Exploring the role of BDNF DNA methylation and hydroxymethylation in patients with Obsessive Compulsive Disorder

Claudio D'Addario[1,2,], Fabio Bellia[1,*], Beatrice Benatti[3], Benedetta Grancini[3], Matteo Vismara[3], Mariangela Pucci[1], Vera De Carlo[3], Daniela Galimberti[6,7], Chiara Fenoglio[6,7], Elio Scarpini[6,7], Mauro Maccarrone[8], Bernardo Dell'Osso[3,4,5]*

Affiliations:

[1]University of Teramo, Bioscience, Teramo, Italy

[2]Karolinska Institutet, Department of Clinical Neuroscience, Stockholm, Sweden

[3]University of Milan, Department of Psychiatry, Milano, Italy

[4]CRC "Aldo Ravelli", University of Milan, Milano Italy

[5]Department of Psychiatry and Behavioral Sciences, Stanford University, CA, USA

[6] University of Milan, Dino Ferrari Center, Milan, IT

[7] Fondazione IRCCS Ca' Granda, Ospedale Policlinico, Neurodegenerative Diseases Unit, Milan, IT

[8] Bio-Medico Campus University of Rome, Department of Medicine, Rome, Italy

**equal contribution*

Corresponding authors:

Claudio D'Addario: cdaddario@unite.it

Bernardo Dell'Osso: bernardo.dellosso@unimi.it

Short title: BDNF gene regulation in OCD

Abstract

Obsessive-compulsive disorder (OCD) is a clinically heterogeneous neuropsychiatric condition associated with profound disability, whose susceptibility, stemming form interplaying genetic and environmental factors, is still under investigation. In this perspective, we sought to explore the transcriptional regulation of Brain Derived Neurotrophic Factor (BDNF), a promising candidate biomarker in both development and etiology of different neuropsychiatric conditions, in peripheral blood mononuclear cells from OCD patients and healthy controls. In particular, we focused on BDNF gene expression and interrogated in depth DNA methylation and hydroxymethylation at gene promoters (promoter exons I, IV and IX) in a sample of OCD patients attending a tertiary OCD Clinic, receiving guidelines recommended treatment, and matched controls. Our data showed a significant increase in BDNF gene expression and a significant correlation with changes in the two epigenetic modifications selectively at promoter exon I, with no changes in the other promoters under study. We can conclude that transcriptional regulation of BDNF in OCD goes through epigenetic mechanisms and suggest that this is possibly evoked by the long-term pharmacotherapy. Further studies are needed to investigate epigenetic mechanisms at early stages of the disease and in drug naïve patients. Of note, we provide unprecedented evidence for the importance of analyzing 5-hydroxymethylcytosine levels to correctly evaluate 5-methylcytosine changes.

Key words: BDNF; Obsessive Compulsive Disorder; DNA methylation; DNA hydroxymethylation

Abbreviations

OCD – Obsessive-Compulsive Disorder

BDNF – Brain Derived Neurotrophic Factor

SNP – Single Nucleotide Polymorphism

5mC – 5-methylcytosine

5hmC – 5-hydroxymethylcytosine

TET – Ten-Eleven-Translocation proteins

BD – Bipolar Disorder

MDD – Major Depressive Disorder

PBMCs – Peripheral Blood Mononuclear Cells

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

ß-ACT – beta actin

BS – Bisulfite conversion

oxBS – oxidative Bisulfite conversion

INTRODUCTION

Obsessive-Compulsive disorder (OCD) is a condition with frequent early onset and chronic course (Dell'Osso *et al.*, 2013) characterized by recurrent, unwanted, time-consuming obsessive and compulsive behaviors that cause distress and/or impairment (Milad and Rauch, 2012). The World Health Organization classifies OCD as one of the 10 most disabling conditions for decreased quality of life and loss of income, with a lifetime prevalence of 2-3% of the general population (Milad and Rauch, 2012).

OCD diagnosis is complex and several studies investigated its genetic etiology with mixed and sometimes conflicting results mainly addressing serotonergic, glutamatergic and dopaminergic pathways (Carlsson 1977; Grunblatt *et al.*, 2014; Muneer 2016).

Currently, the most effective pharmacological intervention for OCD is represented by the selective serotonin reuptake inhibitors (SSRIs) and, due to the delayed onset of their clinical efficacy, downstream molecular targets might be responsible for their therapeutic efficacy (Martinowich and Lu, 2008). One possible mechanism might involve brain-derived neurotrophic factor (BDNF) (Monteggia *et al.*, 2004), well-known for its role in neuronal development, proliferation and survival, as well as in synaptic plasticity (Cattaneo *et al.*, 2016; Duman *et al.*, 2000). Indeed, the association of BDNF gene polymorphisms with OCD has been investigated, mainly focusing on the Val/Met polymorphism at codon 66, due to the Single Nucleotide Polymorphism (SNP) at nucleotide 196 (196G/A; rs6265), showing a protective effect of the rarer Met allele was shown to be protective (Alonso *et al.*, 2008, Hall *et al.*, 2003), yet with conflicting results (Da Rocha *et al.*, 2011; Wendland *et al.*, 2007) in OCD patients.

