, and pre-trauma symptom severity of the relevant subdomain. Pre-trauma DNAm of *DNMT3B* CpG 9 (hyperarousal: p=0.249; avoidance: p=0.137; intrusion: p=0.071) did not predict change in subdomain symptom severity.

Trauma induces PTSD-associated DNAm modifications

DNAm differences may arise following trauma and be associated with PTSD development. To test this, we compared pre-trauma DNAm with post-trauma DNAm within PTSD cases and within trauma-exposed, healthy controls. Both PTSD-associated and PTSD-independent changes in DNAm following trauma were observed at *DNMT* loci (Figure 3). *DNMT1* DNAm increased (Figure 3A; t=3.887, 29 df, p=0.001) following trauma in the PTSD group, but not the control group (t=1.903, 29 df, p=0.067). At *DNMT3A* (Figure 3B) and *DNMT3B* (Figure 3C) loci, DNAm increased following trauma in both PTSD case (*DNMT3A*: t=2.806, 29 df, p=0.009; *DNMT3B*: t=4.286, 29 df, p<0.001) and control (*DNMT3A*: t=3.421, 29 df, p=0.002; *DNMT3B*: t=3.938, 29 df, p<0.001) groups. No change was observed in *DNMT3L* (Figure 3D) DNAm in either cases (t=1.551, 29 df, p=0.132) or controls (t=1.146, 29 df, p=0.261). Table 3 presents a summary including uncorrected p values, Bonferroni-corrected p values, and FDR values, as well as accompanying effect sizes, of our results described above.

Transcription factor binding site prediction

DNAm is associated with gene expression. One mechanism by which increased DNAm can lead to decreased gene expression is by affecting the binding of trans-activating factors to *cis*-regulatory elements. To contextualize our DNAm findings, we used bioinformatic methods to identify putative TFBS that overlap CpG sites showing PTSD-associated DNAm differences. In total, we identified 24 putative TFBS, including 2, 3, 14, and 5 that overlap *DNMT1*, *DNMT3A*, *DNMT3B*, and *DNMT3L* CpG target sites, respectively (Table 2). Notable among these 24 TFBS are those that overlap with CpG sites at which we identified PTSD-associated differential methylation (2 overlap the *DNMT1* CpG; 3 overlap *DNMT3B* CpG 9). Binding sites for heat shock factor 1 and E2F-4/DP-2 heterodimeric complex were identified to overlap with the *DNMT1* CpG site at which an increase in DNAm was observed in PTSD cases, but not controls. Overlapping with *DNMT3B* CpG site 9, at which increased pre-trauma DNAm was associated with PTSD development and predictive of worsening of PTSS, we identified binding sites for Human motif ten element, ZF5 POZ domain zinc finger, and the insulator protein CTCF.

Discussion

Our data represent preliminary findings suggesting that pre-trauma DNAm states and post-trauma DNAm modifications differ between those who develop PTSD following trauma and those who display resiliency. While baseline PTS symptoms did not differ between cases and controls, baseline DNAm at a *DNMT3B* CpG site was higher in resilient individuals compared to those who eventually developed PTSD. Additionally, longitudinal change in DNAm at a *DNMT1* CpG site was associated with PTSD, with an increase in DNAm being observed in those with PTSD but not controls. Finally, increases in DNAm were observed

following trauma at *DNMT3A* and *DNMT3B* loci that were independent of PTSD outcome, being observed in both PTSD cases and trauma-exposed controls. Although some of these results were attenuated following correction for multiple hypothesis testing, our findings suggest that epigenetic variation plays a complex regulatory role in PTSD risk and etiology.

