Changes of BDNF exon IV DNA methylation are associated with methamphetamine dependence

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Changes of *BDNF* exon IV DNA methylations are associated with methamphetamine dependence

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AIM: we investigated the DNA methylation of brain-derived neurotrophic factor *(BDNF)* exon IV in both human methamphetamine (METH) dependence and METH-administered rats. Also, the effects of METH on *Bdnf* expressions in rats were determined and evaluated for the correlation between the methylation and gene expression levels.

MATERIALS & METHODS: *BDNF* exon IV DNA methylation was determined in blood DNA samples of 100 METH dependent (53 METH with psychosis, and 47 METH without) and 102 control subjects by using bisulfite pyrosequencing. In METH-administered rats, *Bdnf* methylation in an equivalent sequence and gene expression in brain tissues of 10 METH injected rats (0.1-4 mg/kg METH, 3 times a day for 14 day and 4 times of 6 mg/kg METH at day15) and 10 controls were assessed by pyrosequencing and TaqMan® real time gene expression assay respectively.

RESULTS: There were significant increases of *BDNF* exon IV DNA methylation in human METH dependence, and at one site in prefrontal cortex (PFC) of METH-administered rats, while rat hippocampus showed a significantly reduced *Bdnf* methylation at this site, with an equivalent increase in gene expression. The methylation increases in humans were greatest in subjects with METH psychosis.

CONCLUSION: our findings demonstrate that *BDNF* exon IV DNA methylation is abnormal in METH psychosis. METH has neurotoxic effects on changes of both *Bdnf* DNA methylation and gene expression. Although a direct relationship between *Bdnf* methylation and its expression has not been proven, changes of *Bdnf* exon IV DNA methylation are associated with the neurobiology underlying METH dependence, especially METH dependent psychosis.

Keywords: methamphetamine, psychosis, drug dependence, METH-induced psychosis, METH administration, *BDNF*, exon IV, DNA methylation, gene expression, rs6265

Background and aim

Methamphetamine (METH) is an addictive psychostimulant that is a social and medical concern due to its increasing abuse worldwide. Long term or high dose METH can induce psychosis [1]; 40-60% of METH abusers who frequently

experience psychotic symptoms [2, 3] closely related to the positive symptoms of schizophrenia [4]. METH can induce neurotransmitter dysregulation, notably of the dopamine, glutamate and GABAergic systems. One consequence of the action of METH on the dopaminergic pathway is an excessive release of neurotransmitter glutamate in the cortex [3]. Excessive cortical glutamate can damage GABAergic neurons similar to that seen in schizophrenia and can lead to the development of psychosis [3]

The addictive and psychotogenic effects of METH have also implicated neurotrophic proteins, including that of brain derived neurotrophic factor (BDNF) [5]. BDNF plays crucial roles in various neuronal processes including neuronal growth, survival, maintenance and synaptic plasticity [6, 7]. Alterations of BDNF proteins are crucial for pathology of diseases. Previous studies have reported that deficits in BDNF signaling are associated with mood disorder, schizophrenia and addiction [5, 7, 8]. Infusions of BDNF in nucleus accumbens and ventral tegmental area are related to drug seeking behavior and sensitization [9]. Alterations of BDNF expression were observed in rats exposed to cocaine [9] and METH [10, 11] as well as in human METH-dependent patients [12].

Recently, a large number of studies providing that there are many mechanisms that can regulate the BDNF expression [13]. In pharmacogenetic studies of BDNF gene polymorphism, BDNF gene has the functional polymorphism in rs6265 (Val66Met). The rs6265 is substitution of valine (G) to methionine (A) and implicates in the level of BDNF secretion [14, 15], synaptic location, intracellular traffic, poorer working memory performance and cognitive ability [16, 17]. It also has been related to different psychiatric conditions [18] including METH dependence and METH psychosis in the Thai population [19].

In addition, epigenetic mechanism as DNA methylation can regulate the expression of BDNF [18, 20-22]. DNA methylation is an epigenetic process that is important in the control of gene transcription by inhibiting transcription factor binding to promoter sequences [23]. Much evidence has suggested that disturbances in the status of DNA methylation of genes are related to the prevalence of schizophrenia [24-26], suicide [27], depression [28], and drug addiction [29]. In the BDNF gene, changes in *BDNF* DNA methylation have been considered as potential biomarkers of psychiatric disorders [30]. The human *BDNF* has a complex gene structure consisting of 11 exons. Among 11 exons contains 9 functional promoters (I–V, Vh, VI-VII, IX). The BDNF promoter IV provides important sequences that related with the degree of BDNF gene expression [31, 32]. Increase of BDNF exon IV DNA methylation was correlating with decreased *BDNF* expression in postmortem brain of patients with major depressive disorder [27]. Although the BDNF rs6265 dost not existed in the sequences of this BDNF exon IV, both BDNF exon IV methylation and the rs6265 have been implicated in the BDNF expression. The BDNF exon IV methylation may associated with the rs6265, implicating to the pathology mechanism underlying METH dependence and METH psychosis.

Therefore, the hypothesis of this study was that METH exposure may induce changes in DNA methylation of the BDNF gene, resulting in dysregulation of *BDNF* transcription and contributing in turn to the neuropathology underlying METH dependence and METH dependent psychosis. **The objectives of this study were to investigate the DNA methylation levels of *BDNF* exon IV in both human METH-dependent subjects and METH-administered rats. DNA methylation profile of long interspersed element-1 (LINE-1), a measure of global DNA methylation, was also determined to prove a gene specific effect of METH on *BDNF* DNA methylation. Furthermore, we examined the**

effects of METH on *Bdnf* expressions and assessed the correlation between DNA methylation and gene expression. Additionally, in this cohort, the functional polymorphism of BDNF gene (rs6265) has been reported to be associated with vulnerability to METH dependence and METH dependent psychosis [19], we hypothesized that rs6265 might also be related to the extent of *BDNF* DNA methylation. Thus, we also investigated the association of the rs6265 with the DNA methylation status of *BDNF* exon IV in human METH dependent subjects.