Despite solid evidence that genetic contribution is important for OCD, environmental factors as well as gene x environment interactions are also involved in disease development (Türksoy *et al* 2014; Faravelli *et al.*, 2012; Nestadt *et al.*, 2010). Environmental signals are integrated by epigenetic processes in order to activate or

repress gene expression regulating DNA accessibility for transcription factors (Jaenisch and Bird, 2003). The most stable epigenetic mechanism is DNA methylation, well-investigated in different psychiatric conditions (Nestler *et al.*, 2016). DNA methyltransferases are the enzymes responsible of the transfer of a methyl group to the fifth carbon of cytosine residues to form 5-methyl-cytosine (5mC) usually occurring at CpG dinucleotides (Jurkowska *et al.*, 2011). Presence of 5mC has been classically associated to gene silencing and formation of repressive chromatin (Schubeler 2015). Only recently, more attention has been given to the oxidative reaction catalyzed by the ten-eleven-translocation (TET) proteins (Ito *et al.*, 2010, Tahiliani *et al.*, 2009, Zhang *et al.*, 2010) and converting 5mC into 5-hydroxymethylcytosine (5hmC) (Dahl *et al.*, 2011). This is an intermediary step in the process of DNA demethylation (Guo *et al.*, 2011, Hashimoto *et al.*, 2012, Iqbal *et al.*, 2011) and is generally associated with increased gene expression (Chen *et al.*, 2012, Jin *et al.*, 2011, Song *et al.*, 2010). The relevance of 5hmC in neural function has already been suggested (Szulwach *et al.*, 2011), however, as yet only a few reports have interrogated its role in brain disorders (Feng *et al.*, 2015).

Human BDNF gene is located on chromosome 11p13-14, is formed by 11 exons (I-IX, Vh and VIIIh) and 9 promoters (Pruunsild *et al.*, 2007). Alterations of DNA methylation in BDNF promoter exon I and IV have been already observed in peripheral blood mononuclear cells (PBMCs) of patients with different psychiatric conditions such as Schizophrenia (Roth *et al.*, 2009; Ikegame *et al.*, 2013; Kordi-Tamandani *et al.*, 2012), Depressive Disorders (Chen *et al.*, 2010; Lopez *et al.*, 2013; Tadic *et al.*, 2013; Kang *et al.*, 2013), suicidal deaths (Keller *et al.*, 2010), Bipolar Disorder (BD) (D'Addario *et al.*, 2012; Dell'Osso *et al.*, 2014), and Major Depressive Disorder (MDD) (Fuchikami *et al.*, 2011; Kang *et al.* 2013; Tadic *et al.* 2013; D'Addario *et al.*, 2013). Moreover, Mill and co-workers evaluated DNA methylation in major psychoses, studying the coding exon IX, where the CpG SNP rs6265 is located (Mill *et al.*, 2008).

We here investigated the possible contribution of both 5-mC and 5-hmC at BDNF promoter exons I, IV and IX in relation to the pathophysiology of OCD, analyzing DNA extracted from PBMCs of patients and healthy control subjects.

MATERIALS AND METHODS

Subjects, gene expression, methylation analysis and genotyping

35 OCD outpatients followed up at the OCD tertiary outpatient Clinic of the University Department of Psychiatry of Milan, IRCCS Policlinico Hospital, were included in the study. Diagnoses were assessed by the administration of a semi-structured interview based on DSM-5 criteria (SCID 5 research version, RV) (First *et al.*, 2015). In case of psychiatric comorbidity, OCD had to be the primary disorder and illness severity was measured through the Yale-Brown Obsessive Compulsive Scale (Goodman *et al.*, 1989). Exclusion criteria were presence of medical condition and/or drug abuse. All patients were for at least one month on stable pharmacological treatment chosen according to International guidelines in the field (Koran *et al.*, 2007). Control subjects (n=32) were volunteers without any psychiatric disorder, as determined by the nonpatient edition of the SCID and no positive family history for major psychiatric disorders in the first-degree relatives (Maxwell, 1992). The study was conducted with the appropriate ethical approval, and all subjects provided written informed consent before enrollment. Demographic and clinical characteristics for the study sample as well as psychotropics used by OCD subjects are shown in Table 1.

Preparation of nucleic acids from PBMCs and analysis of BDNF gene expression paralleled methods described in detail elsewhere and primers sequence are reported in supplementary Table 1 (D'Addario *et al.*, 2012).

To study DNA methylation/hydroxymethylation, two aliquots of 500 ng of genomic DNA samples were processed through bisulfite conversion (BS) or oxidative bisulfite conversion (oxBS) (Matsubara *et al.*, 2015; Qu *et al.*, 2015) and amplified by Pyromark PCR Kit (Qiagen) in accordance with the manufacturer's protocol. BDNF primer sequences are reported in Table 2 and provided by Qiagen. The sequences were designed to target regions within the CpG islands located upstream BDNF exon I (4 Cpg sites) and exon IV (6 CpG sites) as well as in within exon IX (2 CpG sites including the one created by the SNP rs6265) (see Figure 1 for details). PCR conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and, finally, 72 °C for 10 min. PCR products were verified by agarose electrophoresis. Pyrosequencing analysis was conducted using the PyroMark Q24 (Qiagen); primers used for DNA methylation/hydroxymethylation analysis are shown in supplementary Table 2. Data obtained for DNA methylation levels obtained by BS treatment is the combination of 5mC and 5hmC, while these obtained following oxBS treatment gives the level of 5mC alone (Booth *et al.*, 2012).

SNP rs6265 was genotyped using the pyrosequencing assay designed to interrogate percentage of methylation in this region.