One way in which DNAm may regulate gene transcription is by altering the strength and occupancy of transcription factor binding (Weaver *et al.*, 2004). To provide insight into potential functional consequences of the observed PTSD-associated differences, we conducted a secondary analysis of TFBS overlapping the distinguishing CpG sites. Among the sites identified was a binding site for CTCF, a transcription factor known to be involved in chromatin remodeling(Barkess and West, 2012). We identified this binding site overlapping with *DNMT3B* CpG site 9, at which higher DNAm was identified as a protective/risk factor for PTSD and symptom severity change following trauma exposure. Differential methylation at this site is particularly compelling as a determinant of PTSD risk, given that DNAm at CTCF binding sites has been shown to significantly affect CTCF occupancy(Wang *et al.*, 2012) and downstream levels of gene transcription(Renaud *et al.*, 2007). Due to the nature of our samples, we are unable to test directly whether DNAm at these identified TFBS influences gene expression. Where available, we have utilized ENCODE data(Consortium, 2011) to provide evidence for or against transcription factor binding at the PTSD-associated sites in blood-derived cell types. Among the TFBS identified that overlap PTSD-associated CpG sites (*DNMT1* and *DNMT3B* CpG 9), ENCODE data includes binding of CTCF and E2F4. ENCODE data supports the binding of CTCF to *DNMT3B* in blood tissue (specifically b-lymphocyte cell lines: GM12864 and GM12874), but does not support the binding of E2F4 to *DNMT1*. This supports the potential functionality of observed DNAm differences at *DNMT3B* CpG 9 in pre-trauma samples in cases vs. controls.

*DNMT*s have been previously implicated in PTSD, anxiety, and fear conditioning. In suicide completers relative to controls, *DNMT3B* was upregulated in the frontopolar cortex, hypothalamus, and dorsal vagal complex and down regulated, along with *DNMT1*, in the hippocampus(Poulter *et al.*, 2008). Additionally, de novo methyltransferases have been shown to be upregulated during contextual fear conditioning, also in the hippocampus (Miller and Sweatt, 2007); DNMTs are required for fear conditioning and memory consolidation as demonstrated, respectively, by administration of DNMT inhibitors (Miller and Sweatt, 2007) and the creation of mice with the combined knockout of *DNMT1* and *DNMT3A*(Feng *et al.*, 2010). Our results thus add to the growing evidence implicating DNMTs in phenotypes of relevance to PTSD, and of psychiatric phenotypes more broadly.

The expression of *DNMT*s at the mRNA(Goto *et al.*, 1994; Veldic *et al.*, 2004; Kang *et al.*, 2011; Sterner *et al.*, 2012) and protein(Inano *et al.*, 2000; Feng *et al.*, 2005; Veldic *et al.*, 2005) levels in post-mitotic neurons of the central nervous system suggests that they are involved in methyltransferase activity that persists into adulthood and that is unrelated to DNA replication(Goto *et al.*, 1994). Indeed, previous work has identified DNMT1 protein expression in multiple brain regions in rodents (e.g. cortex, cerebellum(Inano *et al.*, 2000)), as well as in specific cortical regions in adult humans (e.g. Broadmann's Area 9(Veldic *et al.*, 2005)). Furthermore, recent work suggests that our epigenetic findings in peripheral

blood may be relevant to brain tissue: environmental exposures such as trauma have been shown to induce parallel epigenetic modifications in peripheral blood and brain (McGowan *et al.*, 2011; Klengel *et al.*, 2013). Although the current study, based on living participants drawn from a population-based cohort, precludes such work, future research is needed to address whether the epigenetic determinants of risk observed here in peripheral blood-derived DNA is also found in brain-derived DNA.

Importantly, this study adds to emerging work utilizing a longitudinal study design capable of measuring biological markers prior to disease onset as well as change between pre-disease and post-disease time points(Nieratschker *et al.*, 2012; Rusiecki *et al.*, 2012; Perroud *et al.*, 2013; Rusiecki *et al.*, 2013). Existing longitudinal studies have documented the importance of DNAm to mental health disorder risk, including differential change in DNAm of *BDNF* among individuals with vs. without borderline personality disorder(Perroud *et al.*, 2013), increased *DAT* (*SLC6A3*) DNAm with age that may be driven by alcohol dependence(Nieratschker *et al.*, 2012), and increasing *SERT* DNAm associated with bullying(Ouellet-Morin *et al.*, 2013). Most relevant to the present study is work by Rusiecki et al.(Rusiecki *et al.*, 2012) which provides evidence for increased global DNAm in controls, but not cases following trauma exposure, suggesting that resiliency is associated with increased global DNAm, potentially mediated by increased activity and expression of DNMTs. Indeed, our data presented here is consistent with this scenario, as DNAm of *DNMT1* was observed to increase following trauma in cases, but not controls. In contrast, however, we observed an increase in *DNMT3B* DNAm following trauma in both cases and controls, and a pre-trauma association between higher DNAm pre-trauma and resiliency post-trauma. The presence of a CTCF binding site opens the possibility that increased DNAm at this locus is associated with increased gene expression because CTCF can act as either a transcriptional activator or repressor(Phillips and Corces, 2009), with strength of DNA binding inversely correlated with local DNAm(Barkess and West, 2012). If binding of CTCF to the *DNMT3B* locus results in transcriptional repression, then increased DNAm, and concurrent decreased CTCF binding, would be associated with increased, not decreased, gene expression. If true, this would put these findings in line with the previously published, longitudinal, trauma-associated epigenetic data: decreased DNAm in pre-trauma PTSD cases would result in tighter CTCF binding and reduced *DNMT3B* transcription and lower global DNAm levels, as reported by Ruisecki and colleagues(Rusiecki *et al.*, 2012). Although DNMT1 is typically thought to maintain DNAm in adult tissues, evidence suggests that *DNMT1* and *DNMT3B* cooperatively maintain DNAm, with one or the other, but not both, required for global DNAm(Rhee *et al.*, 2002). More broadly, our data adds to the emerging evidence that longitudinal DNAm changes may contribute to the etiology of mental illness and can be taken as a proof of principle that locus-specific epigenetic variability both pre-exist and arise following disease-onset in biologically meaningful ways.

