Neuropsychopharmacology (2012) 37, 1647–1655

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Selective DNA Methylation of BDNF Promoter in Bipolar Disorder: Differences Among Patients with BDI and BDII

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The etiology of bipolar disorder (BD) is still poorly understood, involving genetic and epigenetic mechanisms as well as environmental contributions. This study aimed to investigate the degree of DNA methylation at the promoter region of the brain-derived neurotrophic factor (*BDNF*) gene, as one of the candidate genes associated with major psychoses, in peripheral blood mononuclear cells isolated from 94 patients with BD (BD I = 49, BD II = 45) and 52 healthy controls. A significant BDNF gene expression downregulation was observed in BD II 0.53 ± 0.11%; $P<0.05$), but not in BD I (1.13 ± 0.19%) patients compared with controls (CONT: 1 ± 0.2%). Consistently, an hypermethylation of the BDNF promoter region was specifically found in BD II patients (CONT: 24.0 ± 2.1%; BDI: 20.4 ± 1.7%; BDII: 33.3 ± 3.5%, $P<0.05$). Of note, higher levels of DNA methylation were observed in BD subjects on pharmacological treatment with mood stabilizers plus antidepressants (34.6 ± 4.2%, predominantly BD II) compared with those exclusively on mood-stabilizing agents (21.7 ± 1.8%; $P<0.01$, predominantly BD I). Moreover, among the different pharmacological therapies, lithium (20.1 ± 3.8%, $P<0.05$) and valproate (23.6 ± 2.9%, $P<0.05$) were associated with a significant reduction of DNA methylation compared with other drugs (35.6 ± 4.6%). Present findings suggest selective changes in DNA methylation of BDNF promoter in subjects with BD type II and highlight the importance of epigenetic factors in mediating the onset and/or susceptibility to BD, providing new insight into the mechanisms of gene expression. Moreover, they shed light on possible mechanisms of action of mood-stabilizing compounds *vs* antidepressants in the treatment of BD, pointing out that BDNF regulation might be a key target for their effects.
Neuropsychopharmacology (2012) **37,** 1647–1655; doi:10.1038/npp.2012.10; published online 22 February 2012

Keywords: brain-derived neurotrophic factor (BDNF); peripheral blood mononuclear cells (PBMCs); DNA methylation; gene expression; bipolar disorder (BD); mood stabilizers and antidepressants

INTRODUCTION

Bipolar disorder (BD) is a prevalent, recurring, and highly disabling mood disorder determined by the interplay of genes and environmental factors. Despite consistent evidence from genetic studies supporting the role for genes in BD, the precise molecular bases of the disorder remain to be unraveled. Actually, genetic investigation has clearly pointed out that no specific gene is incontrovertibly related to the development of BD which, likely, represents a complex condition in which pathological behaviors, and ultimately, patients' symptoms, result from the combination of numerous susceptibility genes, each of which is not necessarily uncommon (Gershon, 2000). Among genes potentially implicated in the pathophysiology of BD, the brain-derived neurotrophic factor (*BDNF*) gene has been extensively investigated over the last few years and associated with neural adaptations to stress, synaptic plasticity, and antidepressant response, and with an influence on serotonergic system and mood regulation

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Received 11 October 2011; revised 21 December 2011; accepted 23 December 2011

(Castrén and Rantamäki, 2010; Grande *et al*, 2010; Hashimoto *et al*, 2004; Henikoff and Matzke, 1997; Shirayama *et al*, 2002). The BDNF gene maps to chromosome 11p13–15, and associations between single-nucleotide polymorphisms (SNPs) and BD have been reported (Sklar *et al*, 2002; Neves-Perreira *et al*, 2002), particularly in relation to childhood onset (Geller *et al*, 2004). However, negative results have been reported as well (Nakata *et al*, 2003) and, even though the influence of genes in BD has an important role, increasing evidence strongly points out that environmental factors are, in turn, determinant (Craddock and Jones, 2001). In addition, gene expression in the nervous system is modulated by epigenetic processes that consist of mitotically heritable, but reversible, changes in gene expression that occur without a change in the genomic DNA sequence (Henikoff and Matzke, 1997; Petronis, 2003; Pidsley and Mill, 2011; Tsankova *et al*, 2007). Indeed, epigenetic mechanisms represent a link between gene expression alterations and environmental factors: they can contribute to phenotypic effects and, moreover, the reversibility of their marks might be of particular clinical importance to elucidate the action of existing pharmacological treatments as well as to develop new therapeutic tools. For instance, epigenetic changes in the brain have been associated with a range of neurobiological processes including the central nervous system development, learning, memory, and neurodegeneration (Pidsley and Mill, 2011). Main epigenetic mechanisms essentially include DNA methylation and histone modifications (Feng *et al*, 2007), which act on chromatine remodeling and gene expression (Levenson *et al*, 2006; Nelson *et al*, 2008) and are potentially implicated in the development of mental illnesses (Das Das *et al*, 1997–1998; Abdolmaleky *et al*, 2005; Grayson *et al*, 2005). DNA methylation, in particular, consists of the transfer of a methyl group to position 5 of the cytosine pyrimidine ring of a cytosine guanine dinucleotide (CpG) which, ultimately, blocks the binding of transcription factors causing chromatin compaction and gene silencing (Pidsley and Mill, 2011; Klose and Bird, 2006). Moreover, DNA methylation is the best characterized and most stable epigenetic mechanism and has been the focus of most epigenetic studies performed in psychiatric disorders over the last decade (Jirtle and Skinner, 2007). In particular, the role of epigenetic factors in patients with major psychoses has been mainly investigated through the assessment of DNA methylation changes in the promoter regions of candidate genes, such as the *catechol-O-methyltransferase* gene (Abdolmaleky *et al*, 2006). More recently, Mill *et al* (2008) observed that frontal-cortex DNA methylation of the *BDNF* gene correlated with genotype at a nearby non-synonymous SNP, previously associated with major psychoses, suggesting that DNA methylation changes are relevant to the etiology of schizophrenia and BD. In order to further investigate the role of DNA methylation in the regulation of BDNF transcription, we conducted this study on peripheral blood mononuclear cells (PBMCs) of patients with BD on stable pharmacological treatment, with the specific intent of assessing eventual differences in terms of methylation between bipolar patients and healthy controls as well as across the diagnostic groups.

