Translational Brain Rhythmicity

Research Article

Norepinephrine transporter gene (*SLC6A2*) polymorphisms and promoter methylation in peripheral blood of veterans with posttraumatic stress disorder

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Abstract

Background: Norepinephrine (NE) dysregulation has been implicated as one of the mechanisms in the development of post-traumatic stress disorder (PTSD). Norepinephrine transporter (NET) plays an important role in regulating of NE level at the synapses. The gene of NET is a potential research target for PTSD. However, few studies have examined the genetic variants and DNA methylation of NET regards to the risk of PTSD.

Methods: We investigated 16 single-nucleotide polymorphisms (SNPs) across the NET whole gene in 506 veteran patients. Using a subset of individuals (161 patients) from our larger genetic dataset, promoter methylation was performed using pyrosequencing analysis.

Results: Significant difference was found in the genotype and allele frequencies for rs168924 and rs192303 by PTSD status, although the significance of rs192303 genotype (p=0.03) disappeared after Bonferroni correction. Neither rs168924 nor rs192303 was a significant predictor of PTSD diagnosis in primary logistic regression. However, significant interaction with Combat Exposure Scale (CES) was observed. The interaction effect was also positively associated with Clinician Administered PTSD Scale (CAPS). Higher CES and lower methylation were significantly associated with risk of PTSD, but no significant interaction effect was observed.

Conclusions: To our knowledge, this is the first study to extensively investigate NET gene Polymorphisms and promoter methylation among combat veteran PTSD. Our findings suggest the interaction of combat trauma and risk alleles of NET may account for the increased risk of PTSD. The NET promoter methylation may also participate in the process of PTSD development.

Introduction

Post-traumatic stress disorder (PTSD) is a maladaptive response to life-threatening events, characterized by symptoms cluster of re-experiencing of the traumatic event, avoidance and numbing, negative alterations in cognitions and mood, and increased vigilance and arousal [1,2]. Trauma exposure is a required risk factor for development of PTSD, but is not, by itself, sufficient to cause PTSD, as not everyone exposed to trauma develops PTSD. Emerging research from various studies has indicated significant genetic contribution to individual susceptibility to PTSD [3]. However, the gene or genes participating in the PTSD process and the mode of inheritance remains undetermined.

Catecholamine norepinephrine (NE), also known as noradrenaline (NA), is the principal chemical messenger in central noradrenergic and peripheral sympathetic synapses, which plays a critical role in the mammalian response to stress [4]. Norepinephrine (NE) dysregulation has been implicated as the cause behind specific symptom clusters in the pathophysiology of PTSD [5,6]. However, the underlying molecular mechanism is not well understood. Approximately 80–90% of released norepinephrine is taken up again through the neuronal norepinephrine transporter (NET) [7,8]. The NET, also known as solute carrier family 6 member 2 (*SLC6A2*), present on the plasma membrane of noradrenergic neurons, limits the action of NE through reuptake into the cytoplasm [4]. The gene, with 14 exons, is located on human chromosome 16q12.2 [9]. NET belongs to the monoamine transporter superfamily and consists of 617 amino acids with 12 membrane-spanning domains [9,10]. The NET not only regulates the longevity of NE in the synapse but also plays an important role in presynaptic and postsynaptic homeostasis [11-13]. Given the fact that substantial evidence has suggested the existence of impaired neuronal norepinephrine reuptake in PTSD patients and the central role of NET in the regulation of central nervous system and peripheral norepinephrine turnover, NET should be a potential research target for PTSD.

Since noradrenergic neurotransmission can be regulated by changes in NET expression, it appears reasonable to suggest that NET genetic variations may confer risk for altered central NE system putatively contributing to affective disorder and /or treatment response [14]. A number of polymorphisms have been reported for the NET gene [15-17]. Some polymorphisms may lead to an altered transcriptional

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activity by changes in the DNA structure or function [16,18-20]. Some polymorphisms have been shown to have a functional effect on *SLC6A2* gene expression [17]. Numerous single nucleotide polymorphisms (SNPS) within the SLC6A2 gene have been studied for association with psychiatric disorders, such as ADHD [15,20-23], major depression [24-26], panic disorder studies [27-29] and bipolar disorder [14], although there are mixed results. However, NET polymorphisms are poorly explored in PTSD. Only one letter report suggested that promoter SNP rs2242446 was independently associated with anxious arousal symptoms of PTSD and no positive association with the risk of PTSD was observed [30].