While our study is one of the first of its kind to compare pre- and post-trauma DNAm levels with regard to the development of PTSD, there is a minimum of four study limitations that should be kept in mind when interpreting our results. First, it is important to recognize that the epidemiological nature of our cohort precludes sample collection with a well-controlled experimental time course; times between pre-trauma data collection, trauma exposure, and

post-trauma data collection differed between each test subject. As such, we are unable to resolve whether observed PTSD-associated post-trauma DNAm changes precede PTSD-development (i.e. occurred within the first four weeks following trauma). As DNMTs are involved in the global regulation of DNAm, it is tempting to conclude from our data that observed changes in *DNMT* DNAm are an upstream process of PTSD development, thereby having the potential to help explain differences in DNAm epigenome-wide reported elsewhere(Uddin *et al.*, 2010; Smith *et al.*, 2011; Rusiecki *et al.*, 2012). However, it is also possible that the observed PTSD-associated DNAm changes are downstream effects of PTSD development, with no or little involvement in epigenetic modifications across the epigenome. Second, the nature of the epidemiological samples collected precluded the assessment of pre- and post-trauma gene expression differences and changes, as well as any analysis of blood cell composition. Third, the DNAm differences and effect sizes reported here are small; however, they are consistent with published work showing functional effects of DNAm variation(Tyrka *et al.*, 2012). High intraclass correlation coefficients between experimental replicates for each of our assays increases confidence of the validity of observed DNAm differences. Indeed, our sample size and observed effect sizes are consistent with published work in the field(Perroud *et al.*, 2011; Byrne *et al.*, 2013). Fourth, our results across the multiple CpG sites within *DNMT3B* are not corrected for multiple testing. Although this is consistent with the current state of the science of DNAm variation in association with psychiatric endpoints(Perroud *et al.*, 2011; Unternaehrer *et al.*, 2012; Perroud *et al.*, 2013; Rusiecki *et al.*, 2013), we do report corrected results (Table 3) to assess the degree to which our findings might be attenuated by multiple hypothesis test correction. Accepting a stringent FDR of 0.05 requires that we reject several findings reported as significant in our study, notably pre-trauma DNAm differences between cases and controls at *DNMT3B* CpG 9. However, it also means that a significant association between DNAm and PTSD emerges as a result of correction, as a significant change in DNAm at *DNMT3A* following trauma is only seen in controls at this stringent FDR cutoff and would therefore be suggestive of a resiliency-associated change in DNAm (Table 3). While we have chosen to utilize a stringent FDR cut-off of 0.05, other DNAm analyses have accepted a cut-off as high as 0.20(Provencal *et al.*, 2013). Overall, we stress the preliminary nature of these findings—both uncorrected and corrected for multiple hypothesis testing—and the importance of replication in an independent cohort.

Individuals exposed to trauma differ in their risk for subsequent PTSD. Our data suggest that variation in pre-trauma DNAm and post-trauma DNAm change may be part of the molecular underpinnings of PTSD risk and resiliency. Future research is needed to determine if the DNAm variation observed here is associated with functional changes that affect the long-term biology of individuals exposed to trauma. The identification of risk markers, including epigenetic markers, is an important step to understanding the biological underpinnings of PTSD risk and may lead to the development of tools to identify those individuals most at risk of developing PTSD as well as to develop evidence-based interventions.

