Acta Psychiatrica Scandinavica

Acta Psychiatr Scand 2017: 135: 170–179

DOI: 10.1111/acps.12675

Epigenetic alterations of the *BDNF* gene in combat-related post-traumatic stress disorder

Kim TY, Kim SJ, Chung HG, Choi JH, Kim SH, Kang JI. Epigenetic alterations of the *BDNF* gene in combat-related post-traumatic stress disorder.

Objective: Brain-derived neurotrophic factor (BDNF) plays a crucial role in modulating resilience and vulnerability to stress. The aim of this study was to investigate whether epigenetic regulation of the *BDNF* gene is a biomarker of post-traumatic stress disorder (PTSD) development among veterans exposed to combat in the Vietnam War.
Methods: Using the Clinician-Administered PTSD Scale, combat veterans were grouped into those with (n = 126) and without (n = 122) PTSD. DNA methylation levels at four CpG sites within the *BDNF* promoter I region were quantified in the peripheral blood using pyrosequencing. The effects of *BDNF* DNA methylation levels and clinical variables on the diagnosis of PTSD were tested using binary logistic regression analysis.
Results: Subjects with PTSD showed a higher DNA methylation of four CpG sites at the *BDNF* promoter compared with those without PTSD. High methylation levels at the *BDNF* promoter CpG site, high combat exposure, and alcohol problems were significantly associated with PTSD diagnosis.
Conclusions: This study demonstrated an association between higher DNA methylation of the *BDNF* promoter and PTSD diagnosis in combat-exposed individuals. Our findings suggest that altered *BDNF* methylation may be a valuable biomarker of PTSD after trauma exposure.

T. Y. Kim[1], S. J. Kim[2], H. G. Chung[1], J. H. Choi[1], S. H. Kim[3], J. I. Kang[2]

[1]Department of Neuropsychiatry, Veterans Health Service Medical Center, Seoul, [2]Department of Psychiatry and Institute of Behavioral Science in Medicine, Yonsei University College of Medicine, Seoul and [3]Department of Psychiatry and Institute of Clinical Psychopharmacology, Dongguk University Ilsan Hospital, Goyang, Gyeonggi, South Korea

Key words: post-traumatic stress disorder; *BDNF*; epigenetics; DNA methylation; trauma

Jee In Kang, Department of Psychiatry, Yonsei University College of Medicine, Yonsei-ro 50-1, Seodaemun-gu, Seoul 03722, South Korea.
E-mail: jeeinkang@yuhs.ac

Accepted for publication November 7, 2016

Significant outcomes

- Subjects with PTSD showed a higher DNA methylation at the *BDNF* promoter I compared with those without PTSD among individuals exposed to combat.
- Hypermethylation at the CpG sites of the *BDNF* promoter I, high combat exposure and alcohol use were factors significantly associated with PTSD status.

Limitations

- It remains unclear whether the observed *BDNF* DNA methylation is a causal factor or a consequence of PTSD in the present cross-sectional study findings.
- Some potential confounding factors, such as early-life adversity, which can affect *BDNF* methylation, were not considered.
- The present sample consisted of older male veterans exposed to combat approximately 45 years ago, which limits generalizability of findings to other PTSD populations.

Introduction

Post-traumatic stress disorder (PTSD) is a debilitating mental disorder with characteristic symptoms including long-lasting re-experience of traumatic memory that can develop after exposure to a life-threatening traumatic event (1). Although precipitating trauma represents the major environmental factor of PTSD development, the biological mechanisms involved in the manifestation of PTSD following exposure to trauma are still not certain. Epigenetic alterations, representing key mechanisms by which environmental factors, such as life events, elicit enduring changes in gene expression, are proposed to contribute to the pathophysiology of PTSD (2–5).

Brain-derived neurotrophic factor (BDNF) is a key regulator of memory formation and stress response in the brain (6–8). BDNF and its receptor tyrosine kinase B (TrkB) regulate neuronal survival, growth, and differentiation during development, as well as synapse formation and neuroplasticity underlying acquisition and consolidation of memory in the adult brain, which is implicated in playing a crucial role in modulating stress resilience and vulnerability (9, 10). Several lines of evidence have implicated BDNF as a critical candidate biomarker underlying PTSD pathophysiology. First, stress has been demonstrated to contribute to the dysregulation of BDNF level. For example, in animal studies, early maternal separation reduced expression of hippocampal *BDNF* in adulthood (11), and re-exposure to cues previously associated with footshock decreased expression of *BDNF* mRNA in the hippocampus (12). In addition, findings from knockout and transgenic studies showed that *BDNF* was associated with impaired fear memory consolidation and extinction of conditioned fear, closely linked in the pathophysiology of PTSD (13, 14). While *BDNF*-heterozygous knockout mice exhibit reduced hippocampal long-term potentiation (15) and impaired contextual fear learning (16), transgenic mice overexpressing TrkB show improved contextual fear conditioning and reduced anxiety (17).