The complexity of the association between *SLC6A2* and PTSD may be related, in part, to emerging evidence that not only genetic, but also epigenetic factors shape risk of mental illness. Epigenetic dysregulation has been implicated in pathogenesis of several psychiatric disorders such as depression [31], schizophrenia [32], eating disorders [33], and PTSD [34,35]. DNA methylation, an emerging epigenetic mechanism, may explain the potential mechanisms that accounts for how trauma exposure leads to sustained PTSD symptoms. The DNA methylation is often linked to regulation of gene expression – both transcriptional silencing and activation [35,36], and can be influenced by environmental factors [37,38]. The NET gene promoter region is extraordinarily rich in CpG islands and so is a primer target for DNA methylation [39]. There are no earlier reports regarding the association between NET promoter methylation and PTSD, although studies have reported findings in postural orthostatic tachycardia syndrome [39,40], major depression [41] and panic disorder [39].

To better elucidate the molecular basis shaping risk of PTSD at the *SLC6A2* locus, we investigated whether variants within NET gene and promoter methylation are associated with the development of PTSD in veteran patients. We predicted that NET polymorphism and promoter methylation would differ by PTSD status and alleles' differences and altered promote methylation of NET would be associated with PTSD symptom clusters.

Materials and methods

Participants

A total of 506 combat veterans were enrolled in the study, recruited through two Veterans Affairs Medical Centers (VAMC), the Cincinnati VAMC, Cincinnati, OH and Charleston VAMC, Charleston, SC (Table 1). Of the 506 participants in the study, 64.4% (n = 326) met criteria for PTSD. The majority of the participants were male (83.0%) and Caucasian (68.6%). The research protocol was approved by the Institutional Review Boards (IRB) of both the University of Cincinnati and Medical University of South Carolina. Subjects were recruited using similar methods at the two sites.

Procedure

Participants were assessed for the presence of PTSD and other major psychiatric diagnoses by a board-certified psychiatrist with either the Structured Clinical Interview for DSM-IV (SCID) [42] or the Mini-International Neuropsychiatric Interview (MINI) [43]. Full diagnostic level data and demographic data are available for all 506 participants. The Combat Exposure Scale (CES) is used to obtain information regarding exposure to wartime stressor events, with a higher number reflecting a higher severity of combat exposure. Participants were also interviewed using the Clinician Administered PTSD Scale (CAPS) [44] to assess symptoms' frequency and intensity. All participants from the Charleston VAMC were recruited following this addition and a majority of participants from the Cincinnati VA in this present dataset. CAPS data is available for 404 of 506 participants and CES data is available for 478 out of 506 participants.

Table 1. Demographic and Clinical Characteristics. PTSD: posttraumatic stress disorder; CES: combat exposure scale; CAPS: clinician administered PTSD scale.

Variable	Combat Control (N= 180)	PTSD (N=326)
Age (years)	M=43.22	M= 43.07
	SD=14.34	SD= 14.88
Gender		
Male	143 (79.7%)	277(85.2 %)
Female	37 (20.3%)	48 (14.8 %)
Race		
EA	133 (73.9%)	218(66.9%)
AA	30 (16.7%)	94 (28.8%)
Others	17 (9.4%)	14 (4.3%)
CES	M=18.71	M=22.08
	SD=8.70	SD=8.21
CAPS	M=11.67	M=78.13
	SD=16.52	SD=17.83
Major Depression, N (%)		
No Depresion	180 (100%)	115 (35.3%)
Depression	0 (0.00%)	211 (64.7%)

Inclusion/Exclusion criteria

Inclusion criteria included: 1) a history of combat exposure, as evidenced by a DD214 (*i.e.,* official record of service) and 2) a report of combat exposure during the interview with a psychiatrist. In order to match the trauma-exposure severity between the PTSD subjects and controls, the majority of participants in the second half of this study were required to have CES score of 10 or above. Exclusion criteria included: current or lifetime DSM-IV schizophrenia, other psychotic disorders, bipolar disorder, and active substance abuse or dependence in the past six months. Combat veterans with comorbid major depression and anxiety disorders (as assessed during the SCID) were included. Individuals with a past history of substance abuse and dependence were also included if the last use of the substance was over 6 months prior to the enrollment.

