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ORIGINAL ARTICLE

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

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Background. Epigenetic differences exist between trauma-exposed individuals with and without post-traumatic stress disorder (PTSD). It is unclear whether these epigenetic differences pre-exist, or arise following, trauma and PTSD onset.

Method. In pre- and post-trauma samples from a subset of Detroit Neighborhood Health Study participants, DNA methylation (DNAm) was measured at DNA methyltransferase 1 (*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*: 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 pre-existing epigenetic states that differentiate individuals who are resilient *versus* susceptible to PTSD. These distinctive DNAm differences within *DNMT* loci may contribute to genome-wide epigenetic profiles of PTSD.

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Introduction

Post-traumatic stress disorder (PTSD) is a prevalent and debilitating mental health disorder that may arise following exposure to a potentially traumatic event (APA, 2013). While the lifetime prevalence of traumatic exposure is 50–90% (Kessler *et al.* 1995), PTSD in the general US population is estimated to be only 6.8% (Kessler & 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 & 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 (2012), Department of Defense (CDMRP, Department of Defense, 2010) and the National Institute of Mental Health (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 & 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,

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2004; Brewin *et al.* 2000; Koenen *et al.* 2003; 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 Genome-wide Association Study (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 × 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 are 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* DNA methyltransferases DNMT3A, DNMT3B and DNMT3L following fertilization (Bourc'his *et al.* 2001; Bourc'his & 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 & 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, 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 & Sweatt, 2007; Feng *et al.* 2010). Together, these findings suggest that DNAm and DNMTs 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 *versus* 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 US military personnel deployed to Iraq and Afghanistan (Rusiecki *et al.* 2012, 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 analysed DNAm from individuals pre- and post-trauma to identify differences that characterize individuals who are susceptible *versus* resilient to PTSD following trauma. To assess potential functional consequences of examined DNAm differences, we then performed a bioinformatic search for the presence of putative TFBS (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.

Method

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, USA. The current study draws on peripheral blood samples and survey data obtained at two time points from 60 DNHS participants; 46 were female and 14 male; 46 were African-American and 12 were Caucasian, and two 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 that the participant regarded as their worst and one randomly selected traumatic event from the remaining traumas that the participant experienced. Lifetime PTSD cases met all six Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria (APA, 1994) 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 venepuncture when available from DNHS participants selected for inclusion in this study. Blood spots were used as an alternative source of whole blood-derived DNA when venepuncture samples were unavailable. The exact tissue type was shared between matched case–control pairs in all instances. Venepuncture- and bloodspot-derived whole blood represents 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, USA) and the QuickGene DNA Whole blood Kit S (Lifesciences, FujiFilm, Japan) using the manufacturers' recommended protocols.

Blood spots. DNA was isolated using the QIAamp DNA Micro kit (Qiagen, USA) using the manufacturer's recommended protocol. For each sample, one 6 mm punch was taken from dried blood spots using a disposable, sterile biopsy punch (Miltex, USA) within a sterile field and placed immediately into a sterile 1.7 ml 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, about 750 ng of DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen, USA) 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, USA). 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 DNMT 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 HM450 K methylation bead chips from Illumina (see below for actual HG19 nucleotide location). The capacity for each assay to capture DNAm levels ranging from 0 to 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). Polymerase chain reaction (PCR) amplification of target sequences was performed on 20 ng of bisulfite-converted DNA samples using the PyroMark PCR kit (Qiagen, USA).

