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Epigenetic mechanism of maternal post-traumatic stress disorder in delayed rat offspring development: dysregulation of methylation and gene expression

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ABSTRACT. Maternal post-traumatic stress disorder (PTSD) increases the risk of adverse neurodevelopmental outcomes in the child. Epigenetic alternations may play an essential role in the negative effects of PTSD. This study was aimed to investigate the possible epigenetic alterations of maternal PTSD, which underpins the developmental and behavioral impact. 24 pregnant Sprague-Dawley (SD) rats were randomly grouped into PTSD and control groups. Open-field tests (OFTs), elevated pull maze (EPM) assays, gene expression profile chip tests, and methylated DNA immunoprecipitation sequencing (MeDIP-Seq) were performed on the offsprings 30 days after birth. The results

showed that PTSD offsprings had lower body weights and OFT scores than control offsprings. Enzyme-linked immunosorbent assays showed that serotonin receptor (5-HT) and dopamine levels were significantly lower in PTSD offsprings than in control offsprings. In contrast, corticosterone levels were higher in the PTSD group than in the control group. In a comparison of the PTSD group versus the control group, 4,160 significantly differentially methylated loci containing 30,657 CpGs were identified; 2,487 genes, including 13 dysmethylated genes, were validated by gene expression profiling, showing a negative correlation between methylation and gene expression ($R = -0.617$, $P = 0.043$). In conclusion, maternal PTSD could delay the physical and behavioral development of offsprings, and the underlying mechanism could contribute to changes in neurotransmitters and gene expression, owing to dysregulation of whole-genome methylation. These findings could support further clinical research on appropriate interventions for maternal PTSD to prevent methylation dysregulation and developmental retardation.

Key words: Post-traumatic stress disorder; Epigenetics; Behavior; Environmental enrichment; Neurodevelopment

INTRODUCTION

Post-traumatic stress disorder (PTSD) is characterized by symptoms of intrusion, avoidance, and hyperarousal after exposure to traumatic incidents, and has a prevalence of 7-12% (Labonté et al., 2014). It is believed that alterations in the activity of the hypothalamus-pituitary-adrenal (HPA) axis, which plays an essential role in the stress regulating system, are induced by severe stress stimulation in both animals and humans. Moreover, dysfunctional HPA axis activity is a well-characterized characteristic in PTSD (Baumeister et al., 2014; Wilson et al., 2014). The HPA axis secretes glucocorticoids to maintain biological homeostasis and adapt to chronic stress in response to stress (Wilson et al., 2014). Fenchel et al. (2015) reported that gonadal steroid hormones (GSHs) are involved in the neurobiological response to predator scent stress, modulating anxiety-like responses in an animal model. Interestingly, the HPA and HPG axes could be intermediary mechanisms underlying the life adversity symptoms observed in patients with PTSD. Dismukes et al. (2015) reported that coupling of the HPA and HPG axes can be observed in incarcerated adolescents, particularly in those with the greatest life stresses.

Prenatal maternal effects have been reported as a primary factor in offspring development in various species. Zhang et al. (2012) found that PTSD increases the risk of adverse neurodevelopmental outcomes, including hypodevelopment and behavioral disorders, in offsprings. PTSD arises from interactions between traumatic stresses and various genetic factors (Skelton et al., 2012). Epigenetic alterations are thought to be the core causes of these interactions, mediating the continuing effects of the environment on gene regulation (Provencal and Binder, 2015; Zannas et al., 2015). Many studies in both humans and animals have suggested that the epigenetic regulation of distinct genes play roles in the pathogenesis of PTSD (Labonté et al., 2014; Byun et al., 2015). Vukojevic et al. (2014) reported that an epigenetic alteration in the glucocorticoid receptor (*GR*) gene promoter is linked to individual

and gender-specific differences in memory function and risk of PTSD. Additionally, evidence of this epigenetic regulation has been found in an animal model of fear conditioning (Kwapis and Wood, 2014). Although these reports offer promising prospects for future epigenetic studies, crucial limitations exist, including tissue specificity, the phenomenological definition of the disorder, and the trans-species translation of molecular findings. Prenatal maternal exposure to PTSD can cause lifelong psychological dysfunctions, including development of mental disorders and behavioral problems. Previous studies have shown that these effects may result from transgenerational epigenetic programming of genes operating in the HPA axis, such as *GR* (Radtke et al., 2011; Rodgers et al., 2013).

Many studies have focused on the dysregulation of genes in the HPA axis in response to site-specific methylation; however, to the best of our knowledge, few reports have described the relationships between whole-genome methylation dysregulation and maternal PTSD. Since PTSD is a complicated psychological disorders with typical environmental and genetic interactions, elucidation of changes in methylation is needed to clarify the related mechanisms. This study may be one of the first animal studies to investigate regulation of whole-genome methylation to assess the epigenetic mechanisms by which maternal PTSD influences offsprings, and the relationships between these mechanisms and development, behavior, and neurotransmitters. Furthermore, identification of specific features of methylation dysregulation could facilitate interventions that may prevent the negative effects of maternal PTSD.

MATERIAL AND METHODS

Animals

Twenty-eight female rats (body weight, 230-280 g) and 14 male Sprague-Dawley (SD) rats (body weight, 222-280 g) were included in this study. The rats were acclimated for 1 week in an experimental room at a constant temperature of 22°C, a 12-h light/dark cycle, and food and water available ad libitum. After acclimation, females and males were mated. Pregnancy was confirmed by observing the presence of vaginal plugs. Next, 24 pregnant rats were randomly selected and separated into two groups, the control group (N = 12) and PTSD group (N = 12). During pregnancy, all rats were transported with equivalent stress. No significant differences in pregnancy periods or body weights were found between the two groups on the fifth day of pregnancy [control group: 392.337 ± 21.783 g, PTSD group: 390.341 ± 10.949 g; *t*-test, P = 0.736 (two-tailed), t = 0.345, d.f. = 11]. All offsprings were housed and fed with their mothers until the 30th day after birth.