SNP selection and genotyping

Genomic DNA was extracted from peripheral blood using a Wizard Genomic DNA purification kit (Promega, Madison, Wisconsin) following the manufacturer's protocol. 16 SNPs was randomly selected with minor allele frequencies of more than 0.1 to cover a region of 60 kb in the NET gene using a software tool (SNPbrowser version 4.0; Applied Biosystems) and a review of the literature with two exceptions: rs1805065 (MAF=0.0006) and rs5564 (MAF=0.07) since these two SNPs are located in special regions and has been studied in other disease (Bayles *et al.,* 2012). The average spacing between SNPs across the gene was 3.7 kilobases (kb). The SNPs were genotyped by the TaqMan, probe-based SNP genotyping assay on the ABI StepOnePlus™ Real-Time PCR System ((Applied Biosystems, Foster City, CA).

Sodium Bisulfite Conversion and Pyrosequencing Analysis

Sodium bisulfite conversion of genomic DNA (20 μg) was obtained using Epitect Bisulphite kit (Qiagen), following the manufacturer's instructions. A total of 10 ng of modified DNA was subjected to PCR amplification of the specific promoter. Specially, two promoter regions and total 9 CpG sites across *SLC6A2* CpG island were selected to do the PCR application and Pyrosequencing: Hs_SLC6A2_01_PM (ABI cat#:

PM00173922) and Hs_SLC6A2_03_PM (ABI cat#: PM00173936). The primer sequences, the genomic location of the bisulfite pyrosequencing assays and the number of CpG sites investigated in each assay are shown in supplement 1. Following amplification, the biotinylated PCR products were purified and incubated with the sequencing primer designed to bind adjacent to the CpG sites of interest. Pyrosequencing was conducted using a PyroMark Q24 instrument (Qiagen), with subsequent quantitation of methylation levels determined with the PyroMark Q24 advance 3.0.0 software. Relative peak height differences were used to automatically calculate the percentage of methylated cytosines at each given site. The percent methylation fraction, i.e., the C/T ratio, was displayed above each CpG site in the analyzed sequence. Non-CpG cytosine residues were used as internal controls to verify efficient sodium bisulfite DNA conversion.

Statistical analysis

Statistical analysis was performed with SPSS software 21.0 (SPSS Inc. USA). Data distributions of all study variables were assessed for normality using the Shapiro-Wilk tests. Demographic and clinical variables were compared using chi-square χ^2 test for categorical variables and nonparametric Independent Mann-Whitney U Test for continuous variables (age, CES, CAPS). Allelic and genotypic associations were analyzed using chi-square and chi-square linear-by-linear association test respectively. Main and interaction model of logistic regression analysis, adjusting for demographics, was used to assess the effect of *SLC6A2* polymorphisms and methylation on the risk of PTSD. Main and interaction model of linear regression was also conducted to predict CAPS scores, controlling for demographics. The total methylation percentage across the 9 CpG sites was dichotomized based on the median value (median-split; 36.035) to improve the estimation stability of the logistic regression models [45]. Continuous variables including age, CAPS, and CES were centered to the mean. Given that we examined two related outcomes (one diagnostic and one quantitative phenotype) a Bonferroni correction was employed to adjust for multiple testing, and our *p*-value for determination of significance was $p<0.025$.

Haploview software (version 4.2; Broad Institute, Cambridge, Massachusetts, USA) was also used to analyze the status of pairwise linkage disequilibrium (LD) and haplotype frequencies.

Results

Demographic data and clinical features

A total of 506 subjects (180 combat controls, 326 PTSD) were enrolled in the study (Table 1). The combat control and PTSD groups did not differ significantly in regards to race (χ^2 [1, n = 506] = 2.288, p = 0.13) or gender χ^2 [1, n = 506] = 2.509, p = 0.113. Average age was also not significantly different between combat control (M=43.22, SD=14.34) versus PTSD (M=43.07, SD=14.88), z=-0245, p=0.807). Of the PTSD cases, 211 (64.7%) patients also had a diagnosis of MDD. Due to study selection resulting in a quasi-complete separation (*i.e.,* there were no cases of MDD in the control group), MDD was unable to be included as a covariate in analyses. Additionally, among participants with data for the CES (n=478), the level of combat trauma was significantly different between combat control (M=18.71, SD=8.70) versus PTSD (M=22.08, SD=8.21, Z=-3.95, p<0.001). Among participants with data for the CAPS (n=499), CAPS score was significantly different between combat control (M=11.67, SD=16.52) versus PTSD (M=78.13, SD=17.43), Z=-18.31, p<0.001).