In particular, emerging evidence suggests that differential epigenetic regulation of the *BDNF* gene during the lifespan could play a crucial role in the pathophysiology of PTSD (5, 18–20). An animal model of PTSD showed that enhanced hippocampal BDNF and increased TrkB signaling in response to fear conditioning are associated with epigenetic regulation of the *BDNF* gene during fear memory consolidation, suggesting that the *BDNF* epigenetic alteration underlies long-lasting re-experience of traumatic memory in patients with PTSD (18). Another animal study showed that adulthood psychosocial stress can induce increased *BDNF* DNA methylation and decreased levels of *BDNF* mRNA in the dorsal hippocampus, a cellular mechanism underlying the pathophysiology of PTSD (19). Further, an epigenetic study in adult rats reported that hippocampal *BDNF* DNA methylation is regulated in response to contextual fear conditioning and that an infusion of DNA methyl-transferase inhibitor causes *BDNF* DNA demethylation and increased mRNA levels of exons I, IV, and VI with a corresponding increase in total *BDNF* gene expression in the hippocampus *in vivo* (20). Another study using a fear conditioning and extinction paradigm in mice showed that chronic fluoxetine treatment enhances synaptic plasticity and fear extinction through increased *BDNF* exon I and total *BDNF* mRNA levels in the amygdala and *BDNF* exon I level in the hippocampus (21). While a variety of animal models of fear memory formation and PTSD-like symptoms have revealed epigenetic regulation of *BDNF*, only a few studies in human clinical populations have reported that altered BDNF levels may be implicated in the development and recovery of PTSD, suggesting the potential role for epigenetic regulation of *BDNF* in the pathophysiology of PTSD (22, 23).

The aim of this study was to investigate whether epigenetic regulation of the *BDNF* gene is a biomarker of PTSD status among veterans exposed to combat in the Vietnam War. In addition, the influence of several potential factors, including combat exposure levels and alcohol use, was examined in the pathophysiology of PTSD. Our main hypothesis was that higher *BDNF* DNA methylation status might be related to the risk of PTSD among trauma-exposed individuals.

Material and methods

Participants

The study was approved by the institutional review board of the Veterans Health Service (VHS) Medical Center in Seoul, South Korea, and all subjects gave their written informed consent before participating. All subjects were Korean male veterans who served on active duty during the Vietnam War. They were administered a structured, face-to-face interview by a trained psychiatrist (T. Y. Kim). The exclusion criteria for the study included a history of head trauma, organic brain syndrome including cerebrovascular accidents or dementia, psychosis or bipolar disorder, or dependence on substances other than alcohol and nicotine.

A total of 116 male patients with a principal diagnosis of combat-related PTSD who met DSM-IV-TR (1) diagnostic criteria for PTSD were recruited from an out-patient mental health clinic at the VHS Medical Center. A total of 140 potential control subjects were recruited through advertisements from non-psychiatry out-patient clinics in the same hospital. Of these, 12 were diagnosed with PTSD based on the DSM-IV-TR and reclassified to the PTSD group. In total, 128 subjects with PTSD and 128 (non-PTSD) control subjects were included.

Assessment of clinical characteristics

The Clinician-Administered PTSD Scale (CAPS), which is one of the most useful measures of PTSD (24, 25), was applied for assessing symptoms of the disorder. CAPS is a structured clinical interview designed to assess the presence of PTSD and the frequency and intensity of each PTSD symptom dimension, ranging from 0 (never) to 4 (daily or almost every day) and 0 (none) to 4 (extreme) respectively. PTSD status was determined by symptom frequency and intensity based on the 'F1/I2' rule (liberal scoring rule; frequency $\geq$1 and intensity $\geq$2 for each item) (26).

In addition, the Combat Exposure Scale (CES) was used for assessing the level of wartime stressors experienced by combatants (27). The CES, a widely used 7-item, 5-point Likert self-rating scale, has demonstrated good internal consistency and reliability in previous PTSD research with veterans (27). The total CES score (ranging from 0 to 41) was calculated by using a sum of weighted score, which can be divided into six categories of combat exposure: no combat (0), light (1–8), light–moderate (9–16), moderate (17–24), moderate–heavy (25–32), and heavy (33–41). The responses to the CES were classified into dichotomous categories of high combat exposure ($\geq$25, 'moderate-to-heavy' or 'heavy') vs. light-to-moderate levels of combat exposure (<25) based on a previous result that showed 'moderate-to-heavy' or 'heavy' levels of combat exposure associated with high risk of PTSD, compared with light-to-moderate levels (28).

The Alcohol Use Disorders Identification Test (AUDIT) was also used to identify participants with problematic alcohol use (29). This screening questionnaire includes 10 items relating to hazardous and harmful alcohol use. The AUDIT score of 8 or higher was considered to indicate the presence of problematic alcohol drinking (29).

DNA methylation analysis of the *BDNF* promoter region

Among *BDNF* promoter regions, we targeted *BDNF* promoter I region, which has been implicated in fear memory formation and antidepressant responses (18, 21). In particular, four CpG sites were tested in the CpG-rich region of the *BDNF* promoter I, based on previous epigenetic clinical studies of depression in Korean samples (30, 31). The DNA of the subjects was isolated from whole blood cells using standard techniques for DNA methylation analysis. The *BDNF* promoter region for analyzing methylation status was positioned between −694 and −654, relative to the translation start site considered as +1 in *BDNF* exon I (CpG1 = −688, CpG2 = −686, CpG3 = −682, CpG4 = −675) (Fig. 1a). The position was determined according to the Genome Build 38 of the NCBI human reference assembly. *BDNF* DNA methylation was analyzed using the bisulfite pyrosequencing method as previously described (30, 32). The Pyrosequencing Assay Design Software v2.0 (Qiagen, Hilden, Germany) was used for designing PCR and sequencing primers. The PCR was performed in a volume of 20 μl with 20 ng or more converted DNA, 2.5 μl of 10 × Taq buffer, 5 U Hot/Start Taq polymerase (Enzynomics, South Korea), 2 μl of each 2.5 mM dNTP mixture, 1 μl of 10 pmol/μl Primer-S, and 1 μl of 10 pmol/μl biotinylated-Primer-As. The amplification was conducted according to the general guidelines of the pyrosequencing method: denaturation at 95°C for 10 min, followed by 45 cycles at 95°C, 55°C, and 72°C, each for 30 s, followed by a final extension cycle at 72°C for 5 min. The PCR product (2 μl) was electrophoresed on a 2% agarose gel (SeaKem LE Agarose; Lonza Rockland, Inc., Rockland, ME, USA) and visualized by staining with ethidium bromide. The single-stranded DNA templates were prepared from 16 to 18 μl of biotinylated PCR products using streptavidin-coated Sepharose® HP beads (Amersham Biosciences, Uppsala, Sweden) and following the PSQ 96 sample preparation guide for multichannel pipettes. Fifteen picomoles of the respective sequencing primers was used for analysis. Sequencing was performed using a PyroMark ID system with the Pyro Gold reagents kit (Qiagen). The percentages of individual methylation at four CpG sites were calculated, as shown in a representative pyrogram (Fig. 1b). The analysis of a non-CpG cytosine during pyrosequencing was included to provide the internal quality control of the completeness of bisulfite treatment. The pyrosequencing procedures were carried out using the service of Genomictree, Inc. (Daejeon, South Korea).