Bisulfite-converted, PCR-amplified DNA was pyrosequenced on the Pyromark Q24 Pyrosequencer (Qiagen, USA) 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: TTTTTTTAGGTGTGA TGGGGATAAAG
PCR reverse primer (biotinylated): CAAAAA CTCTCACAAACCCTTAAA
PCR program (50 cycles):

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

Sequencing primer: GTGATGGGGAT AAAGT
Target sequence: AG**CG**AGAAGCCCCC AAGGGTTTGTGAGA (**CpG target in bold**; hg19: chr19:10,305,909-10,305,936)

DNMT3A:

PCR forward primer: GGTGGGAGGTTGAAT GAAATGA
PCR reverse primer (biotinylated): AATACCC AACCCCAAATCCTAC
PCR program (50 cycles):

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

Sequencing primer: AGTTGGAAGATTT TGTG
Target sequence: TGTGCCTACACAC**CG** CCCTCACCCCTTCACYGTGGGGGCTG TTCTCCTTCCCCATGGAGYGCTCAGG GCTCTAGGTTCCTGACTTGGGGCACC TCTGTCTAATTCCACCAGCACAGCCA CTCACTATGTGCTCATCTCACTCCTCC AGCAGCYGCTGTAGGACTTGGGGCT GGGCACC (**CpG target in bold**; hg19: chr2:25,565,782-25,565,959)

DNMT3B:

PCR forward primer: GGGGTTAAGTGGTTTAA GTAAAT
PCR reverse primer (biotinylated): CCTCCAAAA ATCCCTAAAAAAAATCT CTCC
PCR program (45 cycles):

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

Sequencing primer: GTTAAGTGGTTTAA GTAAATTTAG
Target sequence: CT**CG**G**CG**AT**CG**G**CG**C **CG**GAGATT**CGCG**AGCCCAG**CG**CCCTG CA**CG**GC**CG**CCAGC**CG**GCCTCC**CG**CCA GCCAGCCCCGACCCGCGGCTCCGCCG CCCAGCCGCGCCCCAGCCAGCCCTGC GGCAGGTGAGCGCCCCGGGGCCC (**CpG targets in bold**; hg19: chr20:31, 350,382-31,350,523)

DNMT3L:

PCR forward primer: AGTTTTTTTTATTGGGGT AGTTAGG
PCR reverse primer (biotinylated): CTTAAA ACCAAAAAACCACATTTTAT TCA
PCR program (45 cycles):

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

Sequencing primer: GATTTAGGGATAGAGAG GG
Target sequence: G**CG**GTAGGGAGTGGGAAAT CTGAATAA (**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 (ICCs) 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 & Fleiss, 1979), as implemented in SPSS (IBM, USA). ICCs for the 15 total CpG sites assayed ranged from 0.703 to 0.937, with a mean ICC of 0.855 (S.D.=0.066). This strongly supports the conclusion that these assays are capable of consistently resolving small DNAm differences.

TFBS prediction

Putative TFBS were identified that overlap target CpG sites using the MatInspector (Cartharius *et al.* 2005) tool from Genomatix, with default parameters. Input

Table 1. *Demographic and pre-trauma characteristics of 30 PTSD case–control pairs*

	Controls (n=30)		PTSD (n=30)		t (df)	p
Age, years	55.37	(12.97)	53.71	(12.94)	−0.47 (29)	0.638
Female, n (%)	23	(76.7)	23	(76.7)	N.A. (1)	1.000
African-American, n (%)	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 shocking 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 symptom severity	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 symptom severity	25.41	(7.01)	54.20	(11.59)	11.4 (29)	<0.001

Data are given as mean (standard deviation) unless otherwise indicated.
PTSD, Post-traumatic stress disorder; df, degrees of freedom; N.A., not applicable.

sequence included 200 bp 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 SPSS Statistics for Windows, version 21.0 (IBM Corp., USA). 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 PTSS and pre-trauma symptom severity. 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 PTSS change was tested via the change in R^2 values comparing full with 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, 2013; Unternaehrer *et al.* 2012; 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 & Hochberg, 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 did 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 exist before 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 *versus* without PTSD. Pre-trauma DNAm was higher in controls compared with cases at a single *DNMT3B* CpG site (CpG 9) [Fig. 1; t=2.250, degrees of freedom (df)=29, p=0.032]; no difference in pre-trauma *DNMT3B* regional DNAm mean was observed (t=1.538, df=29, p=0.135). We observed no pre-trauma differences between cases and controls at *DNMT1* (t=0.582, df=29,