Material and Methods

Human subjects and sample preparation

The sample of subjects enrolled in this study has been previously described [19, 33]. The subjects comprised 100 male METH dependence: 47 METH without psychosis and 53 METH dependent psychosis, and 102 matched controls. The METH dependent subjects were diagnosed by a psychiatrist based on Diagnostic and Statistical Manual of Mental Disorders (DSM IV). The control subjects had no history of drug use or psychiatric disorders. Mean age of subjects in controls and METH groups are 28.6±6.3 and 30.1±5.1 year respectively (p=0.065).

Fingertip blood samples (200 µl) were collected on FTA cards (Whatman, Inc., Florham Park, NJ, USA) and extracted for genomic DNA. The procedure for DNA extraction was performed by Chelex®100 method previously described [19]. The DNA solutions were kept at -20°C prior to DNA methylation analysis.

METH administration in animal

Drug preparation

D-Methamphetamine hydrochloride (Lipomed AG, Arlesheim, Switzerland) was dissolved in saline (0.90% w/v of NaCl) and freshly prepared before injection daily. Volume of drug injection was calculated and prepared dependent on rat body weight.

Animal and sample preparation

20 adult male Sprague-Dawley rats (280-350 g) were housed in cages at 24±1 °C under 12:12 light dark cycle with food and water ad libitum. The rats were acclimated in laboratory conditions for 5 day and followed by 2 days habituation. These 20 rats were randomly divided into 2 groups: **(1)** control group **(**C, n=10**)**, which was injected intraperitoneally (i.p.) with 1-2 ml/kg saline 3 times a day with 3-h interval for 14 days and 4 times a day with 2-h interval at day 15; **(2)** escalating dose METH binge group **(**ED, n=10**)**, which was injected 3 times a day at gradually increasing of doses of METH beginning from 0.1 to 4 mg/kg of METH over 14 days and was injected with 4 times 6 mg/kg METH at day15 [34, 35].

7 days after completing METH administration, rats were sacrificed by cervical dislocation and rat brains were dissected for prefrontal cortex (PFC), hippocampus and striatum [36]. Brain tissues were extracted for RNA and DNA using TRIzol™ Reagent (Invitrogen, CA, USA), according to the manufacturing' s standard protocol. The concentrations of DNA and RNA were measured by NANODROP (ND-1000, version 3.8.1) and Qubit® 2.0 fluorometer

respectively. The RNA was converted to cDNA for gene expression analysis. The DNA and cDNA samples were then stored at -20 °C before analysis.

DNA methylation analysis

Sequences to analyze and primers

The sequence to analyze for *BDNF* DNA methylation was designed based on an analogous region of *BDNF* exon IV in human and rat. The region of interest for *BDNF* promoter IV methylation contained 4 CpG sites of *BDNF* sequence in human (Figure 1) and 3 CpG sites of *Bdnf* sequence in rat (Figure 2). *BDNF* primers used in this study were designed by 64 PyroMark Assay Design software version 2.0 (QIAGEN). Primer sequences are displayed in table 1.

The sequence to analyze for long interspersed nucleotide element-1 (LINE1), a measure of global DNA methylation, in rat and human are provided by Fachim et. al. (2016) and Veerasakul et. al. (2017) respectively [29, 37]

Bisulfite modification and pyrosequencing

500-1000 ng DNA from human blood and rat brain were bisulfite-reacted using Epitect® Fast DNA bisulfite kit (QIAGEN AG, Hilden, Germany), according to manufacturing' s protocol.

The sequences containing CpG sites of *BDNF* exon IV and *LINE1* were identified and amplified by PCR. A total volume of 25 µl PCR reaction set up: containing 2 µl of BS-DNA template, 12.5 µl of 2x PyroMark PCR mastermix, 2.5 µl of 10X Corolload concentrate, 0.5 µl - 1 µl of 100 µM forward and biotinylated reverse primers for *BDNF* exon IV or *LINE1* were performed. The PCR cycling conditions were as follows: 95°C for 15 min, 45 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72 °C for 30 s, final extension at 72 °C for 10 min. The BS-PCR products were verified by 2% agarose gel electrophoresis.

BS-PCR products were processed for pyrosequencing analysis based on the QIAGEN manufacturer's standard protocol. Briefly, 80 µl total volume of immobilization reaction containing 20 µl BS-PCR products, 2 µl of Streptavidin sepharose HP beads, 40 µl of 2x PyroMark binding buffer, and 18 µl of high purify water, was set up into PyroMark 24 well-plate. The process was to perform bead-immobilized PCR products. Mixed well bead-immobilized PCR products were denatured and washed by 40 µl of denaturation solution and 50 µl washing buffer under the PyroMark Q24 vacuum workstation processes. Bead-immobilized PCR products were then suspended into annealing buffer containing 0.3 mM sequencing primer, and incubated at 80 °C for 2 min to hybridize DNA templates with sequencing primer. After 5 min cooling down, the hybridized DNA templates were sequenced by PyroMark Q24 pyrosequencer (Qiagen).

The % DNA methylation level calculated by the PyroMark Q24 software (version 2.0.7) was recorded. Samples underwent PCR and pyrosequencing in duplicate and any inconsistencies between samples were repeated.