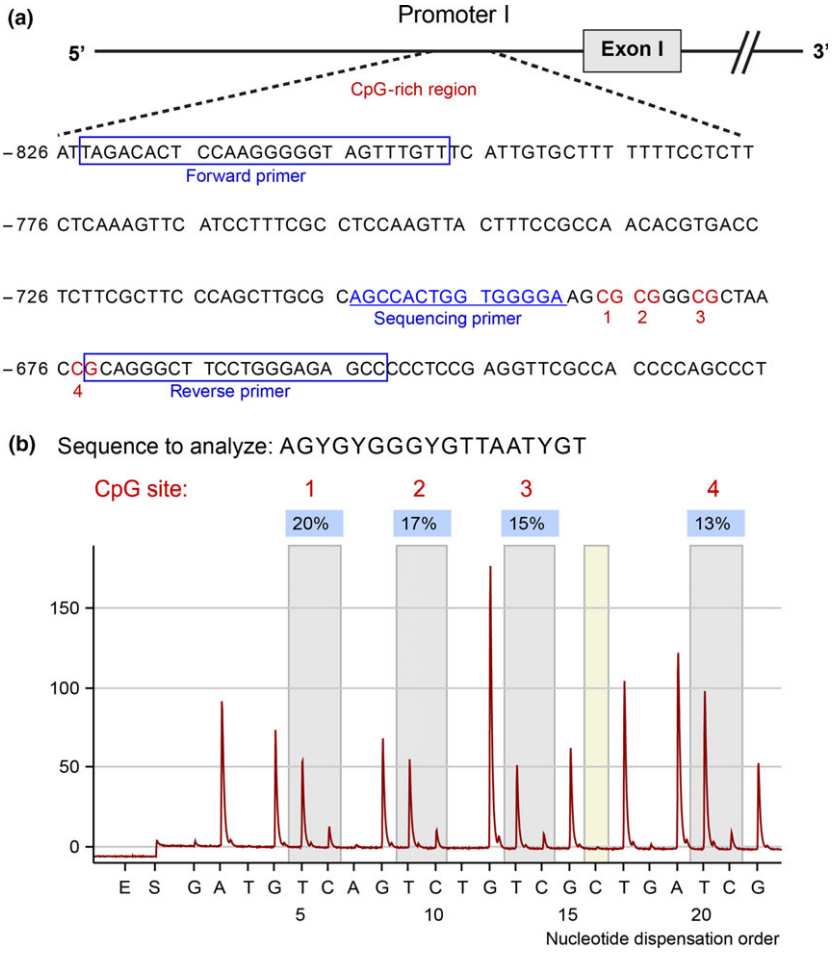

Fig. 1. DNA methylation analysis of the *BDNF* promoter I region. (a) *BDNF* promoter I region sequenced in CpG methylation analysis. (b) A representative pyrogram of the four CpG sites in the *BDNF* promoter. Dispensations corresponding to the potentially methylated cytosine (C or T after bisulfite treatment) are highlighted in light gray. The ratio of the cytosine (methylation) peak height to the sum of cytosine and thymine peak heights within each CpG site in automated DNA sequencing traces is calculated, which are the values in blue boxes on top of each of the CpG sites. The non-CpG C position analyzed is highlighted in yellow as the built-in quality control site to verify bisulfite conversion. [Colour figure can be viewed at wileyonlinelibrary.com]

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA), version 23.0. Significance level was accepted at $P < 0.05$, and all tests were two-tailed.

We assumed that the present data would be normally distributed according to the Central Limit Theorem (33) and then employed parametric statistical tests, although some variables, such as methylation levels, were not perfectly normally distributed. Demographics and clinical characteristics between participants with and without PTSD were compared using the chi-square or Student's *t*-test. Pearson's correlation coefficients (*r*) were used for examining relationships between PTSD symptom scores and possible related factors.

The logistic regression analysis was used to model the dichotomous outcome of PTSD being present vs. absent, and the Hosmer–Lemeshow goodness-of-fit test was conducted for models' performance. *BDNF* DNA methylation levels at four CpG sites within the promoter region were tested using binary logistic regression analysis with the forward conditional method to identify major determinants that best predict PTSD diagnosis. In addition, when statistical significance was found in the Pearson's correlation analyses or when clinical significance has been reported as a predictor of PTSD in previous studies, the potential associated factors were also selected as independent variables of the regression models. The outcome variable was analyzed yielding odds ratios (ORs) with 95% confidence intervals (CIs) and *P* values.