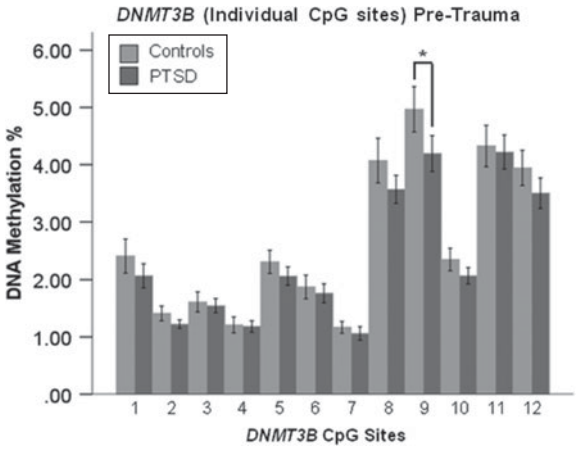

Fig. 1. Pre-trauma DNA methyltransferase 3B (*DNMT3B*) DNA methylation (DNAm) is significantly higher in trauma-exposed controls compared with post-traumatic stress disorder (PTSD) cases at CpG 9. Pre-trauma DNAm did not differ between cases and controls at the other 11 *DNMT3B* CpG sites assessed. Values are means, with standard errors represented by vertical bars. Differences between controls and cases were tested by paired-sample *t* tests (n=60; 30 cases and 30 matched controls).
* Mean values were significantly different (p<0.05).

p=0.565), *DNMT3A* (t=0.579, df=29, p=0.567) and *DNMT3L* (t=1.386, df=29, 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 (Fig. 2; unstandardized B=–2.318, S.E.=1.25, p=0.034) predicted PTSS 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 PTSS 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^2=0.242, p=0.005).

Because the relationship between pre-trauma DNAm and post-trauma changes in PTSS 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 (Fig. 3). *DNMT1* DNAm increased (Fig. 3*a*; t=3.887, df=29, p=0.001) following trauma in the PTSD group, but not in the control group (t=1.903, df=29, p=0.067). At *DNMT3A* (Fig. 3*b*) and *DNMT3B* (Fig. 3*c*) loci, DNAm increased following trauma in both PTSD case (*DNMT3A:* t=2.806, df=29, p=0.009; *DNMT3B:* t=4.286, df=29, p<0.001) and control (*DNMT3A:* t=3.421, df=29, p=0.002; *DNMT3B:* t=3.938, df=29, p<0.001) groups. No change was observed in *DNMT3L* (Fig. 3*d*) DNAm in either cases (t=1.551, df=29, p=0.132) or controls (t=1.146, df=29, p=0.261). Table 2 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.

TFBS 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 3). Notable among these 24 TFBS are those that overlap with CpG sites at which we identified PTSD-associated differential methylation (two overlap the *DNMT1* CpG; three 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 decreased pre-trauma DNAm was associated with PTSD development and predictive of worsening of PTSS, we identified binding sites for human motif