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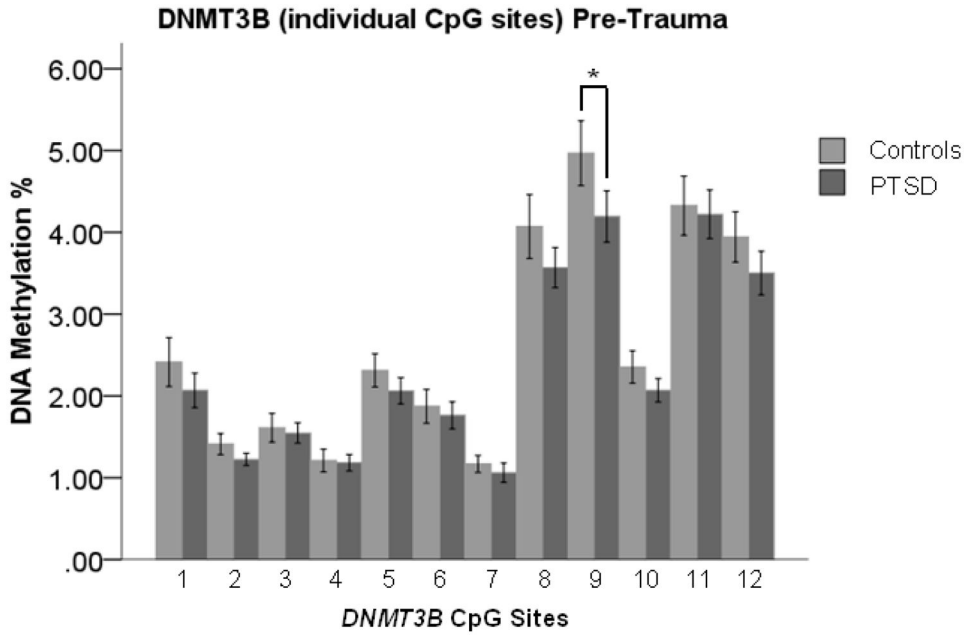

Figure 1.

Pre-trauma "DNA methyltransferase 3B" (*DNMT3B*) DNA methylation (DNAm) is significantly higher in trauma-exposed controls compared to posttraumatic stress disorder (PTSD) cases at CpG 9. Pre-trauma DNAm did not differ between cases and controls at the other 11 *DNMT3B* CpG sites assessed. Light gray bars indicate mean DNAm of controls. Dark gray bars indicate mean DNAm of PTSD cases. Error bars represent standard error of the mean. Difference between controls and cases was tested by paired-sample t-tests (N = 60; 30 cases and 30 matched controls). *: $p<0.05$.