Additionally, a mediation analysis was used to assess whether the *BDNF* DNA methylation level is a mediator between associated factors and PTSD using the R package mediation (34). The mediation effect was estimated using the quasi-Bayesian Monte Carlo method based on normal approximation and the 95% CIs were obtained through 1000 simulations.

Results

Of the 256 people who participated in the interview, data from eight additional veterans were not included in the final analysis: Four veterans in the non-PTSD group who reported their war trauma

Table 1. Demographic and clinical characteristics of participants with and without current PTSD

	PTSD (N = 126)	Non-PTSD (N = 122)	T or χ^2	P
Age	63.16 ± 3.53	62.86 ± 4.39	0.59	0.56
Education (years)	10.40 ± 2.88	10.49 ± 3.18	−0.23	0.82
Marital status: Married/Other, *n*	108/18	113/9	3.05	0.10
Socioeconomic status: High/Medium/Low, *n*	25/56/45	20/58/44	0.54	0.76
Total CAPS	62.53 ± 22.22	9.16 ± 11.52	23.85	<0.001
Re-experience	20.65 ± 8.55	4.31 ± 7.51	16.01	<0.001
Avoidance	21.30 ± 10.17	1.61 ± 3.40	20.58	<0.001
Hyperarousal	20.58 ± 8.15	3.31 ± 4.23	21.03	<0.001
Combat exposure level Light-to-moderate/High, *n*	92/34	110/12	12.06	0.001
AUDIT score	11.76 ± 10.96	6.80 ± 7.58	4.15	<0.001
Alcohol problem: Yes/No, *n*	73/53	45/77	11.01	0.001
Use of psychoactive medication: Yes/No, *n*	91/35	23/99	71.08	<0.001
Use of SSRI: Yes/No, *n*	78/48	18/104	58.08	<0.001
BDNF methylation				
CpG 1	9.63 ± 3.36	8.82 ± 3.18	1.94	0.053
CpG 2	9.34 ± 3.42	8.43 ± 3.52	2.08	0.039
CpG 3	8.29 ± 2.74	7.43 ± 3.04	2.32	0.021
CpG 4	10.91 ± 3.31	9.79 ± 3.46	2.62	0.009
CpG mean	9.54 ± 3.06	8.62 ± 3.22	2.08	0.021

CAPS, Clinician-Administered PTSD Scale; AUDIT, Alcohol Use Disorders Identification Test; SSRI, selective serotonin reuptake inhibitor; *BDNF*, brain-derived neurotrophic factor.

in an interview but scored as 0 on the self-report CES were excluded due to incongruent information related to combat exposure, and another two participants in each group were excluded due to insufficient DNA. Thus, the final analyses included 126 patients with PTSD and 122 control subjects.

Demographic and clinical characteristics of participants are presented in Table 1. The mean age in the final sample was 63.16 years (standard deviation; SD = 3.53 years) for the PTSD group and 62.86 years (SD = 4.39 years) for the non-PTSD group. There were no statistically significant differences between the two groups in demographic characteristics including education, marital status, or socioeconomic status.

Subjects with PTSD were more likely to be associated with harmful alcohol drinking based on the AUDIT score. According to the AUDIT cutoff system (total AUDIT ≥ 8), 57.9% (n = 73/126) of patients with PTSD were classified as probable harmful alcohol users, while 36.9% (n = 45) of people without PTSD were classified.

For combat exposure, the CES scores were 20.44 ± 7.15 in the PTSD group and 14.19 ± 7.74 in the non-PTSD group (P < 0.001). When level of combat exposure was categorized based on the CES score in the PTSD group, 4% (n = 5) reported light combat exposure, 23% (n = 29) reported light-to-moderate exposure, 46% (n = 58) reported moderate exposure, 21.4% (n = 27) reported moderate-to-heavy exposure, and 5.6% (n = 7) reported heavy combat exposure. Among people without PTSD, the corresponding proportions of combat exposure were 27.0% (n = 33), 39.3% (n = 48), 23.8% (n = 29), 9.0% (n = 11), and 0.8% (n = 1) respectively. The distribution of the five CES categories between PTSD and non-PTSD showed a significant difference (X^2 = 46.17, df = 4, P < 0.001), with a higher proportion of high combat exposure in people with PTSD than in those without PTSD.

In Student's t-test, subjects with PTSD showed a higher DNA methylation of four CpG sites at the *BDNF* promoter and higher CAPS scores, compared with those without PTSD (Table 1). Because *BDNF* methylation levels at the four CpG sites were highly correlated with each other (r = 0.87–0.95, P < 0.001), the mean methylation degree at the four CpG sites was applied as a variable in the analyses. There was a weak positive relationship between the *BDNF* DNA methylation level and PTSD symptom scores based on CAPS (r = 0.17, P = 0.008). In addition, *BDNF* DNA methylation levels showed a weak positive correlation with CES scores (r = 0.15, P = 0.019), whereas it had no correlation with AUDIT scores (r = 0.019, P = 0.77).

Binary logistic regression analysis for predicting PTSD included the following independent variables: age, education, marital status, socioeconomic status, the CES level (the dichotomized high [≥25] and light-to-moderate combat exposure ([<25]), the presence of harmful alcohol drinking, and the mean methylation degree of the *BDNF* promoter I region. Table 2 shows the best fit model for the data, as the Hosmer–Lemeshow goodness-of-fit test statistic was 9.527 (P = 0.30). In the regression model, high combat exposure, harmful alcohol drinking, and high

Table 2. Forward conditional logistic regression analysis for predicting PTSD diagnosis in male veterans (N = 248)

	B	Std. Error	Wald test	P	Exp(B)	CI for Exp(B)
BDNF CpG	0.092	0.044	4.340	0.037	1.097	1.005–1.197
Alcohol problem	0.863	0.270	10.235	0.001	2.357	1.388–4.002
CES level	1.096	0.375	8.555	0.003	2.991	1.435–6.234

248 patients (group with PTSD, N = 126; group without PTSD, N = 122) were included in the regression analysis. Model summary: −2 log-likelihood = 316.92, Nagelkerke R^2 = 0.137; overall percentage of correct classification resulting from the model = 62.5%.