Gene expression analysis

1500 ng of RNA templates were converted to cDNA according the protocol of qscript™ XLT cDNA SuperMix (Quanta BioSciences, Perry Parkway, Gaithersburg, USA). The cDNA was used to study Bdnf gene expression by TaqMan®

realtime gene expression assay (assay ID Rn02531967_s1). Briefly, 10 µl of real time PCR reaction: 2X TaqMan®Fast Advanced Master Mix, 20X TaqMan®Assay, and 500 ng cDNA was set up. Each reaction was performed in triplicate. The PCR reaction was amplified in 40 cycles of optimal experiment parameter provided by the assay. *Actb* (assay ID Rn00667869) was run with the Bdnf gene to use as internal control and normalize the data. The results of relative Bdnf gene expression ratio were then calculated by using the Pfaffl method [38].

Statistical analysis

The data of % DNA methylation and gene expression ratio were provided as mean ± S.E.M. All statistically analysis was performed by SPSS version 17. Significant difference comparison between control and METH groups was determined by independent sample-T test. The difference of % DNA methylation in 3 groups including controls, METH with psychosis and METH without psychosis was analyzed by Oneway ANOVA. LINE1 methylation for this cohort was already investigated by a previous study [29]. Mean LINE1 DNA methylation was used to analyze as a covariate. The correlation between DNA methylation and gene expression was determined by Pearson correlation analysis and the association between *BDNF* DNA methylation and gene polymorphism (rs6265) was analyzed by oneway ANOVA. The statistically significant difference was set at $P < 0.05$.

Results

BDNF DNA methylation in human METH dependence and METH-dependent psychosis

Duplication of PCR and pyrosequencing techniques for *BNDF* DNA methylation provided consistent results. Percentages of *BDNF* exon IV DNA methylation in METH dependence and METH-dependent subgroups are shown in figure 3-4. Significant increases of DNA methylation in all 4 CpG sites of *BDNF* exon IV were observed in METH dependence when compared to controls (figure 3). The psychosis subgroup showed strongly significant increases in*BDNF* methylation when compared to controls and METH dependence without psychosis (figure 4). The increase in methylation at CpG4 of *BDNF* exon IV was the greatest difference associated with METH psychosis.

A significant association between the methylation of *BDNF* exon IV and the functional *BDNF* variant (rs6265) was not observed (Table 2).

Previous study of this cohort showed there was no significant difference of LINE1 DNA methylation between control and METH groups [29]. Analysis using mean LINE1 methylation as a covariate showed no apparent influence; *BDNF* DNA methylation at all CpG sites remained significantly elevated in METH dependence.

Bdnf DNA methylation and gene expression in rats

DNA methylation for 3 CpG sites of *Bdnf* promoter IV in the brain of control and METH-administered rats is displayed in figure 5-7. The statistical analysis shows the percentage of prefronto-cortical *Bdnf* methylation at CpG3 is significantly greater in METH-administered rats than controls (figure 5). In contrast, hippocampal *Bdnf* CpG3 methylation was

significantly decreased in METH group (figure 6). There was no significant difference in striatal *Bdnf* DNA methylation (figure 7).

No significant difference in mean *Line1* DNA methylation was observed in 3 brain areas of METH-administered rats (figure 8). However, methylation of *Bdnf* CpG3 in PFC (F=3.160, P=0.101) and hippocampus (F=3.408, P= 0.090) of rats did not reach statistically significant differences after analyzing Line1 as covariate.

Bdnf gene expression is displayed in figure 9. *Bdnf* expression was significantly increased in hippocampus of METH rats when compared controls. Significant differences were not observed in PFC and striatum.

Correlation between *Bdnf* expression and *Bdnf* methylation did not reach significance in any brain areas of study (r = -0.109, p=0.657 in hippocampus, r=0.199, p=0.461 in PFC, r=0.024, p=0.921 in striatum). However, the tendency of METH administration to reduce *Bdnf* expression in PFC and enhance it in hippocampus was consistent with an increase of *Bdnf* methylation in PFC and a decrease in hippocampus of METH-administered rats.

Discussion

METH exposure has the effect of increasing *BDNF* exon IV DNA methylation in human METH dependence, an effect mainly driven by increases in subjects exhibiting psychosis. We also observe a hypermethylation at CpG3 of *BDNF* exon IV in prefrontal cortex (PFC) of rats undergoing a regime of METH administration; the PFC is involved in the pathogenesis of both METH psychosis and schizophrenia [39]. The findings support several previous studies. Increases of BDNF DNA methylation have been reported in both METH [40] and alcohol [41] dependence. Additionally, hypermethylation of the *BDNF* promoter have been proposed in association with psychiatric disorders [18], schizophrenia [26], depression [28, 42], suicide [27, 43], anxiety [42] and early-life adversity [44]. Increases of peripheral *BDNF* methylation are also indicative of a global DNA methylation.

Increase DNA methylation of *BDNF* was identified at exon IV. Moreover, the result in METH-administered rat, the significant change at CpG3 of *Bdnf* exon IV which corresponds to CpG4 in human also showed the greatest effect in METH-induced psychosis. This region of exon IV is rich in TF binding sites including a methylation-sensitive recognition site for cAMP response element binding protein (CREB) [45] which has an activation effect on transcription [46]. CREB is implicated in the regulation of adaptive neuronal responses and gene-mediated synaptic function [46]. A previous study [27] reported that methylation at BDNF promoter IV shows a significant negative correlation with the level of BDNF transcription. Lower BDNF gene expression, corresponding to higher *BDNF* exon IV methylation, was observed in Wernicke's area of suicide subjects [27]. Therefore, enhancement of DNA methylation at CpG3 of *Bdnf* exon IV in PFC of METH-administered rats may imply a METH-induced deficit of BDNF in PFC.