Statistical analysis:

Data are expressed as mean ± standard error of the mean (SEM); differences between OCD and controls in gene expression were calculated with the non-parametric Mann-Whitney t-test. Data analysis of DNA methylation for each region was performed with a multiple t-test using the Sidak-Bonferroni method. All the p values <0.05 were considered statistically significant. All tests were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

RESULTS

Significant increase of BDNF mRNA levels was observed in OCD patients compared to controls (2.32 ± 0.28, P=0.0002 Mann-Whitney test) (Figure 2). We did not observe any difference between OCD patients and control subjects in both 5mC and 5hmC at gene promoters IV and IX (see supplementary Tables 2~~3~~ and 3~~4~~). However, consistently with the gene expression upregulation, we observed at promoter exon I a reduction in 5mC levels (OCD: 1.34 ± 0.15; CTRL: 2.49 ± 0.22; p<0.0002) (Figure 3), as well as an increase in 5hmC levels (OCD: 2.28 ± 0.20; CTRL: 0.82 ± 0.10; p<0.0001) (Figure 3) confirmed by correlation between BDNF gene expression and percentage change in 5mC levels (P=0.0279, Spearman's r =-0.4311) (Figure 4a) and 5hmC levels (P=0.0333, Spearman's r =0.4552) (Figure 4b). No differences among OCD subjects were observed in both 5mC and 5hmC levels when stratifying data based on gender, age, duration of illness, and pharmacotherapies (data not shown). Moreover, genotyping rs6265, we failed to observe any association with OCD in subjects carrying the minor allele T as well as any relevant difference when we compared DNA methylation in OCD patients and control subjects with the different genotypes (see supplementary Tables 4~~5~~, 5~~6~~, 6~~7~~).

DISCUSSION

The first relevant result of this study is the up-regulation in BDNF gene expression observed in PBMCs from OCD patients compared to healthy controls.

To the best of our knowledge, this is the first study reporting changes in BDNF mRNA levels in blood samples of patients with OCD, even though alterations in BDNF protein plasma levels have been previously documented in patients with OCD (Oliveira-Maia and Castro-Rodrigues, 2015), with studies reporting no differences (Yoshimura et al., 2006) or lower levels (Maina *et al.*, 2010; Wang *et al.*, 2011; Fontenelle *et al.*, 2012) in patients compared with controls.

Indeed, altered BDNF gene expression has been reported in patients suffering from other psychiatric disorders showing a down-regulation in depression (Cattaneo *et al.*, 2010, 2013; D'Addario *et al.*, 2013; Pandey *et al.*, 2010) and Bipolar Disorder (D'Addario *et al.*, 2013; Köse Çinar *et al.*, 2016; Lin *et al.*, 2016), consistently with the reduction of BDNF serum levels in these pathologies (Bus *et al.*, 2015; Molendijk *et al.*, 2011; Fernandes *et al.*, 2015) as well as in anxiety disorders (Molendijk *et al.*, 2012). On the other hand, published findings on BDNF role in Schizophrenia are more difficult to interpret, with some studies showing brain down-regulation (Durany *et al.*, 2001; Weickert *et al.*, 2003; Hashimoto *et al.*, 2005) as well as up-regulation (Takahashi *et al.*, 2000; Iritani *et al.*, 2003; Cheah *et al.*, 2016) of gene expression and, consistently with the latter, others reporting increased BDNF mRNA in blood of patients with Schizophrenia as well (Kordi-Tamandani *et al* 2012).

The latter evidence is consistent with present findings and appear of relevance for OCD. Indeed, already in 1878 it was suggested that OC symptoms could be of frequent observation in patients with Schizophrenia (Westphal 1878) and many studies suggested some clinical overlap between OCD and Schizophrenia (Zink 2014). Overexpression of BDNF has been also associated with working memory deficits, increased anxiety-like traits in preclinical models (Govindarajan *et al.*, 2006; Papaleo *et al.*, 2011) as well as with cognitive problems in clinical samples (Yeom *et al.*, 2016; Sayyah *et al.*, 2009; Taha *et al.*, 2017). Indeed, deficits in working memories, and associative learning are commonly encountered in patients with OCD (Chamberlain *et al.*, 2005; Shin *et al.*, 2014). From an animal model perspective, it has been recently reported that hyperactivity of BDNF/TrkB signaling might trigger OCD-like behavior in mice (Ullrich *et al.*, 2018).

It should be also considered that all OCD subjects involved in the study were under long-term treatment with SSRI, and BDNF has been indicated by many reports as a key player in the action of antidepressants (Björkholm and Monteggia, 2016). In particular, we

previously reported that in MDD and BD BDNF transcriptional regulation was likely not affected by antidepressant treatments (D'Addario *et al*., 2012, 2013). However, this might not be case in OCD, whose treatment, according to guidelines, requires higher doses of SSRIs and a longer course of therapy (Koran *et al*., 2007), compared to MDD, and this might have affected the observed increase in BDNF, considered as the main downstream antidepressant treatments mechanism of action (Russo-Neustadt and Chen, 2005).

It is also noteworthy to mention that several other clinical variables collected in our sample, including family history for OCD, early onset, duration of illness, duration of untreated illness, comorbidity with other psychiatric disorders, number of psychotropics, and clinical phenotypes might have somehow influenced the increased BDNF: the limited size of our sample, however, likely prevented us to detect any statistical significance in such regard.

Another objective of this study was to evaluate the role of epigenetic mechanisms in regulating BDNF gene expression. Alterations in 5mC levels and consistent changes in genes expression have been already reported in different psychiatric disorders (Abdolmaleky *et al*., 2006; Kuratomi *et al*., 2008; D'Addario *et al*., 2012, 2013, 2017). 5mC can be oxidized to 5hmC and this modification is environment-sensitive (Wu and Zhang, 2011), and highly enriched in the brain (Kriaucionis *et al*., 2009; Sun *et al*., 2014; Wang *et al*., 2014). To date, DNA methylation methods based on sodium bisulfite treatment could not distinguish between 5mC and 5hmC, and all observed alterations have been attributed to 5mC only. We here used a recently developed pyrosequencing-based assay that allowed us to discriminate between the two modifications (Matsubara *et al*., 2015; Qu *et al*., 2015). The analysis of 5mC and 5hmC levels was performed at BDNF gene promoters' exon I, exon IV and exon IX, and selective alterations were observed only at promoter exon I consistently with the gene expression change.