Linkage disequilibrium and haplotype analysis

The LD map and block structure of the studied NET polymorphisms are presented in Figure 1. Five main haplotype blocks were detected from these 16 SNPs in our veteran population (Figure 1 and Table 2). Block 1 was formed from rs1168924 and rs2242446 in the promoter region , which was in high LD (Figure 1). GT haplotype from rs168924

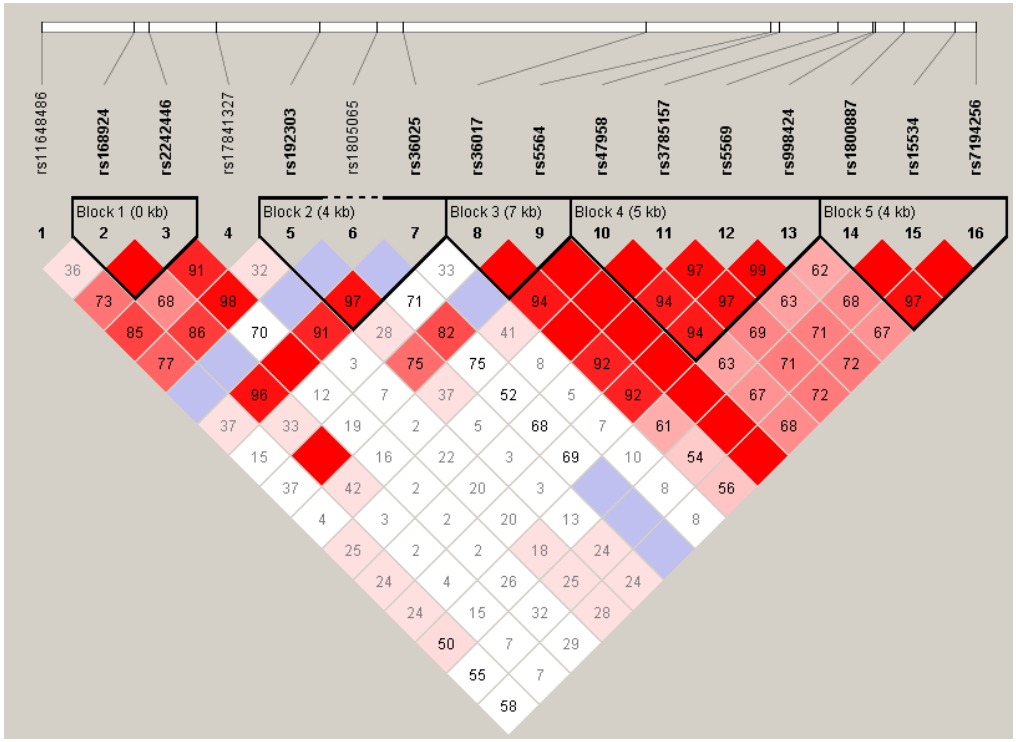

Figure 1. Linkage disequilibrium analyses: D' value of SNPs along the *SLC6A2*gene, illustrating five haplotype blocks. D' value was calculated by Haploview version 4.2

and rs2242446 was more frequent in PTSD than in control (p=0.0216) (Table 2). Block 2 was formed from rs192303 and rs36025 in the region of intron 1 to intron 2, which was in high LD (Figure 1). CC haplotype from rs192303 and rs36025 was more frequent in control than PTSD (p=0.0164) (Table 2). The results show that this region with intron 1 and intron 2 of the NET gene was associated with the development of PTSD. Block 3 was formed from the two loci rs36017 and rs5564 in the region of intron 3 to intron 5. Block 4 was formed rs47958, rs3785157, rs5569 and rs998424. Block 5 was formed rs1800887, rs15534 and rs7194256. Block 3, Block 4, and Block 5 were all in high LD (Figure 1), but no significant difference in haplotype frequencies were found between PTSD patients and combat controls (Table 2).

Allele and Genotype frequency of the SLC6A2 polymorphisms

To assess the influence of each NET variant on the incidence of PTSD, we performed further analyses. rs1800887 has significant deviation from Hardy-Weinberg equilibrium obverted in the populations and were not used in the later analysis (Supplement .2). Two SNPs (rs168924, rs192303) showed nominally significant allelic association with PTSD (χ^2 =5.28, p=0.0216; χ^2 =5.28, p=0.0216,) (Table 3). A significant association was found between the genotype frequency of rs168924 and PTSD (χ^2 =5.244, p=0.022,) and weak genotypic association for rs192303 was also observed (χ^2 =4.724, p=0.03, not significant after Bonferroni correction) (Table 3). No statistically significant differences either in the allele frequencies or in genotype frequencies for other NET polymorphisms were detected. When selecting the subset of participants who were Caucasian (n=351), only rs192303 has weak difference in allele and genotypic frequency by PTSD status (p=0.0325, p=0.027 respectively).