Up to now the etiology of METH-associated psychosis remains poorly understood. Dysfunction in PFC seems to exist for both METH related psychosis and schizophrenia [39, 47]. Chronic amphetamine has been shown to decrease neurotrophins BDNF and NGF in selective brain areas in rats [48]. BDNF reductions in cortical areas [7, 47], as well as in blood plasma and serum [49-52] have been found in patients with schizophrenia, depression and bipolar disorder [53, 54] . In addition, decreases of BDNF after amphetamine exposure in rats can change the behavioral endophenotype relating to the positive symptoms of schizophrenia [55]. Therefore, it is probably that METH-induced

hypermethylation of *BDNF* exon IV may relate to reduction of BDNF. Low BDNF in PFC may suggest dysfunction of BDNF signaling and impair its neuroprotective function against METH-induced neuronal excitotoxicity. Hsieh et al [3] have reviewed that METH-increased dopamine and glutamate outflow in the cortex can damage GABAergic interneurons [34]. The effects can result in reductions in both of GABAergic neuronal populations implicated in schizophrenia [34] and of inhibitory activity in the cortex [3]. Lack of inhibitory control in the bilateral ventrolateral and frontopolar PFC is apparent in METH-dependent psychosis patients [39, 56]. Therefore, changes of prefronto-cortical *BDNF* exon IV methylation might be suggested as one abnormality involved in in the neurobiology of METH-induced psychosis.

We have previously undertaken a genetic study identifying the association of BDNF rs6265 gene polymorphism in vulnerability for METH dependence and METH dependent psychosis. A frequency of *BDNF* (rs6265)-GG genotype was reduced in METH psychosis subjects [19]. Low frequency of BDNF (rs6265)-GG [14, 57] has been suggested with poor BDNF levels. Deficits of BDNF levels are well reported in pathology of schizophrenia (). We have found no evidence for an effect of *BDNF* rs6265 genotype and *BDNF* exon IV DNA methylation. The result might be because that the rs6265-G allele is a sequence near the BDNF promoter IX [58], but not promoter IV. However, the present study supports our association study of the BDNF rs6265 with METH dependence. The *BDNF* rs6265-GG genotype [57, 59] and hypermethylation of the BDNF exon IV have also been reported to be associated with BDNF expression. Our findings indicate both genetic risk and epigenetic consequences may separately contribute to the development of METH-dependent psychosis.

An increase of *Bdnf* expression corresponding to a decrease of *CpG3 Bdnf* exon IV DNA methylation was found in hippocampus of METH-administered rats; this is opposite to the effects seen in the PFC. The results demonstrate regional specific effects of METH on *Bdnf* methylation and expression levels. Such findings are consistent with a previous study which reported selective regional effects of stress on alteration of *Bdnf* DNA methylation. A decrease of *Bdnf* DNA methylation was found in ventral hippocampal CA1 subregion, but a methylation increase was found in dorsal hippocampal CA3 [60]. Moreover, Xiang et al., 2020 reported the difference of DNA methylation at the collapsin response mediator protein 2 (CRMP2) promoter region between the hippocampus and prefrontal cortex in a rat model of depression, suggesting regional different in the regulation of DNA methylation in the CRMP2 promoter between the hippocampus and prefrontal cortex during the development of depression [61]. Based on our results, DNA methylation may response for the changes in *BDNF* expression in both hippocampus and prefrontal cortex of METH-administered rats. The alterations observed in hippocampus are responses to METH exposure. Lower *Bdnf* methylation and higher *Bdnf* transcription show consistent results and indicate compensatory responses to neuronal damage following toxic METH exposure. This is consistent with a previous study which demonstrated upregulation of *Bdnf* mRNA in the rat hippocampus after METH-administration [62]. However, in this study, the correlation between hippocampal DNA methylation and gene expression did not reach a statistical significance. A possible explanation is that changes of BDNF gene expression may be co-regulated by other factors, such as the regulation of other transcription factors and other epigenetic mechanisms (histone modification and non-coding RNA silencing).

There were several limitations existing in the current study. The first is the small sample size which can limit the power of the study. This is an unavoidable consequence of selecting a well-characterized series of METH dependent subjects of Thai ethnicity. Furthermore, we have studied only male METH dependent samples, which reflects METH abuse in Thailand is more prevalent in males than females. In addition, we recognize that methylation can be region-specific, while we have inevitably been restricted in investigating human DNA methylation in peripheral blood instead of brain tissue. However, blood and plasma BDNF has been previously reported to reflect BDNF in the brain [63]. Furthermore, a study in Parkinson disease (PD) also reported concordant effects on methylation in brain and blood samples, indicating that blood may be a surrogate for brain tissue to detect DNA methylation in PD [64]. Therefore, in the present study, the methylation differences in peripheral blood may well indicate equivalent differences in DNA methylation and its functional consequences in the brain. We were also restricted in having small samples of blood to investigate gene and protein expressions. Thus, we have been unable in the human sample to determine the correlation between the blood *BDNF* DNA methylation and gene expression in order to assess the consequence of the DNA methylation change. However, a previous study reported that BDNF exon IV DNA methylation could influence expression of *BDNF* [27]. In an animal model of METH administration, alterations of DNA methylation were not examined in every brain region. However, the areas studied here are particularly implicated in drug addiction [65, 66]. Furthermore, our animal model was not necessarily a proven model of METH dependence or METH dependent psychosis; the pattern of drug administration was to establish a model of human METH abuse [35]. Finally, only methylation in BDNF exon IV was studied. Although this might not reflect DNA methylation of the entire BDNF gene, it is a region that has been widely studied to show a functional effect in multiple disorders [27, 44, 67].