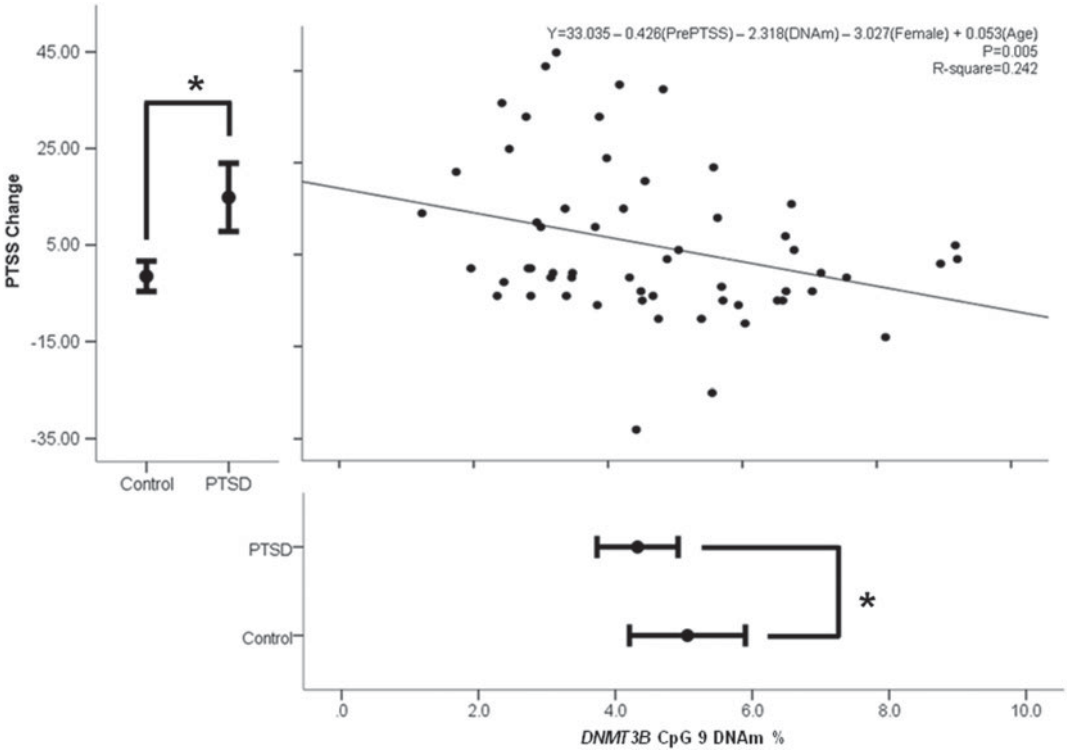

Fig. 2. Linear regression model of post-trauma symptom severity (PTSS) change and pre-trauma DNA methyltransferase 3B (*DNMT3B*) CpG 9 DNA methylation (DNAm), adjusting for age, gender and pre-trauma symptom severity ($n=60$). Only pre-trauma symptom severity and DNAm were significant variables in this model. Values in error bar plots are means, with 95% confidence intervals represented by vertical bars. Differences between post-traumatic stress disorder (PTSD cases and trauma-exposed controls were tested by paired-sample t tests ($n=60$; 30 PTSD cases and 30 matched controls). * Mean values were significantly different ($p<0.05$).

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 symptom severity did not differ between cases and controls, baseline DNAm at a *DNMT3B* CpG site was higher in resilient individuals compared with 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 & 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 Encyclopedia of