The experimental protocol was reviewed and approved by the Experimental Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine. All experiments in this study were performed according to the guidelines of the International Association for the Study of Pain. All efforts were made to minimize animal discomfort and reduce the number of animals used in the experiments. Euthanasia was performed by decapitation.

PTSD simulation

A single prolonged stress (SPS) model was employed to simulate PTSD in pregnant rats (Yamamoto et al., 2009). The SPS model was proposed by Israel Liberzon and replicated the specific neuro-endocrinological abnormalities observed in patients with PTSD (Yamamoto

et al., 2009). Rats in the PTSD group were restrained for 2 h and then immediately underwent forced swimming for 20 min in water (height: 40 cm) at a temperature of 25°C in a plastic tub (diameter: 50 cm, height: 70 cm), with six rats swimming at a time. Intervention was performed from days 8-14 after confirmation of pregnancy confirmation. According to our observations in preliminary experiments, pregnant rats were able to complete the entire process. No rats died or became very weak during the simulation. The 12 rats were housed in three cages, with four rats in each cage.

Body weight measurement and open field tests (OFTs)

The body weights (g) of the offsprings were measured on days 1 (day_0), 10 (day_10), 20 (day_20), and 30 (day_30) after delivery to assess body development.

A whitish-yellow homemade square board (120 x 120 cm) was modified with small squares (15 x 15 cm). Eighteen offspring from each group for day_10, day_15, day_20, day_25, and day_30 were randomly selected and placed in the center square of the board. The number of squares across which each animal crawled in 2 min was then counted. Each additional score number was given only when all four paws of the animal were in the square (Zhang et al., 2012).

Analysis of hormone levels by enzyme-linked immunosorbent assays (ELISAs)

Twenty offsprings were randomly selected from the groups. Blood samples were collected from the arteria femoralis (Zhang et al., 2012). ELISA was performed to determine the serum levels of corticosterone (DZE 30590), dopamine (DZE 30238), and serotonin receptor (5-HT) (DZE 30326).

Gene expression profiling

Gene expression profiling was performed as previously described (Zhang et al., 2012). Briefly, RNA extraction and purification were performed, followed by RNA amplification and labeling. Finally, data were acquired.

Methylated DNA immunoprecipitation sequencing (MeDIP-Seq)

DNA extraction and purification

Total DNA was extracted and purified using a DNeasy Blood & Tissue Kit (#69506; Qiagen, Valencia, CA, USA) following the manufacturer's instructions. DNA was then measured using a NanoDrop ND-1000 spectrophotometer and by 0.8% agarose gel electrophoresis. All samples qualified for the next step (Chavez et al., 2010).

Library preparation

Genomic DNA was fragmented according to the instructions provided in the TruSeq DNA Sample Preparation Guide (Illumina Inc., USA). Covaris shearing generated dsDNA fragments with 3' or 5' overhangs. The overhangs resulting from fragmentation were

converted into blunt ends with an End Repair Mix. The 3' to 5' exonuclease activity of this mix removed the 3' overhangs, and the polymerase activity filled in the 5' overhangs. A single 'A' nucleotide was added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. Indexing adapters were ligated to the ends of the DNA fragments. These products of the ligation reaction were purified on a gel, and the unligated adapters were removed. The size range of the sequencing library was selected to be appropriate for cluster generation. Polymerase chain reaction (PCR) was then used to selectively enrich the specific DNA fragments having adapter molecules on both ends and to amplify the amount of DNA in the library. Enrichment was followed directly by application to a Magnetic Methylated DNA Immunoprecipitation kit (Diagenode, NJ, USA). The size of the DNA library templates was checked using an Agilent Technologies 2100 Bioanalyzer, and the concentration was determined with a Qubit 2.0 Fluorometer. Indexed DNA libraries were normalized to 10 nM in the diluted cluster template (DCT) plate and then pooled in equal volumes in the pooled dilution plate (PDP). DNA libraries that were not intended for indexing were normalized to 10 nM in the DCT plate without pooling.

Clustering and sequencing

Clusters were generated and hybridized with primers on a cBot System following the cBot User Guide (Illumina Inc. CA, USA). Sequencing was performed with a flow cell (Flow Cell V3, single end 1 x 50 nt; Illumina Inc.) with the clusters on an Illumina HiSeq 2500 instrument. Quality control of the sequencing results was carried out using the following parameters: reads quantity ≥20 M and Q20 quantity of base quality ≥90% (Chavez et al., 2010).

Data analysis

Data were analyzed as follows: Raw reads → preprocess reads → mapping genome →BAM/SAM → DMR detection → related gene, related CpG island (Chavez et al., 2010) .

Statistical analysis

Data for body development, OFTs, and ELISAs were analyzed with Statistical Package for the Social Sciences (SPSS) version 19.0. Additionally, *t*-tests were performed to analyze group differences in body weights and hormone levels. A generalized estimating equation (GEE) was used to analyze group differences in the repeated observation of OFTs. The alpha was set to 0.05 for all analyses.