Of relevance, alterations in DNA methylation at BDNF gene exon I promoter have been already observed in depressed and bipolar type II subjects, and were suggested as a

possible biomarker for those disorders (Fuchikami *et al.*, 2011; D'Addario *et al.*, 2012, 2013). Another study reported a reduction in DNA methylation at BDNF gene promoter exon I of treatment-resistant MDD patients subjected to electroconvulsive therapy (Kleimann *et al.*, 2014). Selective modulation of DNA methylation in this particular region of BDNF gene may thus occur under different pathological conditions and might be driven by different therapies, including pharmacological treatment, as we here described for OCD, and somatic ones (e.g., electroconvulsive in MDD).

No association was observed between rs6265 and OCD susceptibility. However, since the way genetic variations might impact genes DNA methylation pattern is not fully understood, we analyzed possible alterations in the epigenetic marks at all BDNF CpG sites under study, based on the presence of the Val or Met allele, which also creates or abolishes a CpG dinucleotide. Again, no relevant alteration was detectable in OCD subjects carrying the minor allele in terms of DNA methylation in any of the CpG sites analyzed. However, the possible interaction between genetic and epigenetic factors is of great relevance and we aim to address this issue in future studies of pathological conditions other than OCD, in line with our recent study on alcoholism and prodynorphin gene regulation (D'Addario *et al.*, 2017).

In conclusion, our preliminary findings confirm the relevance of epigenetic changes in OCD to identify diagnostic and prognostic biomarkers. Moreover, we could hypothesize that pharmacotherapy might be responsible for BDNF gene regulation altering DNA methylation status. It would be of great value to investigate DNA methylation changes at early stages of the disease, in order to identify possible gene-environmental risk factors eventually responsible for OCD development, and thus try to revert such changes not only pharmacologically, but through environmental triggers. Further studies are therefore needed in order to confirm reported findings in larger treated and untreated patients with OCD as well as in drug-naïve patients.

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Contributors

CD and BD conceived and designed the experiments; CD, FB, CF, MP, MV, VDC, BB, BG performed the experiments; CD, BD, FB analyzed the data; CD, BD, DG contributed reagents/material/analysis tools; CD, BD, MM wrote the paper, ES revised critically the manuscript.

Statement of interest.

All authors declare to have nothing to disclose.

Figure legends

Figure 1. Schematic representation of human BDNF promoter and the 5′ upstream region. ATP is the translation start site. In the lower part are shown the three CpG islands (exon I, IV and IX) with their sequences and the position of the SNP (rs6265). In the upper part are shown the location of primers used for mRNA quantification.

Figure 2. BDNF mRNA levels in PBMCs from patients diagnosed with OCD (n = 20). Box plots with whiskers from minimum to maximum represent 2^{-DDCt} values calculated by the Delta-Delta Ct (DDCt) method. Means of mRNA levels are expressed relative to control subjects (CTRL) (n = 20). * $p < 0.05$ Mann-Whitney test.

Figure 3. Comparison of the 5mC and 5hmC levels in human BDNF promoter exon I between OCD and control (CTRL) subjects represented as scattered plot for individual CpG sites under study (see Figure 1) as well as of the average (AVE) of the 4 CpG sites. Significant differences are indicated (Bonferroni corrected). * $p < 0.05$.

Figure 4. Correlation between BDNF gene expression and % change of 5mC (A: P=0.0279, Spearman's r =-0.4311) and 5hmC (B: P=0.0333, Spearman's r =0.4552) considering the average of the 4 CpG sites under study in human PBMCs. Data were compared by Spearman's rank correlation coefficient.

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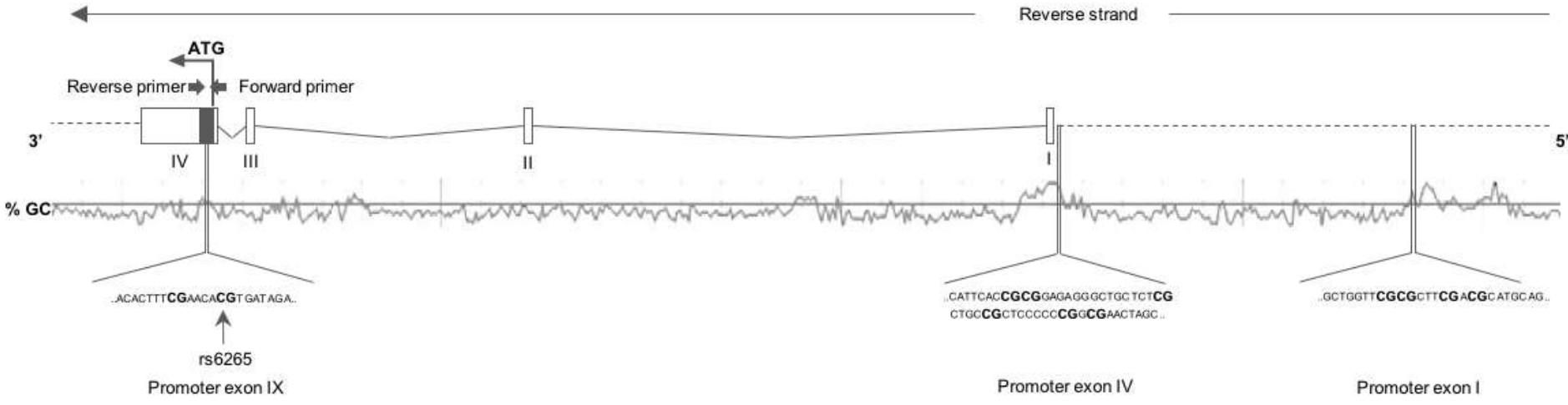