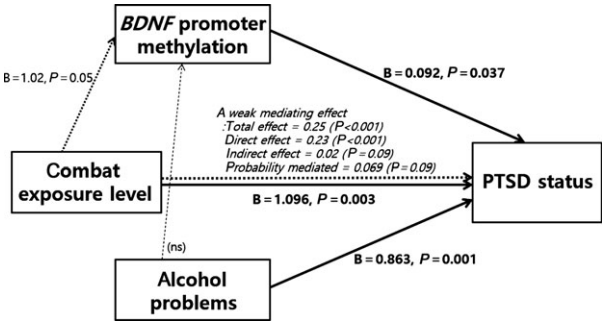

Fig. 2. Logistic regression (solid line) and mediation model (dotted line) of PTSD status in veterans exposed to combat.

BDNF methylation status were significantly associated with PTSD diagnosis (Table 2).

In the additional mediation analyses of the regression model, there were no significant indirect associations from trauma exposure and alcohol consumption to PTSD diagnostic status via *BDNF* methylation levels. *BDNF* methylation levels showed a weak mediating effect in the path from trauma exposure to PTSD status, even though the indirect effect did not reach statistical significance ($P = 0.09$, $B = 0.02$, 95% CI [−0.003, 0.059]). To obtain a better understanding of relationships between the major factors and PTSD status, the results from the regression model and additional mediation analyses are depicted in Fig. 2.

Discussion

The present study demonstrated an association between higher DNA methylation of the *BDNF* promoter region and chronic PTSD in Korean combat veterans of the Vietnam War. The major strength of the present study is that it contributes epigenetic evidence of molecular determinants predicting individual differences in susceptibility to PTSD in a relatively homogenous group of people exposed to similar trauma. A key finding of our study is that *BDNF* promoter methylation status was associated with PTSD diagnosis in combat-exposed individuals, suggesting that traumatic events may influence PTSD pathophysiology via epigenetic modulation of *BDNF*.

To the best of our knowledge, there has been little research in humans of epigenetic regulation of *BDNF* in PTSD. Our observation of altered *BDNF* methylation in patients with PTSD is in agreement with previous reports in animal PTSD models of *BDNF* epigenetic changes in response to experience. An epigenetic study in male rats showed that traumatic stress manipulations in adulthood produced PTSD-like symptoms in rats via significant *BDNF* DNA hypermethylation and *BDNF* mRNA hypoexpression in the dorsal CA1 subregion of the hippocampus (19). Another study using a rodent contextual fear conditioning paradigm showed dynamic epigenetic changes in the adult brain after fear conditioning, including upregulated *BDNF* mRNA in adult hippocampus via DNA methylation and chromatin remodeling during consolidation of fear memory, and *BDNF* DNA de-methylation and subsequently increased *BDNF* mRNA levels by a DNA methyl-transferase inhibitor (20). The extinction of conditioned fear was reported to be associated with histone modifications at *BDNF* promoters and enhanced *BDNF* exon I and IV mRNA expression in the prefrontal cortex, implicating epigenetic regulation of *BDNF* as an important factor in the persistence of pathological fear (35). These findings suggest that traumatic stress occurring in adulthood can induce epigenetic changes of the *BDNF* gene, which may be a molecular mechanism underlying the pathophysiology of PTSD in people exposed to trauma.

In the present prediction model of PTSD, high combat exposure and problematic alcohol use, in addition to *BDNF* methylation levels, were factors significantly associated with PTSD status. Combat exposure, alcohol drinking, and *BDNF* methylation status explained 6.6%, 4.8%, and 2.3% of the variation in PTSD diagnosis respectively. While it is not surprising that high levels of external trauma are a major risk factor for developing PTSD (28), how the noted factors of PTSD work together to influence PTSD in trauma-exposed individuals warrants further consideration. There may be at least two ways external trauma and *BDNF* methylation can work together. One possible explanation is that alterations in *BDNF* methylation may mediate neurobiological changes in the development and persistence of PTSD following trauma exposure. Although its mediating effect in the present mediation analysis only reached a trend level of significance ($P = 0.09$), *BDNF* methylation status may indeed contribute to the relationship between trauma exposure and PTSD. Further studies with greater statistical power to detect subtle differences in methylation changes will help definitively determine a mediating effect of *BDNF* methylation thereon. Another plausible explanation is that *BDNF* methylation may moderate the effect of trauma exposure on PTSD development, if *BDNF* epigenetic modifications temporally precede traumatic events. Previous studies have reported that some factors triggered in early development, such as prenatal exposure to maternal stress (36) and postnatal early-life trauma (32, 37), may affect epigenetic programming and subsequently contribute to the observed *BDNF* methylation levels.

Therefore, preexisting *BDNF* epigenetic modification derived from prior cumulative stress effects, such as early-life trauma, may serve to amplify stress reactions and fear memory formation in response to adulthood trauma, which might ultimately lead to the development and persistence of PTSD. Although their complex relationships of PTSD cannot be inferred clearly from the present data without information on temporal precedence, *BDNF* methylation may be a valuable biomarker of PTSD in trauma-exposed individuals.