RESULTS

Body development and behavior tests

According to the *t*-test results, the body weights of offsprings in the PTSD group were significantly lower than those in the control group on the first day after birth and at 10, 20, and 30 days ($P < 0.05$; Figure 1). According to the GEE analysis, PTSD offsprings showed significantly lower OFT scores than control offsprings (Wald $\chi^2 = 30.514$, d.f. = 1, $P < 0.05$; Figure 2).

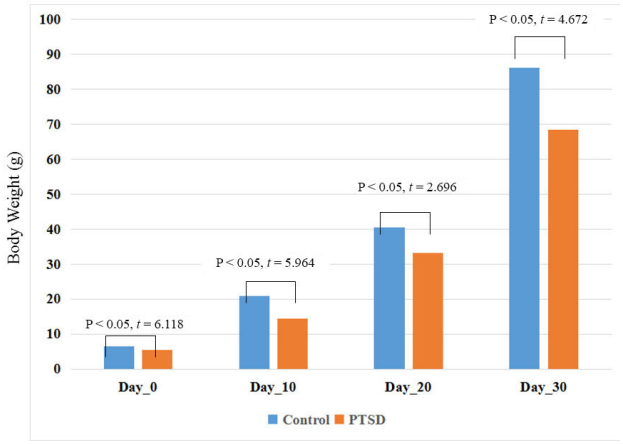

Figure 1. Body weight comparisons between control and PTSD offspring. The body weights of offsprings in the PTSD group on day_0, day_10, day_20, and day_30 after delivery were all significantly lower than those of the control group, with values of 5.389 ± 0.615 versus 6.455 ± 0.408 g; 14.422 ± 1.789 versus 20.965 ± 5.262 g; 33.133 ± 7.380 versus 40.403 ± 9.216 g; and 68.392 ± 12.147 versus 86.139 ± 14.320 g, respectively.

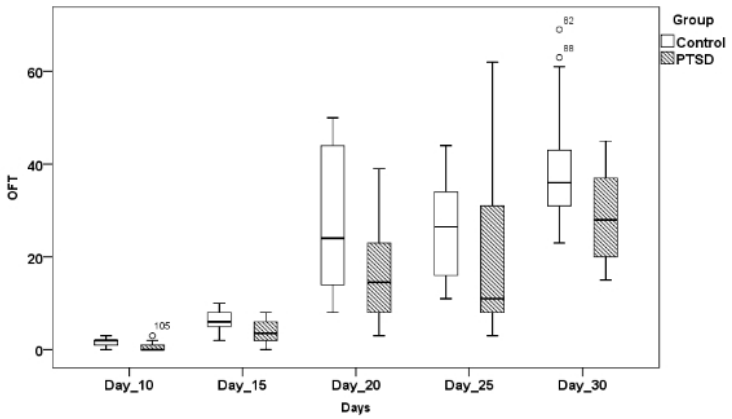

Figure 2. Box plot of OFTs in comparisons between the control and PTSD groups. Since the data were not normally distributed and were from repeated observations, GEE was a more appropriate method for data analysis. OFT comparisons between control and PTSD groups are shown. There was a statistically significant difference between the two groups (Wald χ^2 = 30.514, d.f. = 1, P < 0.05), indicating that the offspring in the PTSD group had lower scores than those in the control group.

Neurotransmitter measurement

According to the ELISA results, 5-HT levels were significantly higher in the control group than in the PTSD group (F = 2.251, t = 2.946, P < 0.05). Moreover, corticosterone levels were significantly lower in the control group than in the PTSD group (F = 0.172, t = -3.561, P = 0.002). Dopamine levels were significantly higher in the control group than in the PTSD group (F = 5.611, t = 3.554, P < 0.05; Figure 3).

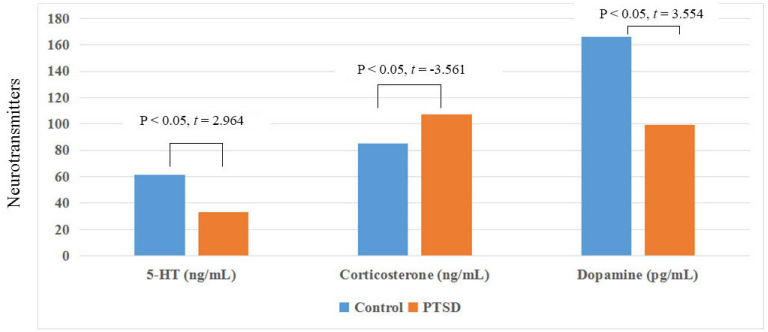

Figure 3. ELISA results for neurotransmitter comparisons between the control and PTSD groups. 5-HT and dopamine levels were significantly higher in the control group than in the PTSD group (F = 2.251, t = 2.946, P < 0.05; F = 5.611, t = 3.554, P < 0.05, respectively). Corticosterone levels were significantly lower in the control group than in the PTSD group (F = 0.172, t = -3.561, P = P < 0.05).

Gene expression profiling

In comparisons of the PTSD group versus the control group, gene expression profiling showed that 70 genes were upregulated, whereas 42 genes were downregulated (fold change ≥2 and P < 0.05; Table 1, Figure 4). These 112 genes were annotated using the Gene Ontology (GO) database, and three GO classes of the first level were obtained (P < 0.05), including cellular component, molecular function, and biological process. Annotations with the KEGG database showed that five significant KEGG pathways were involved, including endocytosis, the mitogen-activated protein kinase (MAPK) signaling pathway, bile secretion, hypertrophic cardiomyopathy, and antigen processing and presentation (P < 0.05, q < 0.05, hits > 2).