Conclusion

BDNF exon IV DNA methylation is associated with METH dependence. Particularly, this *BDNF* methylation is abnormal in METH-induced psychosis. The methylation change is likely a consequence of the toxicity of METH treatment, as confirmed by the results from an animal model of METH administration which demonstrated equivalent changes of *Bdnf* exon IV DNA methylation and gene expression after METH treatment. While a causal effect of changes in *BDNF* DNA methylation on METH dependence and psychosis cannot be proven, these findings are consistent with an influence of DNA methylation on BDNF expression and subsequent neuronal damage and dysfunction associated with METH abuse.

Future perspective

Examination of *BDNF* exon IV DNA methylation might be a useful approach to understanding the biological basis of psychosis in patients with substance abuse. Thus, these epigenetic changes may underlie deficits in BDNF and a subsequent neuronal damage and dysfunction in METH exposure, providing important evidence towards the neurobiology mechanism of drug dependence and drug-dependent psychosis. This information could be valuable in the development of clinical approaches for diagnosis and treatment of psychosis in METH addiction. Also, the present study further defines *BDNF* as a prominent molecular biomarker for psychosis. However, much more needs to be

investigated in order to determine the effects of *BDNF* DNA methylation in METH dependence and its related disorders. It is first important to determine if the methylation changes seen in the specific CpG sites and specific regions are directly related to BDNF changes in the disease.

Ethical conduct of research

The study in human METH dependence was approved by the Ethical Committees of Naresuan University, Thailand (Reference number: COA No.149/2016, IRB No.141/59). Written informed consents were provided by all participants.

All animal procedures followed compliance with Mahidol University Code of Practice and the National Institutes of Health (USA) Guidelines. The protocol was approved by the Animal research committee of Naresuan University (Reference number: 59 01008).

Financial & competing interests disclosure

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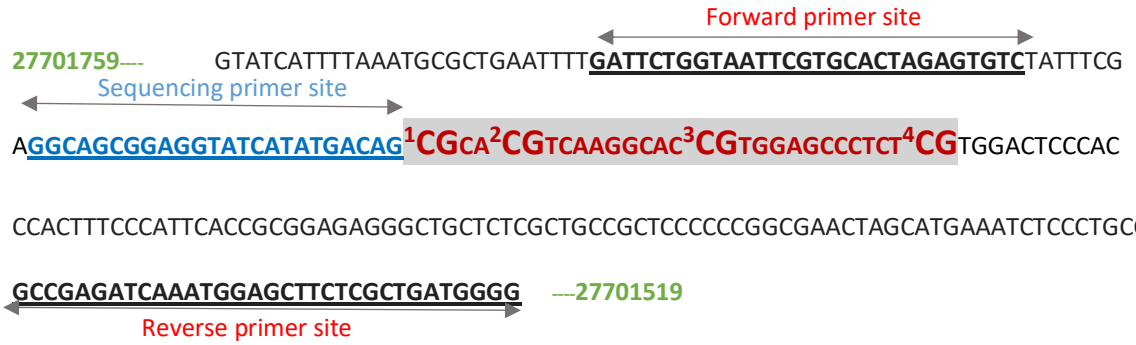

Figure 1 Sequence to analyze for human BDNF exon IV containing 4 CpGs sites

(Homo sapiens chromosome 11, GRCh38.p12, Primary Assembly, Sequence ID: NC_000011.10, Length: 27701519-277759)