Reverse strand
ATG
Reverse primer
Forward primer
3'
5'
IV
III
II
I
% GC
..ACACTTTCGAACACGTGATAGA..
rs6265
Promoter exon IX
..CATTCACCGCGGAGAGGGCTGCTCTCG
CTGCCGCTCCCCCCGGCGAACTAGC..
Promoter exon IV
..GCTGGTTCGCGCTTCGACGCATGCAG..
Promoter exon I

Figure 1

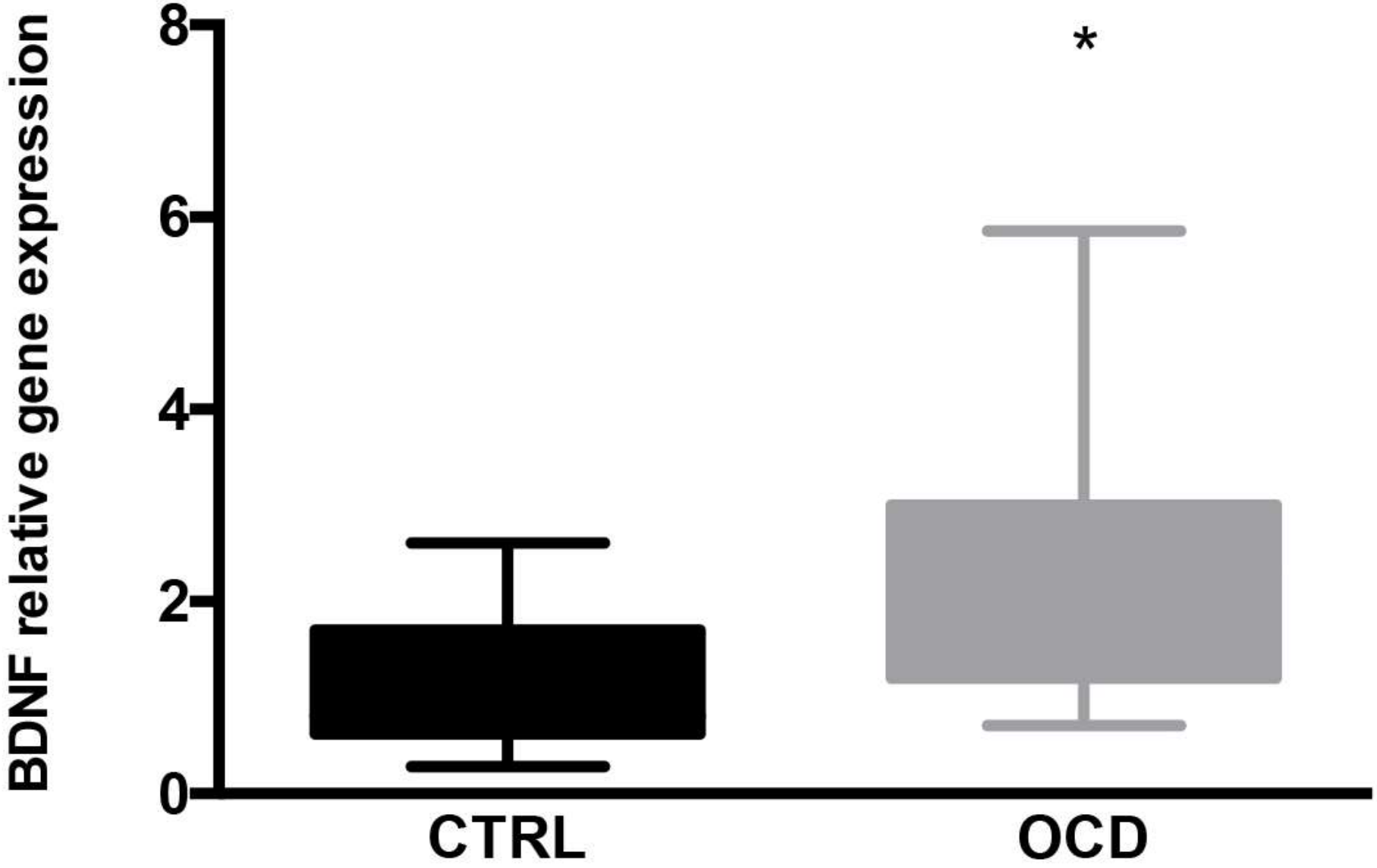
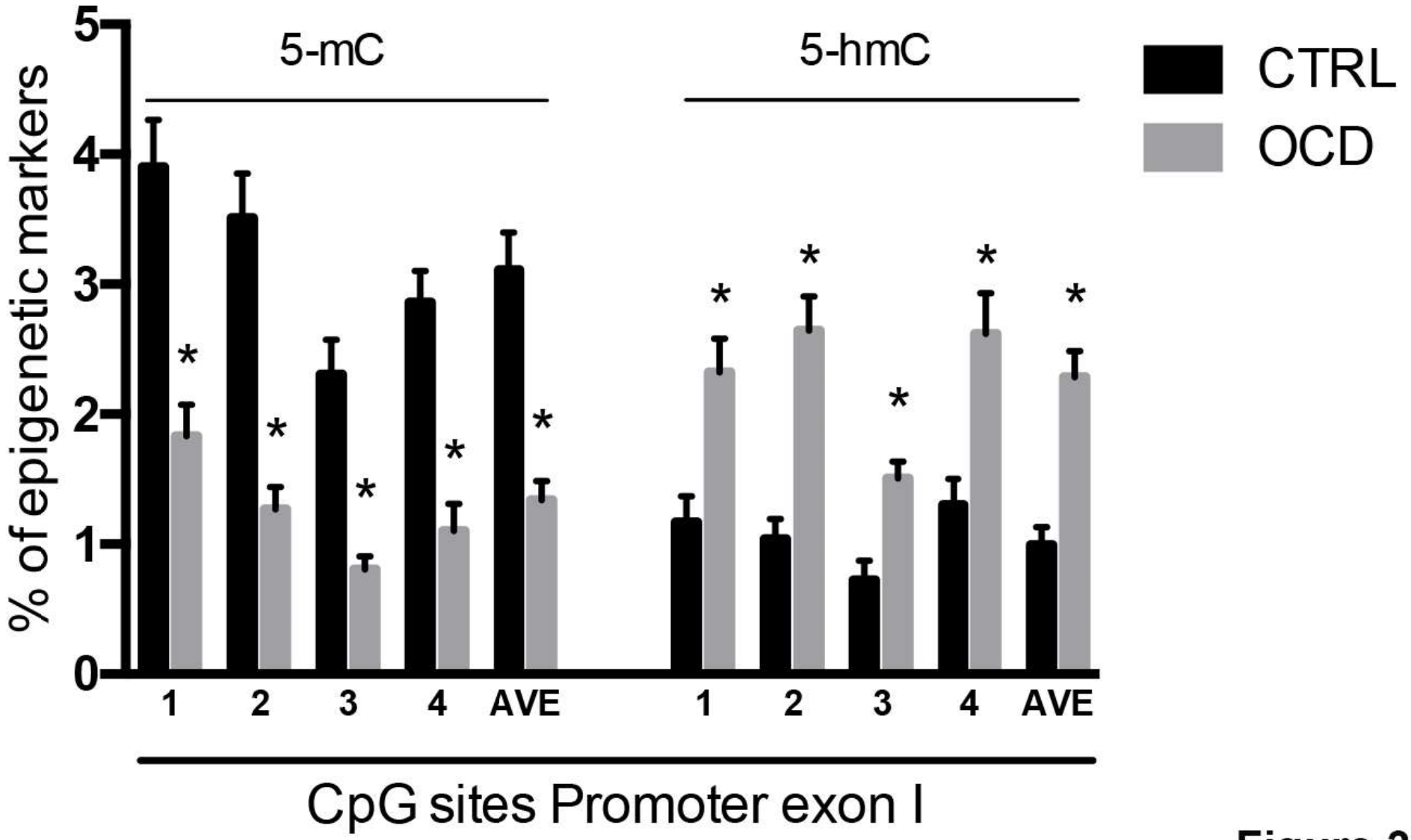