Multivariate logistic regression analysis for the influence of allele types of SLC6A2 and Combat Exposure Scale (CES) on the risk of PTSD diagnosis

Logistic regression analysis was performed to look at if CES and associated SNPs would affect the risk of PTSD after controlling demographics (Table 4). SNP rs168924 and rs192303 was entered into the model separately. In the main effects model, CES was strongly associated with PTSD status (OR=1.052, 95% CI=1.027-1.078, p<0.001), but allele types of rs168924 (p=0.090) and rs192303 (p=0.094) were not significant predictor of PTSD diagnosis after adjusting for age, sex and race. In the interaction model, the interaction effect of SNPxCES was significant (OR=1.075, 95% CI=1.012-1.142, p=0.019; OR=1.056, 95% CI=1.014-1.099, p=0.009 respectively). The interaction model also provided a significantly improved fit over the main effects model (χ^2 (1) =6.213, p=0.013, χ^2 (1) =7.273, p=0.007, respectively). So persons with more traumatic events and with more risk alleles of rs168924 or rs192303 might be at increased risk for PTSD.

Table 2. Haplotype analysis of *SLC6A2* gene in combat control and PTSD. PTSD: posttraumatic stress disorder; *SLC6A2:* solute carrier family 6 member 2.

Haplotype	Frequency of haplotype			Chi Square	P Value
	Total	Combat Control	PTSD		
Block 1					
AT	0.569	0.595	0.554	1.603	0.2055
AC	0.289	0.296	0.285	0.15	0.6982
GT	0.142	0.108	0.161	5.28	0.0216
Block 2					
CC	0.712	0.758	0.687	5.756	0.0164
GC	0.165	0.148	0.174	1.179	0.2775
GT	0.121	0.094	0.136	3.794	0.0514
Block 3					
GA	0.553	0.569	0.543	0.645	0.4218
CA	0.387	0.369	0.397	0.719	0.3966
CG	0.061	0.061	0.06	0.003	0.9552
Block 4					
ACGT	0.417	0.401	0.427	0.655	0.4184
CCGT	0.289	0.294	0.287	0.053	0.8184
CTAC	0.269	0.289	0.258	1.096	0.2952
Block 5					
TCC	0.733	0.747	0.725	0.534	0.4648
CTT	0.187	0.167	0.198	1.485	0.223
CCC	0.076	0.081	0.074	0.183	0.6687

Multivariable linear regression analysis for the association of allele types of SLC6A2 and Combat Exposure Scale (CES) on the PTSD symptom severity

Multivariable linear regression analysis was also conducted to control other variables (table 5). In the main model, rs168924, rs192303 allele types were positively associated with CAPS score (β=0.1, p=0.029, β=0.085, p=0.062), but not statistically significant. In the interaction model, the interaction term for SNP X CES was also positively associated with CAPS Score but rs192303xCES not significant after Bonferroni correction (β=0.114, p=0.024; β=0.128, p=0.034, respectively). Based on the adjusted R^2, both of the interaction models also explained 7% more of the variance in PTSD symptom severity than the main effects models. CES was always a significant predictor of PTSD symptom severity (p<0.0001). So persons with more traumatic events and with more risk alleles of rs168924 or rs192303 may develop more severe symptoms in PTSD.

Effect of SLC6A2 methylation and Combat Exposure Scale (CES) on the risk of PTSD diagnosis

A total of 161 samples (57 combat controls, 104 PTSD) were used to do the DNA methylation sequencing analysis. These patients were all recently recruited from Charleston VAMC and relatively young. Multivariate logistic regression analysis was performed for the influence of *SLC6A2* methylation and CES on the risk of PTSD diagnosis (Table 6). In the main model, Methylation group is a significant predictor of risk of PTSD (OR=0.383, 95% CI=0.171-0.857, p=0.020), indicating that low methylation showed higher risk of PTSD after controlling for demographics. Although higher CES also showed increased risk of PTSD (OR=1.077, 95% CI=1.031-1.125, p=0.001), the interaction model, the interaction term for Methylation X CES was not significant. So persons with low methylation level may independently have higher risk of PTSD.