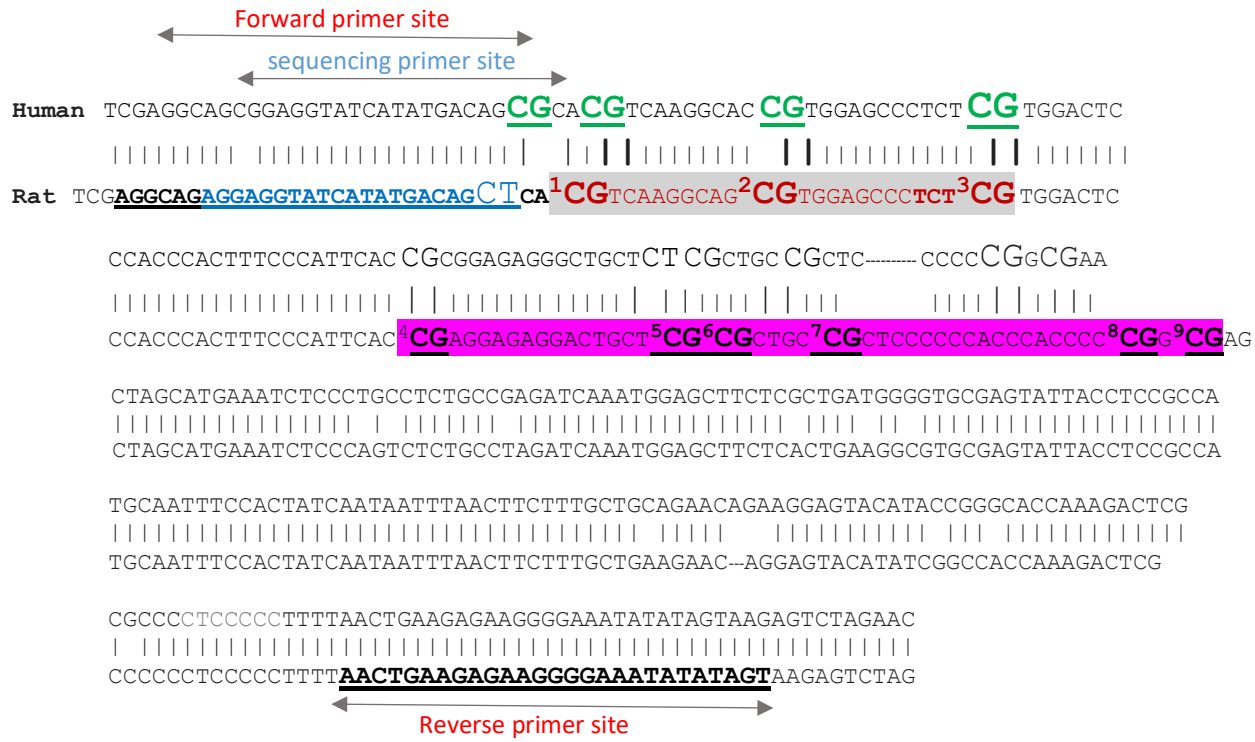

Figure 2 Sequence to analyze for *Bdnf* exon IV in rats containing 3 CpGs sites

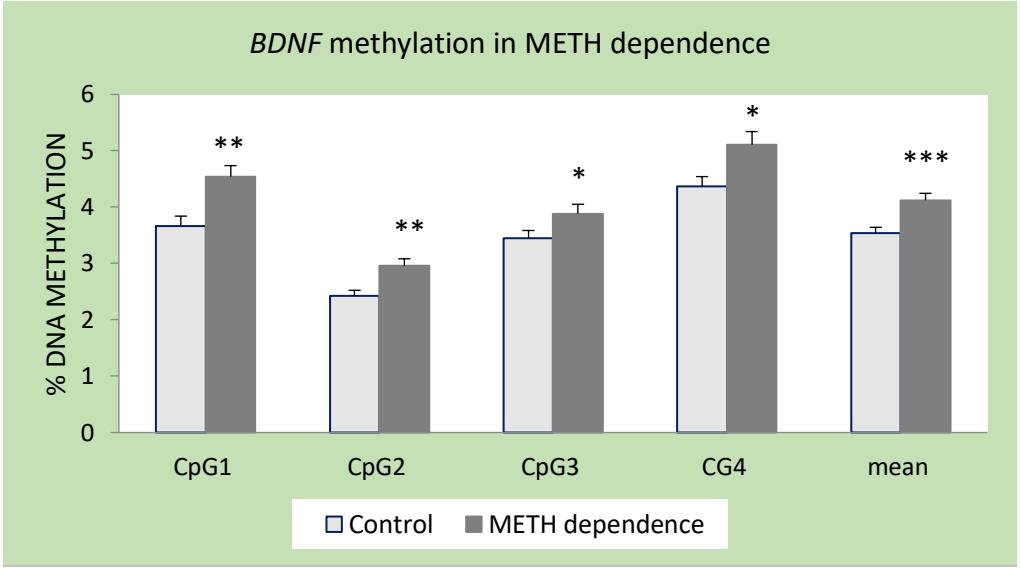

Figure 3 *BDNF* exon IV DNA methylation in METH dependence (n=99-102) and controls (n=100). *p<0.05, **p < 0.01 and ***p<0.001 in comparison between control and METH groups by independent sample T-test

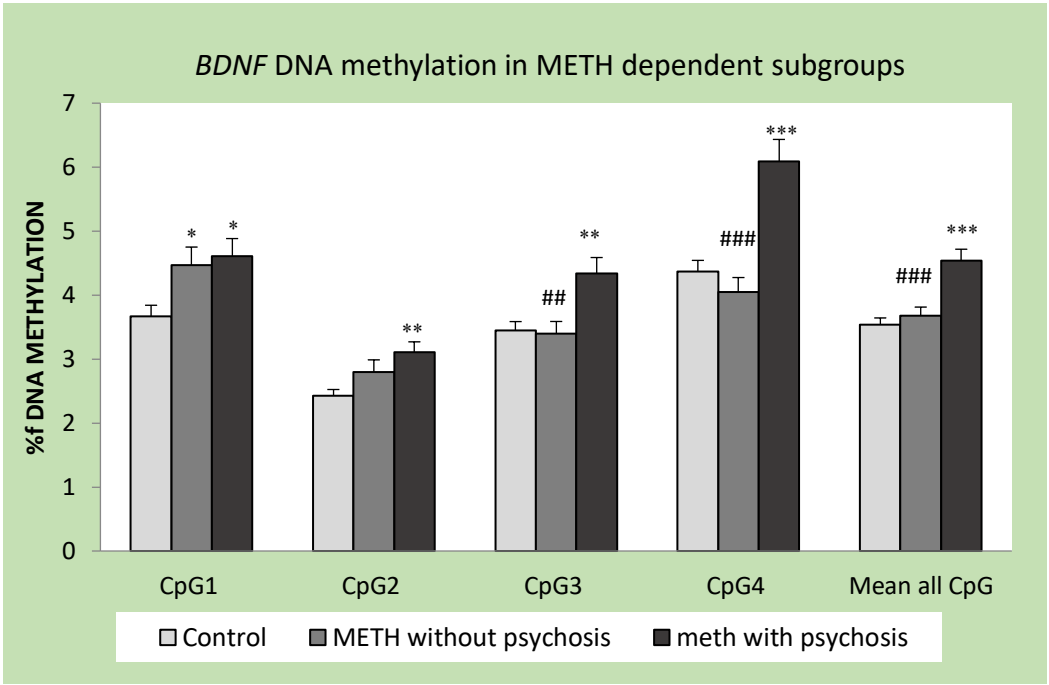

Figure 4 *BDNF* DNA methylation in METH dependent subgroups, *p<0.05, **p < 0.01 and ***p<0.001 in comparison to control, and $^{\#}$P in comparison between METH with and without psychosis by Oneway ANOVA