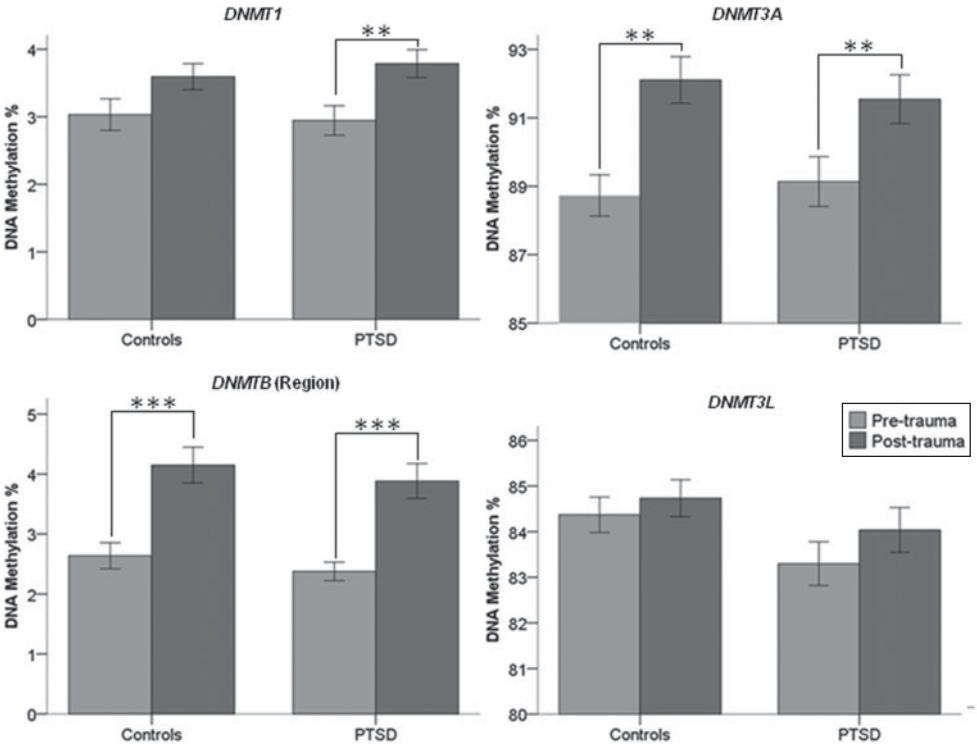

Fig. 3. Longitudinal DNA methylation modifications of DNA methyltransferase (*DNMT*) loci in response to trauma in post-traumatic stress disorder (PTSD) cases and trauma-exposed controls. *DNMT3B* (region) represents the mean of 12 CpG sites. Values are means, with standard errors represented by vertical bars. Differences between PTSD cases and trauma-exposed controls were tested by paired-sample *t* tests ($n=60$; 30 PTSD cases and 30 matched controls). Mean values were significantly different: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

DNA Elements (ENCODE) Project data (ENCODE Project 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 include binding of CTCF and E2F4. ENCODE data support the binding of CTCF to *DNMT3B* in blood tissue (specifically B-lymphocyte cell lines: GM12864 and GM12874), but do 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 *versus* controls.

*DNMT*s have been previously implicated in PTSD, anxiety and fear conditioning. In suicide completers relative to controls, *DNMT3B* was up-regulated 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 up-regulated during contextual fear conditioning, also in the hippocampus (Miller & Sweatt, 2007); DNMTs are required for fear conditioning and memory consolidation as demonstrated, respectively, by administration of DNMT inhibitors (Miller & 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. Brodmann 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 the brain (McGowan *et al.* 2011; Klengel *et al.* 2013). Although the current study, based on living participants drawn from a

Table 2. *Observed and corrected significance values of tests*[a]