Methylation sequencing

In a comparison of the control group versus the PTSD group, performed with MeDIP-Seq, 4160 significantly differentially methylated loci (P < 0.05, $Log_2FC \geq 1$ or $Log_2FC \leq -1$; 1,079 hypermethylated loci and 2390 hypomethylated loci) containing 30,657 CpGs and 2487 genes were screened (943 hyper-regulated genes with $Log_2FC \geq 1$; 1775 hypo-regulated genes with $Log_2FC \leq -1$). All differentially methylated loci were distributed among 20 chromosomes, with none in the sex chromosomes (Figure 5). Interestingly, Chr1 contained the most hypomethylated loci (N = 288) and hypermethylated loci (N = 141), whereas Chr11 contained the fewest hypomethylated loci (N = 45) and Chr16 contained the fewest hypermethylated loci (N = 27).

Relationship between differentially methylated genes and gene expression profiles

A combination linking analysis identified 13 genes from both the 112 differentially expressed genes from gene expression profiling and 2,487 genes from MeDIP-Seq. Among these genes, *Hmha* contained both hypermethylated and hypomethylated loci. A correlation test showed that there was a significant negative correlation of the remaining 11 genes between gene chip analysis and MeDIP-Seq (Pearson correlation r = -0.617, P = 0.043), indicating that hypermethylation led to the downregulation of gene expression, whereas hypomethylation led to the upregulation of gene expression (Figure 6).

Table 1. The 112 differentially expressed genes (PTSD versus control groups).

Up-regulation				Down-regulation			
Probe name	FC*	Gene symbol	P values	Probe name	FC*	Gene symbol	P values
A_44_P175003	25.628	Ttr	0.011	A_44_P1029397	0.494	Bst2	0.038
A_42_P575104	22.497	Sostdc1	0.012	A_44_P405276	0.482	LOC100912860	0.029
A_43_P11759	13.058	Rps26	0.012	A_43_P18361	0.482	Cd7	0.015
A_43_P23092	10.761	F5	0.010	A_44_P536665	0.477	Ggact	0.027
A_43_P13199	9.033	Folr1	0.002	A_44_P226658	0.475	Scnn1g	0.009
A_44_P358110	6.441	Tsg101	0.000	A_42_P541025	0.472	Hspa1l	0.022
A_44_P716655	6.357	RGD1561795	0.015	A_43_P20339	0.471	Hmha1	0.049
A_44_P561510	6.045	Otx2	0.013	A_44_P923195	0.468	Wdfy3	0.040
A_44_P548949	5.314	LOC683753	0.015	A_44_P526184	0.467	LOC102554962	0.006
A_44_P299247	5.235	Aqp1	0.001	A_44_P179996	0.459	Myh2	0.041
A_44_P323892	5.163	RGD1559951	0.000	A_44_P339741	0.452	Mc3r	0.020
A_44_P414582	4.557	Kcne2	0.029	A_44_P101971	0.443	LOC102553736	0.007
A_44_P475564	4.191	Glycam1	0.004	A_44_P445244	0.435	Artn	0.017
A_44_P358654	4.111	Usp17l5	0.005	A_44_P763016	0.429	Adcyap1	0.032
A_44_P416824	3.939	Otx2	0.012	A_44_P233080	0.420	Egr1	0.025
A_44_P1013006	3.434	Steap1	0.003	A_44_P592652	0.417	LOC100362814	0.046
A_44_P746348	3.398	RGD1562658	0.019	A_44_P476830	0.416	Hspa4	0.006
A_42_P755326	3.328	Itgb6	0.009	A_44_P541144	0.416	Igsf9	0.022
A_44_P979417	3.187	Tceal5	0.003	A_44_P655562	0.409	Pcdhb6	0.045
A_44_P791729	3.153	Rtn4rl1	0.001	A_44_P152093	0.402	Pcdhb6	0.036
A_44_P113879	3.103	Slco1a5	0.009	A_44_P468887	0.402	En1	0.001
A_44_P251008	3.074	Efhb	0.013	A_44_P178278	0.401	LOC301839	0.039
A_44_P987286	3.013	Ubr3	0.001	A_44_P391638	0.394	Pcdh12	0.008
A_44_P549075	3.004	Pla2g5	0.049	A_44_P496447	0.390	Ntf3	0.034
A_44_P560541	2.963	Glipr1l1	0.010	A_44_P129187	0.388	Il17ra	0.007
A_44_P113806	2.957	Scn9a	0.003	A_43_P11972	0.380	Fgf17	0.001
A_44_P400494	2.930	Krt18	0.014	A_44_P464237	0.373	RGD1563155	0.006
A_44_P652103	2.913	Pifo	0.004	A_44_P238286	0.360	Olr287	0.024
A_44_P614514	2.899	LOC102556269	0.008	A_42_P788302	0.360	Btnl3	0.002
A_42_P763224	2.825	Scgb1c1	0.046	A_44_P460845	0.360	Casz1	0.006
A_43_P11457	2.812	Ace	0.005	A_44_P210763	0.358	Gprin2	0.001
A_44_P391915	2.797	RGD1561944	0.001	A_44_P182414	0.355	Foxe3	0.039
A_44_P142401	2.783	Fam184b	0.035	A_44_P975437	0.295	Cldn4	0.038
A_44_P175758	2.757	Rsph4a	0.017	A_43_P23375	0.254	Dsg1	0.003
A_43_P18469	2.747	Sytl3	0.002	A_44_P529845	0.245	RGD1562638	0.010
A_44_P1025476	2.648	Krt18	0.014	A_44_P425143	0.243	Grin2d	0.009
A_44_P458987	2.633	LOC364556	0.002	A_44_P290915	0.236	Sema3c	0.022
A_44_P464221	2.625	RGD1566373	0.002	A_44_P912004	0.234	Tlx3	0.024
A_44_P499954	2.603	Kcnip1	0.014	A_44_P496422	0.231	Hrg	0.005
A_42_P694679	2.598	Hdac4	0.023	A_44_P925486	0.226	Cd300a	0.036
A_44_P450107	2.502	RGD1560076	0.000	A_44_P256188	0.207	Rpl39l	0.029
A_44_P191986	2.499	LOC498205	0.001	A_43_P15489	0.179	Il24	0.025
A_42_P678904	2.481	Smim22	0.008				
A_44_P356344	2.474	Ndufc2	0.011				
A_44_P704642	2.456	LOC100361018	0.014				
A_44_P513075	2.453	LOC102556346	0.013				
A_44_P173414	2.436	Eda	0.003				
A_44_P457368	2.426	RGD1561890	0.009				
A_44_P426498	2.317	RGD1564617	0.006				
A_44_P1025667	2.306	Ccdc37	0.046				
A_42_P632813	2.258	Epn3	0.011				