Increased *BDNF* DNA methylation status in PTSD can be also implicated as a potential treatment target for regulating the recovery of pathological fear memory formation. BDNF, a key mediator for neuronal plasticity, is known to be elevated following a course of antidepressant treatment such as selective serotonin reuptake inhibitors (SSRIs), the most common pharmacological treatment for PTSD (38). Previous studies have also shown that altered *BDNF* expression can be reversed by the proper pharmacological intervention such as chronic administration of antidepressants. A study in rat brain showed that the SSRI fluoxetine increased hippocampal *BDNF* mRNA, and it also partially reversed the corticosterone-induced downregulated *BDNF* expression in hippocampus (39). Furthermore, in mice with chronic social defeat stress, increased histone acetylation and downregulated *BDNF* expression in the hippocampus were normalized by chronic imipramine treatment (40). The epigenetic mechanisms of imipramine or paroxetine were reported to be mediated primarily through inhibition of DNA methyl-transferase 1 activity in rat cortex (41). These findings propose that epigenetic modulation of *BDNF* may be a valuable strategy for modifying fear conditioning and preventing and treating PTSD through synaptic plasticity. As DNA methylation may represent a combination of state- and trait-dependent effects, further prospective research that incorporates multiwave longitudinal data may provide a better biological basis for establishing causality and recovery of PTSD.

However, given the difficulty of assessing *BDNF* methylation in the brain tissue directly, we investigated *BDNF* promoter methylation in peripheral leukocytes. The question remains how *BDNF* methylation expressed by blood cells parallels *BDNF* methylation in brain tissue. It has been shown that BDNF may cross the blood–brain barrier (42) and a positive correlation between BDNF levels in blood and hippocampus was reported (43). In addition, peripheral BDNF level was reported to be dysregulated in stress-related psychiatric disorders including PTSD (22, 44). Thus, although we could not answer this question clearly in the present data, we hypothesized that the observed methylation pattern in peripheral blood might be relevant to the brain. This is also supported by several recent studies, in which peripheral *BDNF* methylation status was associated with psychiatric symptoms, such as depression, suggesting that it may be a potential biomarker of psychiatric disorders (45, 46). Moreover, DNA methylation patterns in CpG-rich promoters were shown to be less tissue specific across blood and brain regions (47, 48). On the contrary, several studies reported that DNA methylation markers are inconsistent across tissues (49, 50). Although there is controversy about whether epigenetic changes in the peripheral blood reflect expression changes in the brain or not, our findings suggest that peripheral DNA methylation changes in *BDNF* may be a possible biomarker for PTSD status.

On the other hand, hazardous alcohol use, a significant factor for present PTSD, showed no significant association with *BDNF* methylation status. Considering mounting evidence of a relationship between alcohol consumption and *BDNF* promoter methylation (51), the present negative result may also be attributable to insufficient statistical power to detect a subtle effect and caution should be warranted in interpreting these findings. Alcohol use disorders frequently co-occur with PTSD among both civilians and veterans (52, 53). The causal relationship between PTSD and comorbid alcohol use disorders is complex because some patients with PTSD try to relieve their symptoms via alcohol drinking and consequently develop alcohol use disorders, while others with hazardous alcohol use are susceptible to PTSD and subsequently develop PTSD following trauma exposure (54). According to the common factors hypothesis, the association between PTSD and alcohol use disorders would be attributable to shared biological vulnerability factors (53).

In the present data, 72.2% and 18.9% of participants with and without PTSD have taken any psychoactive drugs, and the most common medication used was SSRI. The proportion of SSRI users in the PTSD group in our data was significantly higher than that in the non-PTSD group. Considering the effects of SSRIs on epigenetic changes in *BDNF* as mentioned above, SSRI use may affect *BDNF* methylation levels in our results. Although this issue requires further confirmation in drug-naïve samples, it seems unlikely that SSRI use contributes to *BDNF* hypermethylation observed in the PTSD group because SSRIs tend to have the opposite effect.

Several potential limitations of this study should be noted. First, although bisulfite pyrosequencing is known as a highly specific and sensitive screening technique for DNA methylation analysis, it may often limit the acquisition of reliable data on DNA methylation status, which may lead to miss detecting a subtle difference or make a false-positive result. Thus, to generalize the present prediction model of PTSD, it should be replicated in independent PTSD samples with larger sample sizes using highly accurate tools. New techniques, such as nanowire transistor and force spectroscopy, in combination with the current pyrosequencing, would be helpful to developing more accurate detection of DNA methylation and more reliable and reproducible results. Second, we employed both groups of trauma-exposed PTSD and trauma-exposed non-PTSD and did not recruit trauma-unexposed healthy control group, because our main purpose was to determine the influence of *BDNF* methylation on PTSD pathophysiology under similar environmental conditions, rather than not to examine the effects of trauma exposure itself. When trauma-unexposed healthy controls are also included and compared with trauma-exposed non-PTSD group, it may enable them to identify a function of trauma exposure on *BDNF* methylation. Third, the present sample consisted of older male veterans (average age 63 years) exposed to combat approximately 45 years prior, limiting generalization of present findings to other PTSD populations, although our sample set is likely to be a more homogeneous group with similar trauma and demographic characteristics of age, educational level, sex, and ethnic origin. Furthermore, the present control group (combat-exposed individuals without PTSD) may include some remitted individuals with a past PTSD diagnosis, who could have different *BDNF* methylation patterns from individuals who have never had a PTSD. Finally, we did not consider some possible confounding factors affecting epigenetic changes of *BDNF*, such as early-life trauma, recent stressors, and amount of physical activity. However, we believe that these limitations do not detract from the major significance of our results, which will require further confirmation in independent PTSD samples with consideration of various confounding factors.