Test	Mean difference[b]	(S.E.)	Observed p[c]	Rank	Bonferroni threshold	FDR thresholds
Pre- *v.* post-trauma *DNMT3B* in cases	1.51	(0.35)	0.000*	1	0.002*	0.002*
Pre- *v.* post-trauma *DNMT3B* in controls	1.51	(0.38)	0.000*	2	0.002*	0.003*
Pre- *v.* post-trauma *DNMT1* in cases	0.84	(0.22)	0.001*	3	0.002*	0.005*
Pre- *v.* post-trauma *DNMT3A* in controls	3.38	(0.99)	0.002*	4	0.002*	0.006*
Pre- *v.* post-trauma *DNMT3A* in cases	2.41	(0.86)	0.009*	5	0.002	0.008
Cases *v.* 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 *v.* controls *DNMT3B* (CpG 2) pre-trauma	0.19	(0.11)	0.057	8	0.002	0.013
Pre- *v.* 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 *v.* controls *DNMT3B* (CpG 12) pre-trauma	0.44	(0.24)	0.122	11	0.002	0.017
Cases *v.* controls *DNMT3B* (CpG 10) pre-trauma	0.28	(0.19)	0.127	12	0.002	0.019
Pre- *v.* post-trauma *DNMT3L* in cases	0.74	(0.47)	0.132	13	0.002	0.020
Cases *v.* 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 *v.* controls *DNMT3B* (CpG 1) pre-trauma	0.35	(0.23)	0.151	16	0.002	0.025
Cases *v.* controls *DNMT3B* (CpG 8) pre-trauma	0.50	(0.33)	0.158	17	0.002	0.027
Cases *v.* controls *DNMT3L* pre-trauma	0.91	(0.65)	0.176	18	0.002	0.028
Cases *v.* 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- *v.* post-trauma *DNMT3L* in controls	0.36	(0.32)	0.261	21	0.002	0.033
Cases *v.* controls *DNMT3L* post-trauma	0.58	(0.56)	0.304	22	0.002	0.034
Cases *v.* controls *DNMT1* post-trauma	−0.19	(0.19)	0.323	23	0.002	0.036
Cases *v.* controls *DNMT3B* post-trauma	0.18	(0.22)	0.351	24	0.002	0.038
Cases *v.* controls *DNMT3A* post-trauma	0.56	(0.66)	0.356	25	0.002	0.039
Cases *v.* controls *DNMT3B* (CpG 7) pre-trauma	0.11	(0.12)	0.487	26	0.002	0.041
Cases *v.* controls DNMT1 pre-trauma	0.09	(0.20)	0.565	27	0.002	0.042
Cases *v.* controls DNMT3A pre-trauma	−0.41	(0.73)	0.567	28	0.002	0.044
Cases *v.* controls *DNMT3B* (CpG 6) pre-trauma	0.11	(0.17)	0.603	29	0.002	0.045
Cases *v.* controls *DNMT3B* (CpG 3) pre-trauma	0.06	(0.15)	0.711	30	0.002	0.047
Cases *v.* controls *DNMT3B* (CpG 11) pre-trauma	0.11	(0.39)	0.773	31	0.002	0.048
Cases *v.* controls *DNMT3B* (CpG 4) pre-trauma	0.03	(0.14)	0.881	32	0.002	0.050

S.E., Standard error; FDR, false discovery rate; DNMT, DNA methyltransferase.

[a] Corrected significance thresholds at $p<0.05$ are listed using two controlling procedures: Bonferroni and FDR using the procedure of Benjamini & Hochberg (1995).

[b] In percent DNA methylation. For regression analyses, 'B' represents unstandardized β values.

[c] The list of observed p values is sorted from smallest to largest (indicated by the rank column).

* Values meet significance at $p<0.05$ for the various correction procedures.

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 are 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 (Rusiecki *et al.* 2012, 2013; Perroud *et al.* 2013; Nieratschker *et al.* 2014). Existing longitudinal studies have documented the importance of DNAm to mental health disorder risk, including differential change in DNAm of *BDNF* among individuals with *versus* without borderline personality disorder (Perroud *et al.* 2013), increased *DAT* (*SLC6A3*) DNAm with age that may be driven by alcohol dependence (Nieratschker *et al.* 2014), and increasing *SERT* DNAm associated with bullying (Ouellet-Morin *et al.* 2013). Most relevant to the present study is work by 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

Table 3. *Putative transcription factor binding sites overlap DNMT CpG sites of interest*[a]

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 hematopoiesis, 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	–

DNMT, DNA methyltransferase.

[a] V$ matrix families indicate Genomatix-annotated transcription factor binding site matrix families. DNMT3B CpG sites are described in the Method section.

presented here are 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 & Corces, 2009), with strength of DNA binding inversely correlated with local DNAm (Barkess & 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 *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 add 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-exists and arises 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 4 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; furthermore, we did not collect data on 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 ICCs between experimental replicates for each of our assays increase 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 primary results presented in this work 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 2) 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 cut-off and would therefore be suggestive of a resiliency-associated change in DNAm (Table 2). 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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Declaration of Interest

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