Discussion

To our knowledge, this is the first report of SNP studies spanning the full spectrum of *SLC6A2* gene and also the first report of *SLC6A2* promoter methylation studies in peripheral blood of veterans with and without PTSD. We tested a total of 16 SNPS and found that promoter SNP rs168924 and intron 1 SNP rs192303 were linked to the susceptibility to PTSD. Our findings indicate that veterans with more traumatic events and with more risk alleles of rs168924 or rs192303 may be at increased risk for PTSD development. Veterans with low methylation level in SLC6A2 promoter region may independently have higher risk of PTSD. To argue against false positive findings, we analyzed the data with different statistic approaches. Our positive findings hold true regardless of analyzing approaches.

Table 3. Comparison of SLC6A2 Gene Genotype and Allele Frequencies among Combat Control and PTSD Individuals.PTSD: posttraumatic stress disorder; *SLC6A2:* solute carrier family 6 member 2.

	Genotype Count (Frequency)							Allele Count(frequency)						
		Non PTSD		PTSD					Non PTSD		PTSD			
		n	%	n	%	χ^2	*p*		n	%	n	%	χ^2	*p*
rs116484836	CC	127	70.6%	218	66.9%									
	TC	47	26.1%	98	30.1%	0.465	0.495	C	301	83.6%	534	81.9%	0.47	0.4932
	TT	6	3.3%	10	3.0%			T	59	16.4%	118	18.1%		
rsA168924	AA	143	79.4%	232	71.2%									
	AG	35	19.4%	82	25.1%	5.244	**0.022**	A	321	89.2%	547	83.9%	5.28	**0.0216**
	GG	2	1.1%	12	3.7%			G	39	10.8%	105	16.1%		
rs2242446	CC	13	7.3%	28	8.6%									
	TC	80	44.7%	129	39.7%	0.149	0.699	C	106	29.6%	185	28.5%	0.148	0.7004
	TT	86	48.0%	168	51.7%			T	252	70.4%	465	71.5%		
rs17841327	CC	61	34.0%	108	33.2%									
	AC	93	52.0%	168	51.7%	0.099	0.753	C	215	60.1%	384	59.1%		
	AA	25	14.0%	49	15.1%			A	143	39.9%	266	40.9%	0.092	0.762
rs192303	CC	104	57.8%	155	47.5%									
	GC	65	36.1%	140	42.9%	4.724	0.03	C	273	75.8%	450	69.0%	5.28	**0.0216**
	GG	11	6.1%	31	9.6%			G	87	24.2%	202	31.0%		
rs1805065	TT	0	0.0%	0	0.0%									
	CT	6	3.3%	7	2.1%	0.650	0.420	T	6	1.7%	7	1.1%	0.643	0.4225
	TT	180	96.7%	326	97.9%			C	354	98.3%	645	98.9%		
rs36025	CC	148	82.2%	245	75.4%									
	TC	30	16.7%	70	21.5%	3.849	0.050	C	326	90.6%	560	86.1%	4.168	0.0412
	TT	2	1.1%	10	3.1%			T	34	9.4%	90	13.9%		
rs36017	GG	59	32.8%	94	28.9%									
	CG	87	48.3%	165	50.8%	0.656	0.418	G	205	56.9%	353	54.3%	0.651	0.4196
	CC	34	18.9%	66	20.3%			C	155	43.1%	297	45.7%		
rs5564	GG	1	0.6%	4	1.2%									
	AG	20	11.1%	31	9.6%	0.003	0.955	G	22	6.1%	39	6.0%	0.003	0.9529
	AA	159	88.3%	289	89.2%			A	338	93.9%	609	94.0%		
rs47958	CC	64	35.6%	104	31.9%									
	AC	86	47.8%	161	49.4%	0.769	0.380	C	214	59.4%	369	56.6%	0.771	0.3799
	AA	30	16.6%	61	18.7%			A	146	40.6%	283	43.4%		
rs3785157	TT	15	8.3%	28	8.7%									
	CT	76	42.2%	113	35.1%	1.128	0.288	T	106	29.4%	169	26.2%	1.191	0.2752
	CC	89	49.5%	181	56.2%			C	254	70.6%	475	73.8%		
rs5569	AA	15	8.4%	31	9.6%									
	GA	77	43.0%	119	37.0%	0.341	0.559	A	107	29.9%	181	28.1%	0.357	0.5502
	GG	87	48.6%	172	53.4%			G	251	70.1%	463	71.9%		
rs998424	CC	16	8.9%	31	9.5%									
	TC	77	42.8%	1120	37.0%	0.58	0.455	C	109	30.3%	182	28.0%	0.586	0.4439
	TT	87	48.3%	174	53.5%			T	251	69.7%	468	72.0%		
rs15534	CC	127	70.5%	212	65.0%									
	TC	46	25.6%	99	30.4%	1.403	0.236	C	300	83.3%	523	82.2%	1.485	0.223
	TT	7	3.9%	15	4.6%			T	60	16.7%	129	19.8%		
rs7194256	CC	126	70.4%	210	64.8%									
	TC	44	24.6%	98	30.2%	1.033	0.310	C	296	82.7%	521	79.9%	1.124	0.2891
	TT	9	5.0%	16	5.0%			T	62	17.3%	127	20.1%		