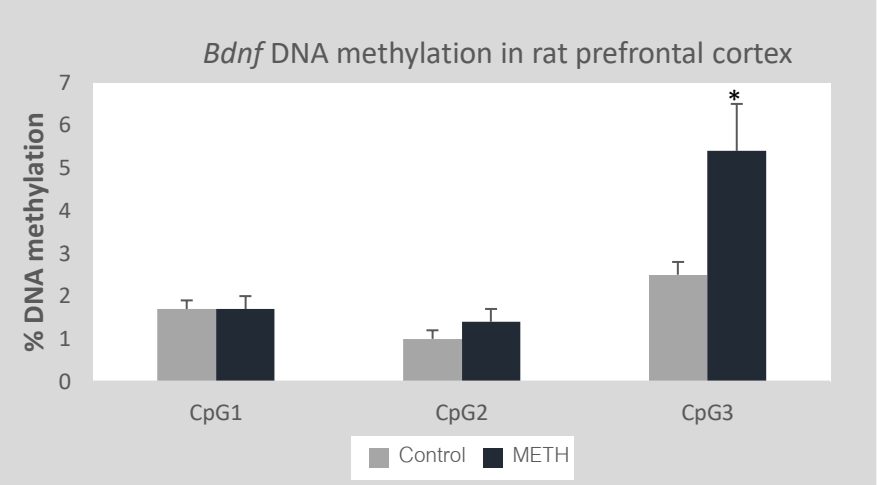

Figure 5 *Bdnf* DNA methylation in rat prefrontal cortex

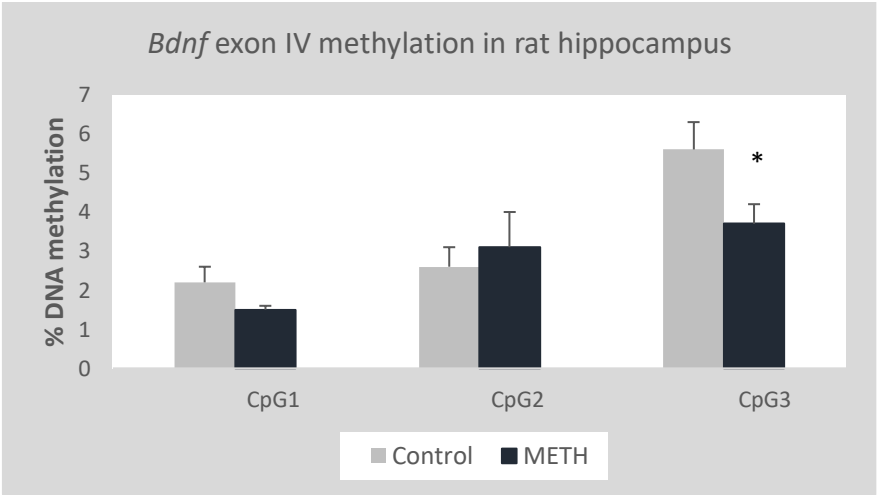

Figure 6 *Bdnf* DNA methylation in rat hippocampus

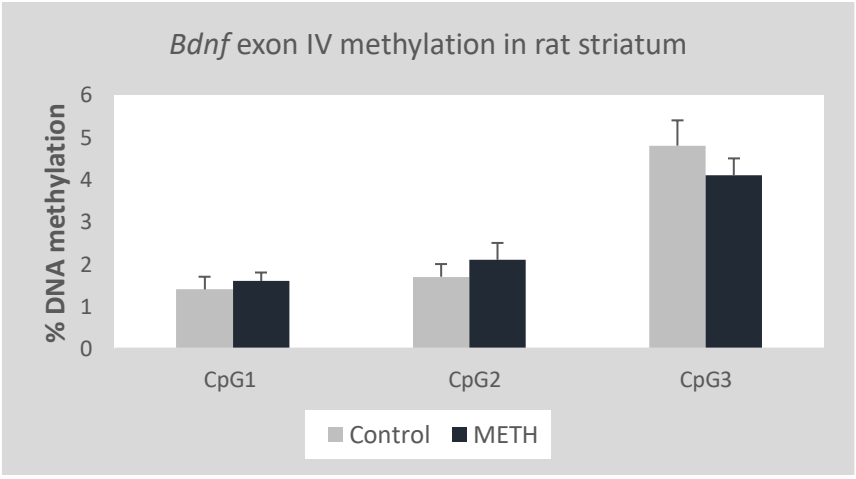

Figure 7 *Bdnf* DNA methylation in rat striatum

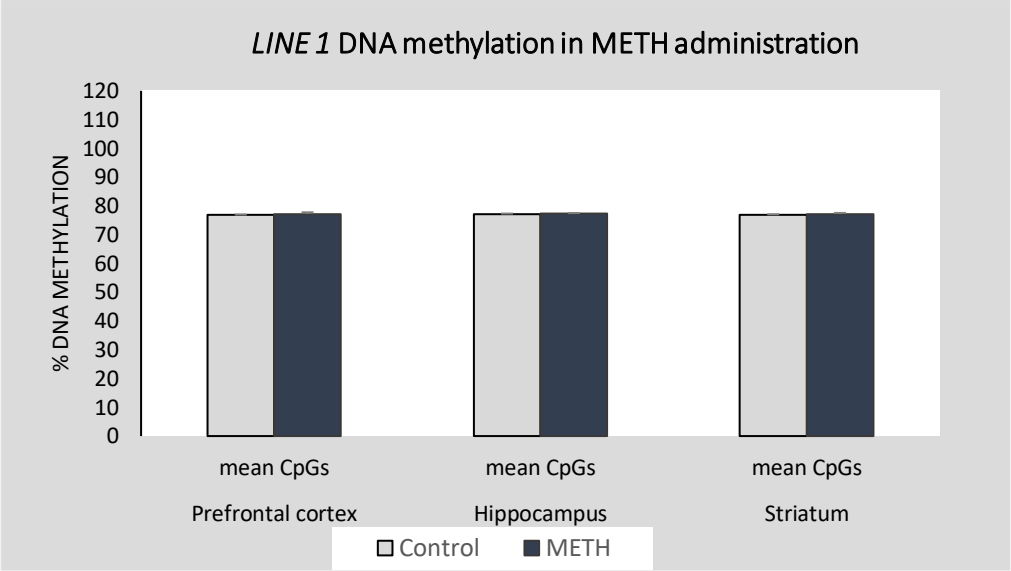

Figure 8 *LINE1* methylation in rat (METH group (n=9), controls (n=9))