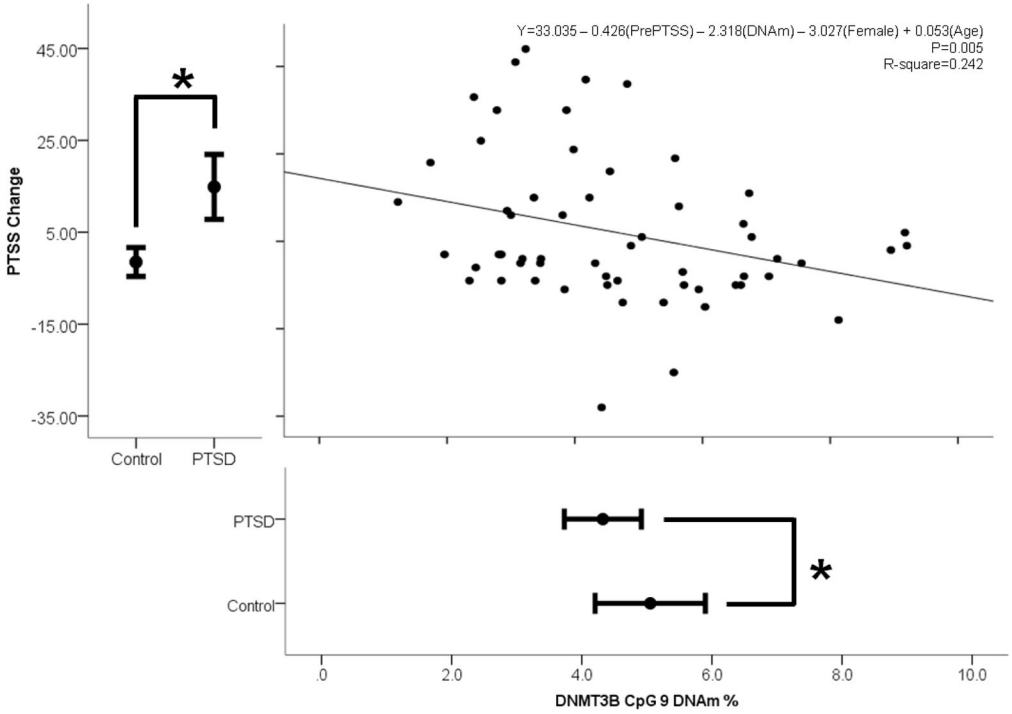

Figure 2.

Linear regression model of symptom severity (PTSS) change post-trauma and pre-trauma "DNA methyltransferase 3B" (*DNMT3B*) CpG 9 DNA methylation (DNAm), adjusting for age, gender, and pre-trauma symptom severity (N=60). Only pre-trauma PTSS and DNAm were significant variables in this model. Error bar plots represent the mean plus/minus the 95% confidence intervals. Differences between posttraumatic stress disorder cases and trauma-exposed controls were tested by paired-sample t-tests (N = 60; 30 PTSD cases and 30 matched controls). *: $p<0.05$.

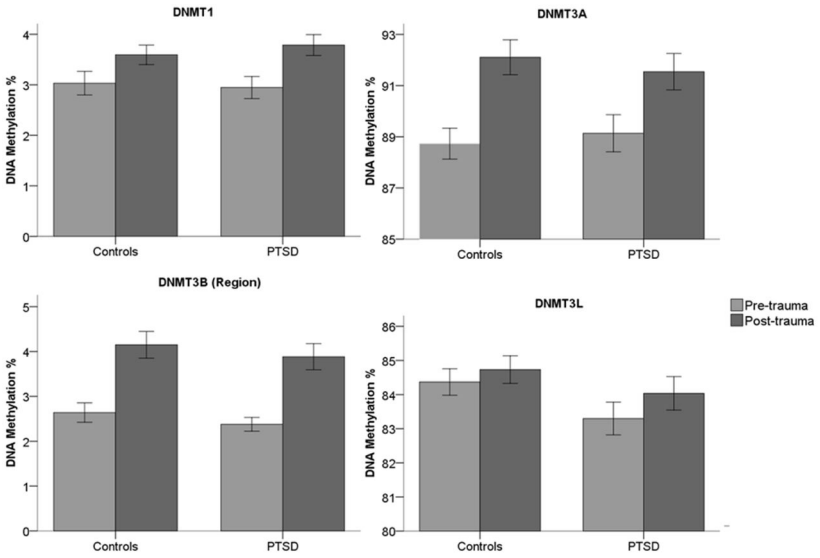

Figure 3.

Longitudinal DNA methylation (DNAm) modifications of DNA methyltransferase (*DNMT*) loci in response to trauma in posttraumatic stress disorder (PTSD) cases and trauma-exposed controls. *DNMT3B* (region) represents the mean of 12 CpG sites. Differences between PTSD cases and trauma-exposed controls were tested by paired-sample t-tests (N = 60; 30 PTSD cases and 30 matched controls). Error bars represent standard error of the mean. *: p<0.05; **:p<0.01; ***:p<0.001.

Table 1

Demographic and pre-trauma characteristics of 30 posttraumatic stress disorder (PTSD) case-control pairs.