Continued on next page

Table 1. Continued.

Up-regulation				Down-regulation			
A_44_P414838	2.229	RGD1561841	0.000				
A_44_P342739	2.227	Ribc2	0.044				
A_44_P825985	2.197	Megf11	0.015				
A_44_P914096	2.195	RGD1560017	0.005				
A_44_P928636	2.188	LOC681006	0.015				
A_42_P711464	2.183	Ccdc19	0.039				
A_44_P651053	2.169	Supt16h	0.006				
A_44_P116898	2.165	Ctsll3	0.001				
A_44_P701913	2.157	Arl4a	0.045				
A_44_P424164	2.133	RGD1563903	0.001				
A_44_P1046205	2.116	Kdm4c	0.002				
A_44_P424218	2.101	Mlf1	0.030				
A_44_P102931	2.096	LOC102547000	0.039				
A_44_P184874	2.074	RGD1563636	0.005				
A_44_P114606	2.070	RGD1563194	0.001				
A_44_P135657	2.027	Ccdc113	0.043				
A_44_P667896	2.026	Uaca	0.015				
A_44_P490092	2.003	LOC102551208	0.018				
A_44_P549840	2.003	Chmp2b	0.007				

*FC: fold change.

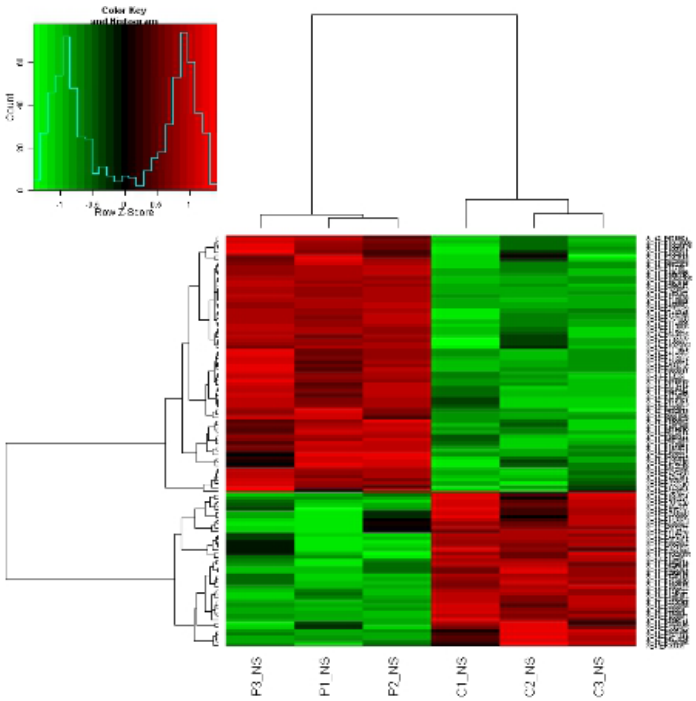

Figure 4. Heat-map of the differentially expressed genes. C1_NS, C2_NS, and C3_NS refer to the control group, and P1_NS, P2_NS, and P3_NS refer to the PTSD group. Each column represents a sample, and each row represents a single gene. The color gradient intensity scale is shown in the upper left-hand corner. Red: upregulation; green: downregulation.