The 5′-flanking promoter region of the NET gene contains several cis-elements that play a critical role in transcription regulation [46,47], so changes in this promoter DNA structure may lead to an altered transcriptional activity responsible for predisposition to PTSD. Therefore, the polymorphisms in this region are very important candidates for genetic studies in PTSD. Three promoter SNPs rs11648486, rs168924 and rs2242446 were detected in our studies. Only rs168924 was found to be significantly associated with risk of PTSD. Although we failed to detect a significant association between rs2242446 and risk of PTSD, we found the rs168924 is in LD with rs2242446, which consistent with other studies [28]. GT haplotype from rs1168924 and rs2242446 was significantly more frequent in PTSD than in controls. The results show that this promoter region of the NET gene was associated with the development of PTSD. SNP rs192303 is located in intron 1, a region reported to be responsible for high-level transcription of NET [46]. In combination with the promoter region, the intron 1 region contributed to the highest level of transcriptional activity within noradrenergic cells [47]. So it is biologically plausible that this SNP or variant that is in high LD with this SNP may affect NET expression and thus increase the risk of developing PTSD.

Since coding polymorphisms in the NET gene are rare, most did not pass the selection criteria as they were less polymorphic (MAF

Table 4. Effect of *SLC6A2* SNP genotype and Combat Severity Score (CES)on risk of lifetime PTSD diagnosis. PTSD: posttraumatic stress disorder; *SLC6A2*: solute carrier family 6 member 2; SNP: single nucleotide polymorphism. Continuous variables were centered to the mean.

	Main effects model				Interaction model			
	OR	**95% CI of OR**		***p***	**OR**	**95% CI of OR**		***p***
Age	0.998	0.985	1.011	0.778	1	0.987	1.013	0.991
Gender	0.913	0.552	1.512	0.724	0.917	0.553	1.522	0.738
Race	1.319	0.849	2.049	0.218	1.304	0.836	2.032	0.242
CES	1.052	1.027	1.078	0	1.037	1.01	1.065	0.006
rs168924	1.425	0.946	2.147	0.09	1.575	1.005	2.467	0.047
rs168924 X CES					1.075	1.012	1.142	0.019
Age	0.999	0.985	1.012	0.828	0.999	0.986	1.013	0.929
Gender	0.899	0.542	1.49	0.679	0.882	0.531	1.465	0.628
Race	1.348	0.871	2.084	0.18	1.382	0.89	2.147	0.15
CES	1.052	1.027	1.078	0	1.024	0.993	1.056	0.125
rs192303	1.303	0.956	1.776	0.094	1.412	1.02	1.954	0.038
rs192303 X CES					1.056	1.014	1.099	0.009

Table 5. Effect of *SLC6A2* SNP genotype and Combat Severity Score (CES) on PTSD symptom severity. PTSD: posttraumatic stress disorder; *SLC6A2:* solute carrier family 6 member 2; SNP: single nucleotide polymorphism. Continuous variables were centered to the mean.