	Controls (N = 30)		PTSD (N = 30)		t (d.f.)	p-value
	Mean/#	s.d./%	Mean/#	s.d./%		
Age (years)	55.37	12.97	53.71	12.94	−0.47 (29)	0.638
Female	23	76.7%	23	76.7%	NA (1)	1.000
African-American	22	73.3%	24	80%	0.01 (1)	0.938
Lifetime traumas	3.80	3.83	4.43	3.70	−0.84 (29)	0.407
Assaultive Violence	0.87	1.33	1.07	1.55	−0.55 (29)	0.589
Other Injury or Shcking Experience	0.83	1.32	1.27	1.48	−1.51 (29)	0.141
Learning about traumas to others	1.20	1.50	1.17	1.37	0.12 (29)	0.909
Sudden Death	0.77	0.43	0.70	0.47	0.63 (29)	0.536
Other Event	0.13	0.35	0.23	0.43	−1.36 (29)	0.184
Pre-trauma PTS	26.90	10.96	39.33	16.20	−1.12 (29)	0.275
Intrusion	9.58	7.90	11.65	6.08	−0.99 (29)	0.333
Avoidance	11.65	11.04	14.69	6.97	−1.14 (29)	0.267
Hyperarousal	8.60	7.98	11.24	5.11	−1.24 (29)	0.226
Post-trauma PTS	25.41	7.01	54.20	11.59	11.4 (29)	<0.001

Table 2

Putative transcription factor binding sites overlap DNA methyltransferase (*DNMT*) CpG sites of interest. V$ matrix families indicate Genomatix-annotated transcription factor binding site matrix families. *DNMT3B* CpG sites are described in the Methods.

Gene	Matrix Family	Matrix Information	Core similarity	*DNMT3B* CpG overlap
DNMT1	V$HEAT	Heat shock factor 1	1.000	-
	V$E2FF	E2F-4/DP-2 heterodimeric complex	0.847	-
DNMT3A	V$SP1F	TGFbeta-inducible early gene (TIEG)/Early growth response gene alpha (EGRalpha)	0.750	-
	V$SP1F	Stimulating protein 1, ubiquitous zinc finger transcription factor	1.000	-
	V$EGRF	EGR1, early growth response 1	0.802	-
	V$GCMF	Glial cells missing homolog 1, chorion-specific transcription factor GCMa	1.000	-
	V$KLFS	Kidney-enriched kruppel-like factor, KLF15	1.000	-
DNMT3B	O$MTEN	Human motif ten element	0.839	1, 2, 3, 4, 5
	V$PAX5	PAX5 paired domain protein	0.789	1, 2, 3, 4, 5, 6, 7
	V$E2FF	E2F transcription factor 3 (secondary DNA binding preference)	1.000	2, 3, 4, 5
	V$E2FF	E2F transcription factor 3 (secondary DNA binding preference)	1.000	2, 3, 4, 5, 6, 7
	V$ETSF	Ets variant 4	1.000	3, 4, 5, 6, 7
	O$MTEN	Human motif ten element	0.961	7, 8, 9
	V$ZF5F	ZF5 POZ domain zinc finger, zinc finger protein 161 (secondary DNA binding preference)	0.775	8, 9, 10
	V$CTCF	Insulator protein CTCF (CCCTC-binding factor)	0.818	9, 10, 11, 12
	V$HDBP	Huntington's disease gene regulatory region-binding protein 1 and 2 (SLC2A4 regulator and papillomavirus binding factor)	1.000	10, 11, 12
	V$EGRF	Collagen krox protein (zinc finger protein 67-zfp67)	1.000	11, 12
	V$PLAG	Pleomorphic adenoma gene (PLAG) 1, a developmentally regulated C2H2 zinc finger protein	0.958	12
	V$ZF02	Transcriptional repressor, binds to elements found predominantly in genes that participate in lipid metabolism	0.776	12
DNMT3L	V$KLFS	Basic transcription element (BTE) binding protein, BTEB3, FKLF-2	1.000	-
	V$SP1F	Stimulating protein 1, ubiquitous zinc finger transcription factor	1.000	-
	V$MYBL	C-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus oncogene v-myb	0.797	-
	V$GLIF	Zinc finger transcription factor, Zic family member 2 (odd-paired homolog, Drosophila)	1.000	-
	V$CP2F	LBP-1c (leader-binding protein-1c), LSF (late SV40 factor, CP2, SEF (SAA3 enhancer factor)	0.875	-

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Table 3

Observed and corrected significance values of tests. The list of observed p-values is sorted from smallest to largest (indicated by the Rank column). All tests above and including those bolded meet significance at $p<0.05$ for various corrections for multiple hypothesis testing. Corrected significance thresholds at $p<0.05$ are listed using two controlling procedures: Bonferroni and False Discovery Rate (FDR) using the procedure of Benjamini and Hochberg (BH, (Benjamini, 1995)). Bolded values meet significance at $p<0.05$ for the various correction procedures.