Figure 2


5-mC
5-hmC
CTRL
OCD
% of epigenetic markers
0
1
2
3
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5
1
2
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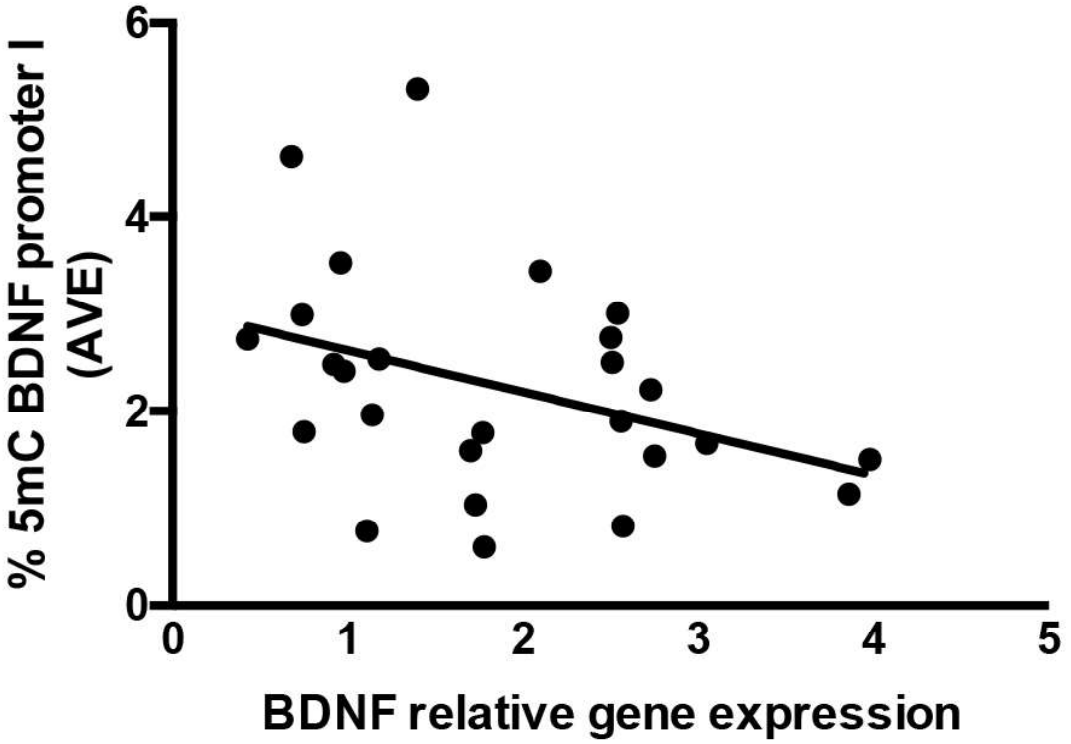
4
AVE
1
2
3
4
AVE
CpG sites Promoter exon I

Figure 3

A.


% 5mC BDNF promoter I (AVE)
0
2
4
6
0
1
2
3
4
5
BDNF relative gene expression

B.