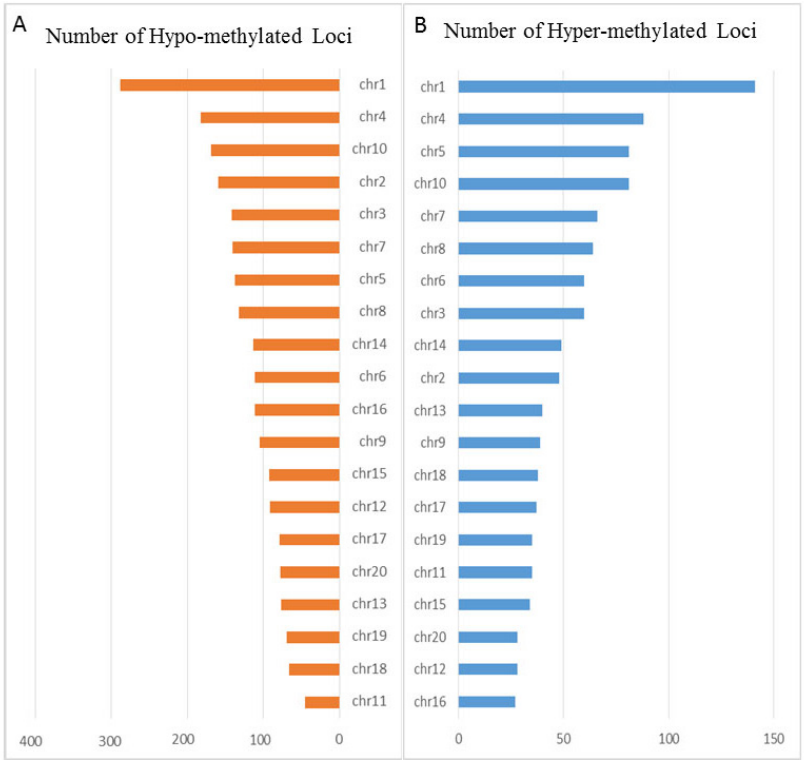

Figure 5. Methylation distribution among 20 chromosomes. **A.** Distribution of hypomethylated loci in the 20 chromosomes; **B.** distribution of hypermethylated loci in the 20 chromosomes.

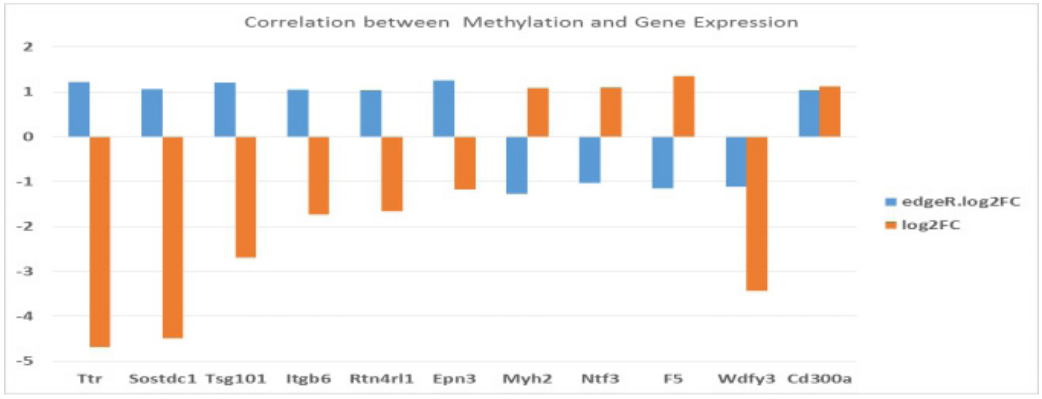

Figure 6. Correlation between gene expression and methylation. There was a negative correlation of 11 genes between gene chip analysis and MeDIP-Seq (Pearson correlation r = -0.617, P = 0.043). FC refers to the fold change in gene expression (control versus PTSD), and edgeR.log_2FC refers to the methylation fold change (control versus PTSD). Log_2FC and edgeR.Log_2FC > 0 refer to upregulation and hypermethylation, Log_2FC and edgeR.Log_2FC < 0 refer to downregulation and hypomethylation. With the exceptions of *Wdfy3* and *Cd300a*, the remaining genes showed opposite regulation of methylation and gene expression.

GO annotations

Seventeen significant GO annotations for the filtered 13 genes were obtained, including two level-2 GO annotations and 15 level-3 GO annotations (enrichment test $P < 0.05$; Table 2). The two level-2 GO annotations were related to the extracellular region.

Table 2. GO annotations of the 13 genes.

GO ID	Level	Name	Hits	Total	Percent	Enrichment test P value
GO:0008289	3	Lipid binding	2	416	0.0048	0.0293
GO:0042562	3	Hormone binding	1	65	0.0154	0.0422
GO:0005576	2	Extracellular region	4	1405	0.0028	0.0103
GO:0044421	3	Extracellular region part	3	848	0.0035	0.0159
GO:0031982	3	Vesicle	3	698	0.0043	0.0094
GO:0044421	2	Extracellular region part	3	848	0.0035	0.0159
GO:0005615	3	Extracellular space	3	633	0.0047	0.0072
GO:0032153	3	Cell division site	1	21	0.0476	0.0143
GO:0032155	3	Cell division site part	1	21	0.0476	0.0143
GO:0001709	3	Cell fate determination	1	31	0.0323	0.0207
GO:0007272	3	Ensheathment of neurons	1	65	0.0154	0.0422
GO:0022415	3	Viral reproductive process	1	67	0.0149	0.0435
GO:0022415	3	Viral reproductive process	1	67	0.0149	0.0435
GO:0007566	3	Embryo implantation	1	28	0.0357	0.0188
GO:0009605	3	Response to external stimulus	3	1097	0.0027	0.0313
GO:0014823	3	Response to activity	1	48	0.0208	0.0315
GO:0065008	3	Regulation of biological quality	4	1724	0.0023	0.0208

FC = 1.206, P = 0.002, CpGs = 5.

KEGG pathway annotations

Nine significant KEGG pathway annotations for the screened genes were obtained (enrichment test $P < 0.05$; Table 3). Only six genes, i.e., *Itgb6*, *F5*, *Epn3*, *Tsg101*, *Ntf3*, and *Myh2*, were involved. Interestingly, most of the KEGG pathways, including complement and coagulation cascades, dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, hypertrophic cardiomyopathy, tight junctions, and viral myocarditis, were related to circulatory diseases. *Itgb6* was hypomethylated at positions 51836001-51836500 in Chr3 ($\log_2$FC = 1.053, P = 0.007, CpGs =11) in PTSD offspring. *Myh2*, which encodes myosin, heavy chain 2, skeletal muscle, was hypermethylated at positions 53485382-53485500 in Chr10 ($\log_2$FC = -1.472, P = 0.007, CpGs =15) in PTSD offspring. *F5*, which encodes coagulation factor V, was hypermethylated at positions 87378001-87378500 in Chr13 ($\log_2$FC = -1.150, P = 0.002, CpGs = 12). *Ntf3*, which encodes neurotrophin 3, was hypermethylated at positions 225679501-225680000 in Chr4 ($\log_2$FC = -1.022, P = 0.023, CpGs = 9). *Epn3*, which encodes epsin 3, was hypomethylated at positions 82047501-82048000 in Chr10 ($\log_2$FC = 1.256, P = 0.000, CpGs = 7). *Tsg101*, which encodes tumor susceptibility gene 101, was hypomethylated at positions 104013001-104013500 in Chr1 ($\log_2$FC = 1.206, P = 0.002, CpGs = 5).