	Main effects model				Interaction model			
	b	**beta**	***t***	***p***	***b***	**beta**	***t***	***p***
Age	0.017	0.007	0.148	0.882	0.034	0.013	0.299	0.765
Gender	-4.215	-0.043	-0.953	0.341	-4.373	-0.045	-0.992	0.321
Race	1.122	0.018	0.4	0.689	1.14	0.019	0.408	0.683
CES	0.926	0.214	4.687	<0.0001	0.701	0.162	3.179	0.002
rs168924	7.221	0.1	2.193	0.029	6.227	0.087	1.883	0.06
rs168924 X CES					0.902	0.114	2.259	**0.024**
Age	0.023	0.009	0.205	0.838	0.025	0.01	0.221	0.825
Gender	-4.635	-0.048	-1.047	0.296	-4.956	-0.051	-1.123	0.262
Race	1.559	0.025	0.56	0.576	2.192	0.036	0.785	0.433
CES	0.927	0.214	4.684	<0.0001	0.554	0.128	2.101	0.036
rs192303	4.861	0.085	1.873	0.062	4.776	0.084	1.846	0.065
rs192303 X CES					0.654	0.128	2.121	0.034

Table 6. Effect of *SLC6A2* Methylation and Combat Severity Score (CES) on risk of lifetime PTSD diagnosis. PTSD: posttraumatic stress disorder; *SLC6A2:* solute carrier family 6 member. Continuous variables were centered to the mean. [a]Median-Split

	OR	**95% CI of OR**		***p***
Age	1.009	0.954	1.068	0.752
Gender	0.793	0.113	5.58	0.816
Race	2.29	1.083	4.84	0.03
CES	1.077	1.031	1.125	0.001
Methylation Group[a]	0.383	0.171	0.857	0.02

>=0.1) and were therefor not included in our studies. One SNP (rs1805065), located in exon 2 was selected in our studies according to other research findings [40]. Another SNP, rs5564, located in the spice site consensus sequence of exon 5 were included, because T allele was found to be significantly associated with the diagnosis of Postural Orthostatic Tachycardia Syndrome (POTs) [40]. However, we did not find significant association of both SNPs with PTSD.

A silent rs5569 polymorphism, located at exon 9 of the NET gene, was a particularly interesting candidate because it has higher heterozygosity than the other markers [19,29]. The G/G genotype was found to be correlated with the concentration of 3-methoxy-4-hydroxyphenylglycol (MHPG), a primary NE metabolite in the cerebrospinal fluid, consequently attributing to the increased reuptake of NE [48]. So we also first time studies this SNP in PTSD. However, no apparent relations supposed were observed between this polymorphism and PTSD. We found that rs3785157, rs5569, rs998424 are in high LD which was in line with other studies [22]. But the results for these three SNPs were not significant in our studies.

We also determine the differences in NET gene promoter methylation between traumas exposed individuals with and without PTSD for the first time. We found that lower NET promoter methylation may be a risk for the development of PTSD. Our study is consistent to other studies, which reported hypo-methylation in depressed and panic disorder patients [39,41]. However, we didn't find that *SLC6A2* promoter methylation was correlated with PTSD symptom severity scales. We didn't find that combat trauma had influence on DNA methylation neither and there's no interaction effect of CES and NET promoter methylation on risk of PTSD in our studies. The region around the transcription start site is transcriptionally important, and contains multiple putative transcription factor binding sites [40,49,50]. The analyzed region we chose is Chr16: 55690108 to Chr16: 55690516, which encompasses transcription start site (TSS) chr16:55690555. It is possible that this methylation island could have impact on gene expression as the methylation site is just located at the promoter region.

Our results indicate that considering both genetic variants and DNA methylation will improve the explanation of PTSD even though we didn't find the direct interaction effect of these two factors in our sample size. Genetic variant and DNA methylation may jointly contribute to gene expression or alternative splicing [51].

Limitations

First, the selected16 SNPs markers may not provide complete

coverage of the NET gene because the r^2 value between some contiguous markers was less than 0.8 [52]. In addition, our random SNP selection method may decrease the power of the study compared with tag SNP selection method [53]. Second, we used the whole blood to carry out the methylation studies in PTSD. Despite DNA methylation patterns between different people, and between different tissues within an individual were found to be highly conserved [54], it cannot be ruled out that *SLC6A2* DNA methylation may differ specifically in diseased central neurons. Third, our sample size is relatively small. The positive findings have to be considered explorative and findings need to be further verified in larger samples.

Conclusion

This is the first study to determine the role of both genetic and epigenetic factors within the genomic region of the *SLC6A2* gene on the risk of PTSD. Our results suggest that genetic variants and promoter methylation in the *SLC6A2* might be associated with the susceptibility of PTSD. Further in-depth analysis of SNPs and DNA methylation on transcriptional regulation of the *SLC6A2* gene would be of value.

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Conflict of interest

The authors have no conflicts of interest to disclosure.

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