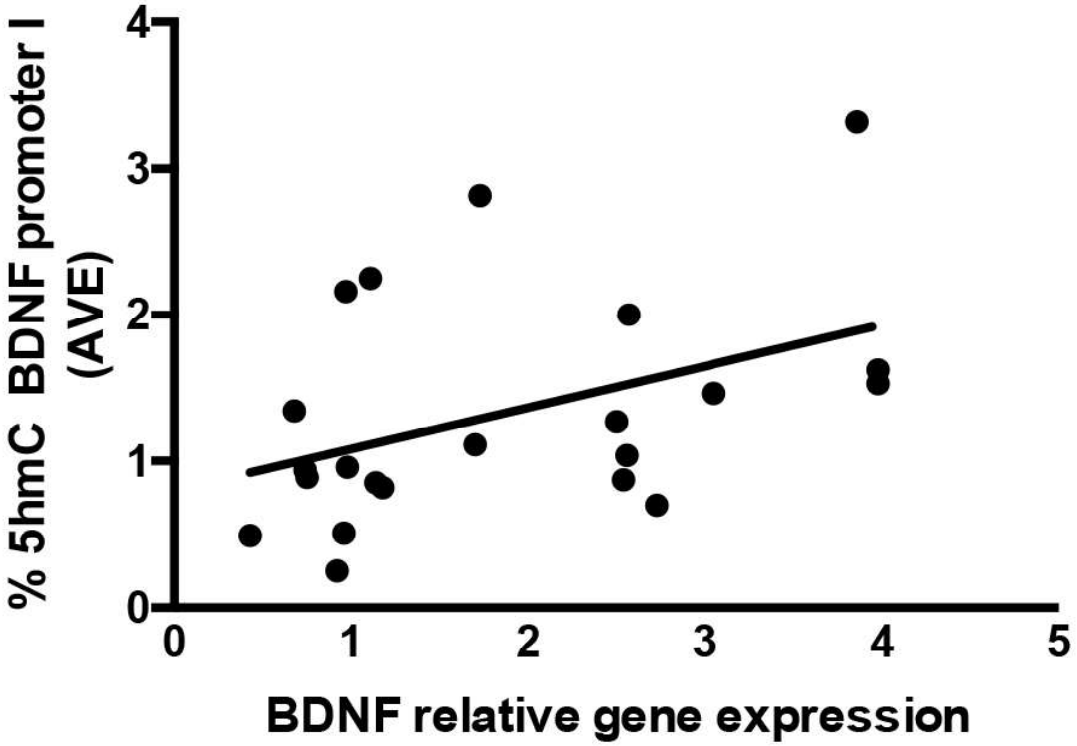

% 5hmC BDNF promoter I (AVE)
0
1
2
3
4
0
1
2
3
4
5
BDNF relative gene expression

Figure 4

Table 1. Demographic, social and clinical variables of OCD patients

Social variables	
Gender (%)	
Males	54.29
Females	45.71
Mean Age (years old)	36.96 ± 12.99
Education (%)	All patients
Secondary school	15.2
High-school	57.5
University	27.3
Employment (%)	
Employed	39.3
Unemployed	48.5
Student	6.1
Retired	6.1
Married (%)	30.3
Age at onset (years, mean ± SD)	21.8 ± 10.4
Duration of illness (years, mean ± SD)	17.5 ± 10.5
Duration of Untreated Illness (months, mean ± SD)	56.3 ± 72.0
Family history of psychiatric disorder (%)	63.6
Psychiatric comorbidity (%)	60.6
Drug naive patients (%)	8.6
Current treatment (%)	
Antidepressants	85.7
Antipsychotics	57.1
Mood stabilizers	34.3
Benzodiazepines	42.9
Psychotropic compounds (number, mean ± SD)	2.39 ± 1.6
≥ 1 psychotropic compounds (%)	74.3

Table 2. BDNF primer sequences used for Pyrosequencing analysis.

Promoter	***Primer sequences***	
I	***Forward***	Hs_BDNF_08_PM PyroMark CpG assay (PM00155540)
	Reverse	
	Sequencing	
IV	***Forward***	AGGTAGGGAGATTTTATGTTAGT
	Reverse	ACCCTAAAACCAAACTCTTCTAATAAAAAA
	Sequencing	AATGGGAAAGTGGGT
IX	***Forward***	TGGGTTTAAGGTAGGTTTAAGAGGTTTGAT
	Reverse	TCTAATCCTCATCCAACAACTCTTC
	Sequencing	GGTTTGATATTATTGGTTGATATTT

Supplemental Material:

Table S1.

Gene		Primer sequences
GAPDH	Forward	CAGCCTCAAGATCATCAGCA
	Reverse	TGTGGTCATGAGTCCTTCCA
ß-ACT	Forward	GACCCAGATCATGTTTGAGACCT
	Reverse	CCATCACGATGCCAGTGG
BDNF	Forward	AAGAAGCAAACATCCGAGG
	Reverse	AAGGCACTTGACTACTGAGC

Table S2.

CpG site	CTRL	OCD
	Promoter exon IV	
1	2.66 ± 0.18	2.09 ± 0.27
2	4.19 ± 0.32	3.17 ± 0.36
3	3.75 ± 0.15	2.96 ± 0.32
4	2.43 ± 0.24	2.04 ± 0.26
5	4.93 ± 0.16	4.92 ± 0.38
6	1.28 ± 0.15	0.91 ± 0.15
Average	3.21 ± 0.12	2.70 ± 0.19
	Promoter exon IX	
1	95.56 ± 0.28	86.71 ± 3.26
2	79.14 ± 3.61	71.34 ± 3.87
Average	87.35 ± 1.82	79.03 ± 3.28

Table S3.