Table 3. KEGG pathways of the 13 genes.

KEGG Pathway	Hits	Total	Percent (%)	Enrichment test P value	Gene
Arrhythmogenic right ventricular cardiomyopathy	1	72	1.39	0.020	Itgb6
Complement and coagulation cascades	1	69	1.45	0.020	F5
Dilated cardiomyopathy	1	88	1.14	0.025	Itgb6
ECM-receptor interaction	1	76	1.32	0.021	Itgb6
Endocytosis	2	231	0.87	0.002	Epn3,Tsg101
Hypertrophic cardiomyopathy (HCM)	1	80	1.25	0.023	Itgb6
Neurotrophin signaling pathway	1	129	0.78	0.036	Ntf3
Tight junction	1	133	0.75	0.037	Myh2
Viral myocarditis	1	100	1.00	0.028	Myh2

DISCUSSION

Current studies have proven that maternal PTSD passes on negative traits to offsprings. Moreover, epigenetic factors, rather than a single gene mutation, essentially underlie the interactions between maternal stress and postnatal phenotypes. In particular,, epigenetic regulation of the HPA axis is considered the "window to the brain", i.e., dysregulation of the HPA axis has been shown to be frequently correlated with psychiatric and trauma-related disorders (Videlock et al., 2009; Boscarino et al., 2011; Perroud et al., 2014; Zannas et al., 2015). Another growing field of interest is immune dysregulation associated with PTSD. PTSD dysregulates peripheral immune function through lasting effects on the HPA axis (Uddin et al., 2013). Immune dysregulation results in vulnerability to PTSD via alterations in brain function (Lanius et al., 2010).

Prenatal maternal stress predicts a large number of behavioral and physical symptoms in offsprings (Weinstock, 2008). Our findings suggested that maternal PTSD could delay the physical and behavioral development of offsprings, with evidence of lowered body weight and poorer performance in OFT testing, consistent with a previous report (Zhang et al., 2012). Mulligan et al. (2012) reported that extreme maternal psychosocial stress stimulation could alter locus-specific epigenetic marks in neonate and found significant correlations among maternal prenatal stress, birth weight, and promoter methylation of the glucocorticoid receptor NR3C1.

A growing body of evidence has suggested that maternal PTSD and other stressors also influence the behavioral development and hormone levels of offsprings (Mulligan et al., 2012). Maternal PTSD contributes to alterations in the stress-induced responsivity of the HPA axis; the transportation and distribution of regulatory neurotransmitters, such as dopamine, acetylcholine, and serotonin; the modification of key limbic structures; and the retardation of intrauterine growth (Kofman, 2002). In this study, significant differences were observed between the control and PTSD groups in terms of body weight and OFT performance, suggesting developmental retardation resulting from maternal PTSD, in accordance with previous studies (Mulligan et al., 2012).

The 5-HT and dopamine levels in offsprings in the PTSD group were lower than those in the control group, whereas corticosterone levels were higher in the PTSD group than in the control group. In rat models, maternal PTSD disrupts offspring physiology, leading to dysfunction of the HPA axis and induction of anxiety-related behaviors in adulthood (Cottrell and Seckl, 2009). Serum corticosterone levels were higher in PTSD offsprings than in control offsprings. Similar findings have been reported in other studies (Kotozaki and Kawashima, 2012; Huang et al., 2012). Previous studies have indicated that maternal PTSD alters the activity of the placental barrier enzyme 11-βHSD2, which metabolizes corticosterone (Seth

et al., 2015). Moreover, maternal PTSD increases corticosterone levels in the fetus and mother, which may result in behavioral disorders and insulin resistance in the offspring. Abnormalities in the central 5-HT system play an important role in behavioral disorders due to maternal stress (Brown and Luo, 2009; Spinelli et al., 2010). Within the course of pregnancy, the 5-HT system contributes to development of the central nervous system in the fetus. Moreover, 5-HT neurotransmission is involved in the activation and feedback of the HPA axis (Davidson et al., 2009). Our findings suggested that maternal PTSD reduced 5-HT levels in the offspring, in accordance with previous studies. A previous report showed that 5-HT levels were lower in the rat hippocampus and prefrontal cortex (PFC) in a predator exposure/psychosocial stress model of PTSD (Wilson et al., 2014). The role of dopamine in the pathogenesis of PTSD has been supported by outcomes of elevated urinary and plasma levels in dopamine-related disorders (Chang et al., 2012). Additionally, there is a significant link between dopamine concentration and symptom severity (Chang et al., 2012). Our findings showed that maternal PTSD reduced the dopamine levels in the offspring, in accordance with previous reports. Geracioti et al. (2013) found that the acute decline in central nervous system homovanillic acid (HVA) concentrations is associated with laboratory-induced symptoms in patients with chronic PTSD. Another study showed that the nine-repeat allele in the 3' untranslated region variable number of tandem repeat (VNTR) of the *SLC6A3* gene increases the risk of PTSD (Chang et al., 2012).