In summary, the present study of Korean combat veterans demonstrates an association between higher DNA methylation of the *BDNF* promoter I region and PTSD. Hypermethylation at the CpG sites of the *BDNF* promoter I, high combat exposure, and alcohol use were factors significantly associated with PTSD status. Although we cannot definitively conclude how the related factors work together to affect PTSD pathophysiology from the present data, the findings suggest that altered *BDNF* methylation may be a valuable biomarker of PTSD after trauma exposure. Further prospective research examining dynamic epigenetic changes in the *BDNF* locus in people exposed to trauma might shed light on the role and the biological basis of *BDNF* in susceptibility to PTSD.

Acknowledgements

This work was supported by grants from the Disease Oriented Translational Research of Korea funded by the Ministry of Education, Science, and Technology (HI14C0202). The funding source did not give any influences on the study design, data collection, analysis and interpretation of data, the writing of the report, and the decision to submit the manuscript for publication.

References

1. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 4th edn, text revision. Washington: DC American Psychiatric Association, 2000.
2. Voisey J, Young RM, Lawford BR, Morris CP. Progress towards understanding the genetics of posttraumatic stress disorder. J Anxiety Disord 2014;**28**:873–883.
3. Zannas AS, Provencal N, Binder EB. Epigenetics of posttraumatic stress disorder: current evidence, challenges, and future directions. Biol Psychiatry 2015;**78**:327–335.
4. Malan-Muller S, Seedat S, Hemmings SM. Understanding posttraumatic stress disorder: insights from the methylome. Genes Brain Behav 2014;**13**:52–68.
5. Zovkic IB, Sweatt JD. Epigenetic mechanisms in learned fear: implications for PTSD. Neuropsychopharmacology 2013;**38**:77–93.
6. Licinio J, Wong M. Brain-derived neurotrophic factor (BDNF) in stress and affective disorders. Mol Psychiatry 2002;**7**:519.
7. Vollmayr B, Faust H, Lewicka S, Henn FA. Brain-derived-neurotrophic-factor (BDNF) stress response in rats bred for learned helplessness. Mol Psychiatry 2001;**6**:471–474, 358.
8. Gray JD, Milner TA, McEwen BS. Dynamic plasticity: the role of glucocorticoids, brain-derived neurotrophic factor and other trophic factors. Neuroscience 2013;**239**:214–227.
9. Park H, Poo MM. Neurotrophin regulation of neural circuit development and function. Nat Rev Neurosci 2013;**14**:7–23.
10. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 2003;**4**:299–309.
11. Roceri M, Hendriks W, Racagni G, Ellenbroek BA, Riva MA. Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. Mol Psychiatry 2002;**7**:609–616.
12. Rasmusson AM, Shi L, Duman R. Downregulation of BDNF mRNA in the hippocampal dentate gyrus after re-exposure to cues previously associated with footshock. Neuropsychopharmacology 2002;**27**:133–142.
13. Heldt SA, Stanek L, Chhatwal JP, Ressler KJ. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. Mol Psychiatry 2007;**12**:656–670.