CpG site	CTRL	OCD
	Promoter exon IV	
1	2.66 ± 0.18	2.09 ± 0.27
2	4.19 ± 0.32	3.17 ± 0.36
3	3.75 ± 0.15	2.96 ± 0.32
4	2.43 ± 0.24	2.04 ± 0.26
5	4.93 ± 0.16	4.92 ± 0.38
6	1.28 ± 0.15	0.91 ± 0.15
Average	3.21 ± 0.12	2.70 ± 0.19
	Promoter exon IX	
1	95.56 ± 0.28	86.71 ± 3.26
2	79.14 ± 3.61	71.34 ± 3.87
Average	87.35 ± 1.82	79.03 ± 3.28

Table S4.

	OCD		CTRL	
CpG site	C/T	C/C	C/T	C/C
	Promoter exon I			
1	1.55 ± 0.30	1.46 ± 0.20	3.95 ± 0.68	4.33 ± 0.43
2	0.88 ± 0.18	1.30 ± 0.19	3.42 ± 0.58	3.77 ± 0.41
3	0.76 ± 0.12	0.83 ± 0.15	2.26 ± 0.37	2.48 ± 0.30
4	0.99 ± 0.38	0.77 ± 0.15	2.67 ± 0.39	3.24 ± 0.30
Average	1.40 ± 0.26	1.28 ± 0.18	3.08 ± 0.45	3.33 ± 0.31
	Promoter exon IV			
1	1.42 ± 0.30	1.71 ± 0.34	1.95 ± 0.10	2.50 ± 0.25
2	2.82 ± 1.08	3.11 ± 0.64	3.84 ± 0.40	4.13 ± 0.16
3	2.08 ± 0.30	3.01 ± 0.54	3.93 ± 0.17	3.73 ± 0.10
4	1.30 ± 0.31	2.473± 0.60	2.10 ± 0.22	2.72 ± 0.25
5	4.24 ± 0.39	3.87 ± 0.73	4.05 ± 0.31	4.70 ± 0.24
6	0.72 ± 0.33	0.70 ± 0.18	0.92 ± 0.07	1.37 ± 0.10
Average	2.10 ± 0.32	2.38 ± 0.37	2.80 ± 0.13	3.19 ± 0.14
	Promoter exon IX			
1	93.00 ± 0.65	93.32 ± 0.45	91.54 ± 0.95	91.98 ± 0.86
2	55.68 ± 10.05	86.34 ± 1.09	70.27 ± 11.82	67.77 ± 6.30
Average	74.34 ± 4.75	87.58 ± 2.22	80.91 ± 5.48	79.87 ± 3.39

Table S5.

	OCD		CTRL	
CpG site	C/T	C/C	C/T	C/C
	Promoter exon I			
1	2.55 ± 0.47	2.33 ± 0.30	1.29 ± 0.23	1.21 ± 0.26
2	3.24 ± 0.50	2.32 ± 0.28	0.75 ± 0.21	1.20 ± 0.24
3	1.80 ± 0.21	1.27 ± 0.12	0.66 ± 0.25	0.83 ± 0.22
4	2.77 ± 0.72	2.64 ± 0.32	1.51 ± 0.27	1.36 ± 0.27
Average	2.51 ± 0.37	2.11 ± 0.25	0.94 ± 0.16	1.10 ± 0.19
	Promoter exon IV			
1	0.99 ± 0.29	1.08 ± 0.17	0.24 ± 0.03	0.60 ± 0.22
2	2.19 ± 0.45	1.75 ± 0.39	0.58 ± 0.21	0.33 ± 0.16
3	1.23 ± 0.50	1.01 ± 0.40	0.46 ± 0.44	0.60 ± 0.14
4	2.04 ± 1.16	1.55 ± 0.39	0.57 ± 0.32	0.49 ± 0.20
5	1.43 ± 0.73	1.66 ± 0.67	0.51 ± 0.22	0.70 ± 0.22
6	0.83 ± 0.20	0.74 ± 0.17	0.19 ± 0.01	0.61 ± 0.17
Average	1.39 ± 0.21	0.93 ± 0.32	0.25 ± 0.07	0.41 ± 0.14
	Promoter exon IX			
1	3.22 ± 0.63	4.68 ± 0.87	4.76 ± 0.79	3.71 ± 1.35
2	11.55 ± 5.76	4.12 ± 1.59	26.41 ± 17.13	25.86 ± 7.49
Average	7.39 ± 3.06	2.00 ± 0.75	15.58 ± 7.88	12.93 ± 4.06

Table S6.

SNP	Allele						
	Major	Minor		Genotype Frequency			
rs6265 Chr.11:27658369	C	T	TOT	C	CT	T	p value
			OCD	0,6389	0,3333	0,0278	0,826
			CTRL	0,6667	0,2778	0,0556	

Supplementary Table legends:

Table S1. Sequences of primers used for gene expression analysis.

Table S2. % of 5mC at BDNF gene promoters exon IV and IX in DNA from PBMCs of OCD subjects and controls (CTRL).

Table S3. % of 5hmC at BDNF gene promoters exon IV and IX in DNA from PBMCs of OCD subjects and controls (CTRL).

Table S4. % of 5mC at BDNF gene promoters exon I, IV and IX in DNA from PBMCs of OCD subjects and controls (CTRL) carrying C/T or C/C genotype.

Table S5. % of 5hmC at BDNF gene promoters exon I, IV and IX in DNA from PBMCs of OCD subjects and controls (CTRL) carrying C/T or C/C genotype.

Table S6. Association of BDNF SNP rs6265 with OCD.

Conflict of Interest

All authors declare that they have no conflicts of interest.