Dysregulation of DNA methylation has been found to be related to PTSD pathogenesis. Cao-Lei et al. (2014) found that prenatal and maternal objective hardship was associated with DNA methylation levels in 1675 CGs of 957 genes; this was primarily correlated with various immune functions, although maternal subjective distress was not correlated. Altered DNA methylation in *SCG5* and *LTA* is highly associated with maternal objective stress and is comparable in peripheral blood T cells, mononuclear cells, and saliva cells (Onose et al., 2015). Our whole-genome methylation sequencing found widespread dysregulation of DNA methylation loci distributed from Chr1 to Chr20. It is difficult to analyze the huge number of methylation loci and related genes. In order to narrow down the range of specific significantly dysregulated genes, the two high-throughput method was used with 13 genes identified in both gene chip analysis and MeDIP-Seq. A negative correlation was obtained, further supporting that hypermethylation of a gene results in downregulation of gene expression, and vice versa.

After careful screening, 2487 genes were significantly differentially methylated. These genes shared the same GO of extracellular region, suggesting the possibility that modification of the extracellular region in maternal PTSD affects offspring. Interestingly, KEGG pathway analysis of methylated genes showed a predominance of circulation-related pathways rather than HPA axis or immune pathways. Clinically, PTSD is considered as predictor and negative prognostic factor for heart disease (Onose et al., 2015). A large-scale meta-analysis (N = 402,274) showed that PTSD is independently related to increased risk of incident coronary heart disease after adjusting for covariates, e.g., depression and other factors (Edmondson et al., 2013). To the best of our knowledge, few reports have examined the relationship between the circulation system and gene methylation.

Itgb6 was the most frequently identified gene in the identified pathways (4/9). The *Itgb6* encodes integrin beta 6, which is involved in transforming growth factor (TGF)-beta activation and regulation of macrophage metalloproteinase (Mmp12) expression. According to recent studies, Itgb6 is related to multiple carcinogenesis-related pathways, including migration, invasion, and survival (Moore et al., 2014). Liang et al. (2015) reported that Itgb6-targeted immunoliposomes could provide a highly efficient method for targeted-drug

transportation in colon cancer and therefore may represent a promising clinical anticancer therapy. To the best of our knowledge, this is the first report of Itgb6 hypomethylation in prenatal PTSD intervention. Myh2 has been reported to be associated with hereditary myosin myopathies owing to mutations in the skeletal muscle myosin heavy chain genes (D'Amico et al., 2013). However, no studies have shown whether Myh2 is involved in maternal PTSD intervention. F5 has been reported to be involved in embolization and coagulation. Friso et al. (2012) found that promoter methylation in the coagulation *F7* gene can influence the concentrations of plasma F5II and is associated with coronary artery diseases. PTSD may elicit hypercoagulability, suggesting possible psychobiological characteristics, through which atherosclerosis progression and clinical cardiovascular disorders are attributed to PTSD (von Känel et al., 2006). However, the role of F5 in hypercoagulability and maternal PTSD stress in offspring remains unknown (von Känel et al., 2006). Neurotrophic factors (NTFs) and cytokines play important roles in supporting brain equilibrium in stressogenic situations and are essential to many nervous system functions, e.g., regulation, development, maintenance, survival, and neuronal and glial cell death (Stepanichev et al., 2014). Ntf3 is reported to function in the development of the enteric nervous system and acts as a candidate causative gene for Hirschsprung disease (Ruiz-Ferrer et al., 2008). Additionally, in the development of the mouse neocortex, Ntf3 acts as a feedback signal between postmitotic neurons and progenitors, which promote cell fate switches from apical progenitors to basal progenitors and from deep layer cells to upper layer cells (Parthasarathy et al., 2014). Ntf3 is also related to the pathophysiology of attention-deficit/hyperactivity disorder (ADHD), mood disorders, and psychostimulant action (Park et al., 2014). Although Ntf3 affects various system functions and mental disorders, no reports have described the potential role of this protein in the crossgeneration effects of maternal PTSD. Epn3 may play a vital role in the activation of tissue morphogenesis in epithelial cells (Spradling et al., 2001); however, the functional role of Epn3 in the stress reaction remains unclear. Tsg101 has been widely reported to be involved in immunological disorders, e.g., human immunodeficiency virus (HIV) infection, Ebola infection, acute myeloblastic leukemia, and papillary thyroid carcinomas (Lin et al., 2000; Martin-Serrano et al., 2001; Liu et al., 2002). However, there is no current evidence to support the correlation between Tsg101 and psychiatric disorders.

There were several limitations to our current study. First, the differential expression of the identified genes has not yet been validated, which may limit the strength of our findings. These genes will be validated by reverse transcript-PCR in our future studies. Additionally, we will perform additional analyses of body development and behavioral tests to determine the negative effects of maternal PTSD in greater detail. Interventions or treatments should also be assessed to support the clinical applicability of our findings.

CONCLUSION

Our results showed that maternal PTSD during pregnancy could delay physical and nervous system development, resulting in long-term negative effects on the offspring. The underlying mechanism could be attributed to changes in neurotransmitters and gene expression resulting from dysregulation of whole-genome methylation. Furthermore, circulatory system disorders in the offspring may be related to maternal PTSD through effects on specific genes such as *Itgb6*, *F5*, *Ntf3*, *Myh2*, and *Epn3*. Clinicians should carefully monitor the possibility of circulation disorders in patients with a family history of maternal PTSD.

Conflicts of interest

The authors declare no conflict of interest.

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