14. Soliman F, Glatt CE, Bath KG et al. A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. Science 2010;**327**:863–866.
15. Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci USA 1995;**92**:8856–8860.
16. Liu IY, Lyons WE, Mamounas LA, Thompson RF. Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. J Neurosci 2004;**24**:7958–7963.
17. Koponen E, Voikar V, Riekki R et al. Transgenic mice overexpressing the full-length neurotrophin receptor trkB exhibit increased activation of the trkB-PLCgamma pathway, reduced anxiety, and facilitated learning. Mol Cell Neurosci 2004;**26**:166–181.
18. Takei S, Morinobu S, Yamamoto S, Fuchikami M, Matsumoto T, Yamawaki S. Enhanced hippocampal BDNF/TrkB signaling in response to fear conditioning in an animal model of posttraumatic stress disorder. J Psychiatr Res 2011;**45**:460–468.
19. Roth TL, Zoladz PR, Sweatt JD, Diamond DM. Epigenetic modification of hippocampal Bdnf DNA in adult rats in an animal model of post-traumatic stress disorder. J Psychiatr Res 2011;**45**:919–926.
20. Lubin FD, Roth TL, Sweatt JD. Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J Neurosci 2008;**28**:10576–10586.
21. Karpova NN, Pickenhagen A, Lindholm J et al. Fear erasure in mice requires synergy between antidepressant drugs and extinction training. Science 2011;**334**:1731–1734.
22. Dell'osso L, Carmassi C, Del Debbio A et al. Brain-derived neurotrophic factor plasma levels in patients suffering from post-traumatic stress disorder. Prog Neuropsychopharmacol Biol Psychiatry 2009;**33**:899–902.
23. Berger W, Mehra A, Lenoci M et al. Serum brain-derived neurotrophic factor predicts responses to escitalopram in chronic posttraumatic stress disorder. Prog Neuropsychopharmacol Biol Psychiatry 2010;**34**:1279–1284.
24. Blake DD, Weathers FW, Nagy LM et al. The development of a clinician-administered PTSD scale. J Trauma Stress 1995;**8**:75–90.
25. Weathers FW, Keane TM, Davidson JR. Clinician-administered PTSD scale: a review of the first ten years of research. Depress Anxiety 2001;**13**:132–156.
26. Weathers FW, Ruscio AM, Keane TM. Psychometric properties of nine scoring rules for the Clinician-Administered PTSD Scale (CAPS). Psychol Assess 1999;**11**:124–133.
27. Keane TM, Fairbank JA, Caddell JM, Zimering RT, Taylor KL, Mora CA. Clinical evaluation of a measure to assess combat exposure. Psychol Assess 1989;**1**:53–55.
28. Wisco BE, Marx BP, Wolf EJ, Miller MW, Southwick SM, Pietrzak RH. Posttraumatic stress disorder in the US veteran population: results from the National Health and Resilience in Veterans Study. J Clin Psychiatry 2014;**75**:1338–1346.
29. Babor TF, Higgins-Biddle JC, Saunders JB, Monteiro MG. The alcohol use disorders identification test: guidelines for use in primary care. Geneva: World Health Organization, 2001.
30. Choi S, Han KM, Won E, Yoon BJ, Lee MS, Ham BJ. Association of brain-derived neurotrophic factor DNA methylation and reduced white matter integrity in the anterior corona radiata in major depression. J Affect Disord 2015;**172**:74–80.
31. Na KS, Won E, Kang J et al. Brain-derived neurotrophic factor promoter methylation and cortical thickness in recurrent major depressive disorder. Sci Rep 2016;**6**:21089.
32. Roth TL, Lubin FD, Funk AJ, Sweatt JD. Lasting epigenetic influence of early-life adversity on the BDNF gene. Biol Psychiatry 2009;**65**:760–769.
33. Field AP. Discovering statistics using IBM SPSS statistics, 4th edn. London: SAGE, 2013.
34. RC Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2014.
35. Bredy TW, Wu H, Crego C, Zellhoefer J, Sun YE, Barad M. Histone modifications around individual BDNF gene promoters in prefrontal cortex are associated with extinction of conditioned fear. Learn Mem 2007;**14**:268–276.
36. Boersma GJ, Lee RS, Cordner ZA et al. Prenatal stress decreases Bdnf expression and increases methylation of Bdnf exon IV in rats. Epigenetics 2014;**9**:437–447.
37. Unternaehrer E, Meyer AH, Burkhardt SC et al. Childhood maternal care is associated with DNA methylation of the genes for brain-derived neurotrophic factor (BDNF) and oxytocin receptor (OXTR) in peripheral blood cells in adult men and women. Stress 2015;**18**:451–461.
38. Sen S, Duman R, Sanacora G. Serum brain-derived neurotrophic factor, depression, and antidepressant medications: meta-analyses and implications. Biol Psychiatry 2008;**64**:527–532.
39. Dwivedi Y, Rizavi HS, Pandey GN. Antidepressants reverse corticosterone-mediated decrease in brain-derived neurotrophic factor expression: differential regulation of specific exons by antidepressants and corticosterone. Neuroscience 2006;**139**:1017–1029.
40. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci 2006;**9**:519–525.
41. Zimmermann N, Zschocke J, Perisic T, Yu S, Holsboer F, Rein T. Antidepressants inhibit DNA methyltransferase 1 through reducing G9a levels. Biochem J 2012;**448**:93–102.
42. Pan W, Banks WA, Fasold MB, Bluth J, Kastin AJ. Transport of brain-derived neurotrophic factor across the blood-brain barrier. Neuropharmacology 1998;**37**:1553–1561.
43. Klein AB, Williamson R, Santini MA et al. Blood BDNF concentrations reflect brain-tissue BDNF levels across species. Int J Neuropsychopharmacol 2011;**14**:347–353.
44. Matsuoka Y, Nishi D, Noguchi H, Kim Y, Hashimoto K. Longitudinal changes in serum brain-derived neurotrophic factor in accident survivors with posttraumatic stress disorder. Neuropsychobiology 2013;**68**:44–50.
45. Ikegame T, Bundo M, Murata Y, Kasai K, Kato T, Iwamoto K. DNA methylation of the BDNF gene and its relevance to psychiatric disorders. J Hum Genet 2013;**58**:434–438.
46. Zheleznyakova GY, Cao H, Schioth HB. Bdnf, DNA methylation changes as a biomarker of psychiatric disorders: literature review and open access database analysis. Behav Brain Funct 2016;**12**:17.
47. Masliah E, Dumaop W, Galasko D, Desplats P. Distinctive patterns of DNA methylation associated with Parkinson disease: identification of concordant epigenetic changes in brain and peripheral blood leukocytes. Epigenetics 2013;**8**:1030–1038.
48. Davies MN, Volta M, Pidsley R et al. Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. Genome Biol 2012;**13**:R43.
49. Beery AK, McEwen LM, Macisaac JL, Francis DD, Kobor MS. Natural variation in maternal care and cross-tissue patterns of oxytocin receptor gene methylation in rats. Horm Behav 2016;**77**:42–52.

50. Huang YT, Chu S, Loucks EB et al. Epigenome-wide profiling of DNA methylation in paired samples of adipose tissue and blood. Epigenetics 2016;**11**:227–236.
51. Heberlein A, Buscher P, Schuster R et al. Do changes in the BDNF promoter methylation indicate the risk of alcohol relapse? Eur Neuropsychopharmacol 2015;**25**:1892–1897.
52. Jacobsen LK, Southwick SM, Kosten TR. Substance use disorders in patients with posttraumatic stress disorder: a review of the literature. Am J Psychiatry 2001;**158**:1184–1190.
53. Norman SB, Myers US, Wilkins KC et al. Review of biological mechanisms and pharmacological treatments of comorbid PTSD and substance use disorder. Neuropharmacology 2012;**62**:542–551.
54. McLean CP, Su YJ, Foa EB. Posttraumatic stress disorder and alcohol dependence: does order of onset make a difference? J Anxiety Disord 2014;**28**:894–901.