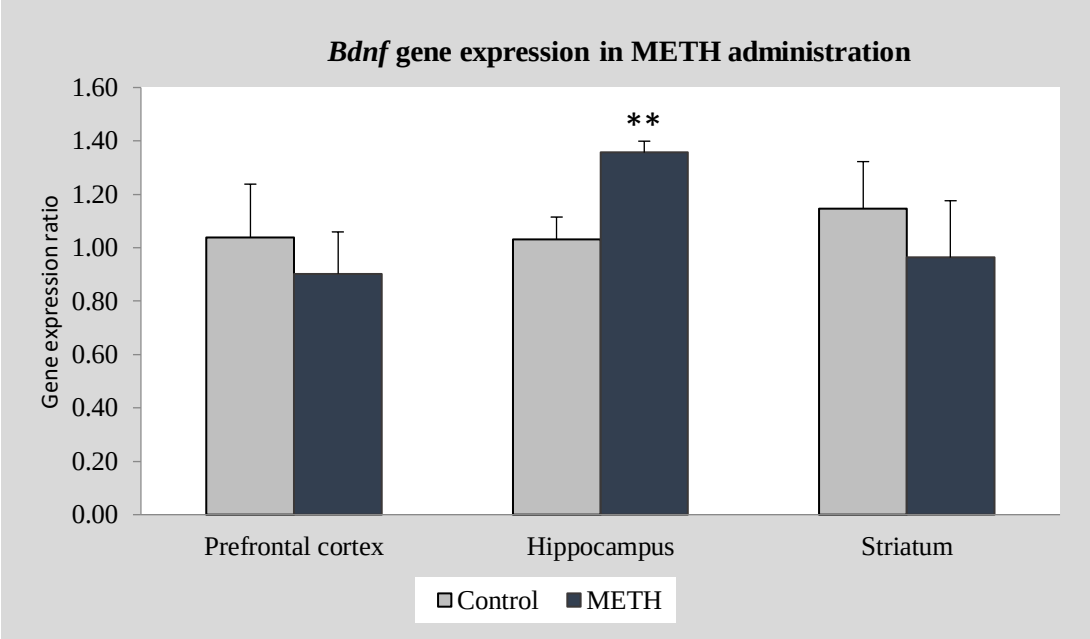

Figure 9 Bdnf gene expression in rat (METH group (n=9), controls (n=9)), **p < 0.01 in comparison to control

Table 1 Set of primer sequences used in DNA methylation study

Gene	Primer	Sequence	Tm, ºC	Amplicon length
1. *Bdnf* exon IV in rat (CpG1-3)	Fwd	5'AGGTAGAGGAGGTATTATATGATAGT3'	59.7	324
	Rev (biotin)	5'ACTATATATTTCCCCTTCTCTTCAATT3'	58.5	
	Seq	5'AGGAGGTATTATATGATAGTT3'	44.8	
	Sequence to analyse	5'TAYGTTAAGGTAGYGTGGAGTTTTTTYGTGGATTTTTATTTATTTTTTTATT3'		
2. *Line* 1 in rat	Fwd	5'TTGTTGTAAGAAAGTTGTTTGGTGAGTT 3'	63	182
	Rev (biotin)	5'ACCTCAAAAATACCCACCTAACC3'	62.1	
	Seq	5'GGTGAGTTTGGGATA	44.4	
	Sequence to analyse	5'TAYGGAAGTAGAATTTTTTTAGAATYGGGTAYGTTTTGTGTTTATYGGAA3'		
3. *BDNF exon IV in human*	Fwd	5' GATTTTGGTAATTAGTGTATTAGAGTGTT 3'	56.8	215
	Rev (biotin)	5' CCCCATCAACCAAAAACTCCATTTAATCTC 3'	59.5	
	Seq	5' GGTAGAGGAGGTATTATATGATAG 3'	45.2	
	Sequence to analyse	5' YGTAYGTTAAGGTATYGTGGAGTTTTTTYGTGGATT 3'		

Table 2 The association of *BDNF* gene polymorphism rs6265 with *BDNF* DNA methylation in controls and METH dependent patients

Mean *BDNF* exon IV DNA methylation in each group	Genotype			ANOVA	Independent sample test	
	GG	GA	AA			
	mean±S.E.M. (n)	mean±S.E.M. (n)	mean±S.E.M. (n)	*p*	p^a	p^b
Control (n=101)	3.7± 0.2(23)	3.4± 0.1(53)	3.6± 0.2(25)	0.624	0.730	0.450
METH (n=100)	4.1± 0.2(38)	4.0± 0.2(42)	4.2± 0.3(20)	0.943	0.810	0.896
- with psychosis (n=53)	4.9± 0.3(15)	4.1±0.3(27)	4.8 ± 0.5(11)	0.119	0.34	0.159
- without psychosis (n=47)	3.6± 0.2(23)	4.0± 0.3(15)	3.4 ± 0.2(9)	0.242	0.233	0.638

Note: p^a A/A homozygotes Vs. G allele carriers

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