Test	Mean Difference	SE	Observed P-value	Rank (i)	Bonferroni threshold	FDR (BH) thresholds
Pre- vs. post-trauma *DNMT3B* in Cases	1.51	0.35	**0.000**	1	**0.002**	**0.002**
Pre- vs. post-trauma *DNMT3B* in Controls	1.51	0.38	**0.000**	2	**0.002**	**0.003**
Pre- vs. post-trauma *DNMT1* in Cases	0.84	0.22	**0.001**	3	**0.002**	**0.005**
Pre- vs. post-trauma *DNMT3A* in Controls	3.38	0.99	**0.002**	4	**0.002**	**0.006**
Pre- vs. post-trauma *DNMT3A* in Cases	2.41	0.86	**0.009**	5	0.002	0.008
Cases vs. Controls *DNMT3B* (CpG 9) pre-trauma	0.77	0.34	**0.032**	6	0.002	0.009
CpG 9 Regression analysis (all symptoms)	B: −2.318	1.251	**0.034**	7	0.002	0.011
Cases vs. Controls *DNMT3B* (CpG 2) pre-trauma	0.19	0.11	0.057	8	0.002	0.013
Pre- vs. post-trauma *DNMT1* in Controls	0.56	0.30	0.067	9	0.002	0.014
CpG 9 Regression analysis (Intrusion symptoms)	B: −0.851	0.458	0.071	10	0.002	0.016
Cases vs. Controls *DNMT3B* (CpG 12) pre-trauma	0.44	0.24	0.122	11	0.002	0.017
Cases vs. Controls *DNMT3B* (CpG 10) pre-trauma	0.28	0.19	0.127	12	0.002	0.019
Pre- vs. post-trauma *DNMT3L* in Cases	0.74	0.47	0.132	13	0.002	0.020
Cases vs. Controls *DNMT3B* pre-trauma	0.27	0.17	0.135	14	0.002	0.022
CpG 9 Regression analysis (Avoidance symptoms)	B: −0.821	0.541	0.137	15	0.002	0.023
Cases vs. Controls *DNMT3B* (CpG 1) pre-trauma	0.35	0.23	0.151	16	0.002	0.025
Cases vs. Controls	**0.50**	**0.33**	**0.158**	**17**	**0.002**	**0.027**

Test	Mean Difference	SE	Observed P-value	Rank (i)	Bonferroni threshold	FDR (BH) thresholds
DNMT3B (CpG 8) pre-trauma						
Cases vs. Controls *DNMT3L* pre-trauma	0.91	0.65	0.176	18	0.002	0.028
Cases vs. Controls *DNMT3B* (CpG 5) pre-trauma	0.25	0.19	0.221	19	0.002	0.030
CpG 9 Regression analysis (Hyperarousal symptoms)	B: −0.466	0.399	0.249	20	0.002	0.031
Pre- vs. post-trauma *DNMT3L* in Controls	0.36	0.32	0.261	21	0.002	0.033
Cases vs. Controls *DNMT3L* post-trauma	0.58	0.56	0.304	22	0.002	0.034
Cases vs. Controls *DNMT1* post-trauma	−0..19	0.19	0.323	23	0.002	0.036
Cases vs. Controls *DNMT3B* post-trauma	0.18	0.22	0.351	24	0.002	0.038
Cases vs. Controls *DNMT3A* post-trauma	0.56	0.66	0.356	25	0.002	0.039
Cases vs. Controls *DNMT3B* (CpG 7) pre-trauma	0.11	0.12	0.487	26	0.002	0.041
Cases vs. Controls DNMT1 pre-trauma	0.09	0.20	0.565	27	0.002	0.042
Cases vs. Controls DNMT3A pre-trauma	−0.41	0.73	0.567	28	0.002	0.044
Cases vs. Controls *DNMT3B* (CpG 6) pre-trauma	0.11	0.17	0.603	29	0.002	0.045
Cases vs. Controls *DNMT3B* (CpG 3) pre-trauma	0.06	0.15	0.711	30	0.002	0.047
Cases vs. Controls *DNMT3B* (CpG 11) pre- trauma	0.11	0.39	0.773	31	0.002	0.048
Cases vs. Controls *DNMT3B* (CpG 4) pre-trauma	0.03	0.14	0.881	32	0.002	0.050

Note: For regression analyses, “B” represents unstandardized Beta values.

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