SUBJECTS AND METHODS

Subjects

In all, 94 bipolar subjects (49 with BD I and 45 with BD II) of either gender and any age, treated and followed up at the mood disorders outpatient clinic within the University Department of Psychiatry of Milan, were included in the study. Diagnoses were assessed by the administration of a semi-structured interview based on DSM-IV criteria (SCID-I and II; First *et al*, 1997 and First *et al*, 2002a). In case of psychiatric comorbidity, BD had to be the primary disorder, causing the most significant distress and dysfunction and providing the primary motivation to seek treatment. Patients were excluded from the study if they had recent or current alcohol or substance abuse (last 3 months) as well as medical conditions including autoimmune diseases due to their potential influence over BDNF expression. For the same reason, lifetime history of trauma (according to DSM-IV-TR) as well as the current presence of relevant psychological stress were considered exclusion criteria as well. Clinical assessments included the collection of the following demographical and clinical variables: gender, age, subtype of BD, current pharmacological treatment, and mood state (ie, euthymic, depressed, manic, hypomanic, and mixed). In addition, continuous measures of episode severity were systematically collected through psychometric scales (ie, Hamilton Depression Rating Scale (Hamilton, 1960), Montgomery Asberg Depression Rating Scale (Montgomery and Asberg, 1979), and Young Mania Rating Scale (Young *et al*, 1978)). Patients had maintained their pharmacological treatment stable for at least one month in order to be enrolled in the study.

Control subjects ($n = 52$) were volunteers matched for gender, age and ethnicity, with no psychiatric diagnosis as determined by the SCID-I Non Patient Edition (First *et al*, 2002b) and no positive family history for major psychiatric disorders in the first-degree relatives (as assessed by the Family Interview for Genetic Studies; Maxwell, 1992). All subjects had given their written informed consent to participate in the study, which included the use of personal and clinical data as well as blood drawing for genotyping and methylation analysis. The study protocol had also been previously approved by the local Ethics Committee. Demographical and clinical characteristics for the study sample as well as drugs used by BD subjects are shown in Tables 1 and 2.

BDNF Gene Expression

PBMCs were separated by density gradient using the Lympholyte-H kit (Cedarlane Laboratories, Canada), and total RNA was isolated using the Chomczynski and Sacchi's modified method (Chomczynski and Sacchi, 2006). The concentration of total RNA was quantified by means of spectrophotometry, and 2 μg was reverse transcribed using the M-MLV Reverse Transcriptase System and oligo (dT; Clontech, Italy). Relative abundance of each mRNA species was assessed by real-time RT-PCR using 1 μl diluted cDNA samples in a final volume of 20 μl using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on an DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). All data were normalized to

Table 1 Demographical and Clinical Characteristics of Patients Enrolled for DNA Methylation Studies

Sample	Total (n)	M	F	Mean age ± SD	Current phase
BD I	49	27	22	49.34 ± 12.65	A = 9
					B = 16
					C = 21
					D = 3
BD II	45	11	34	53.58 ± 12.04	A = 22
					B = 17
					C = 4
					D = 1
Controls	52	21	31	68.99 ± 1.89	

Keys: A, euthymia; B, depression; C, mania/hypomania; D, mixed state.

Table 2 List of Drugs in the Study Divided for Class, Dose, and % of Patients Under Treatment

DRUGS	CLASS	DOSE (mg)	% PATIENTS
Quetiapine	Mood stabilizer	25–1000	53
Valproate	Mood stabilizer	300–2000	51
Lithium	Mood stabilizer	300–1500	24
Aripiprazole	Mood stabilizer	5–25	17
Olanzapine	Mood stabilizer	5–40	17
Gabapentin	Mood stabilizer	300–1200	7
Pregabalin	Mood stabilizer	25–150	6
Venlafaxine	SNRI	75–300	15
Citalopram	SSRI	10–30	10
Duloxetine	SNRI	30–120	10
Clomipramine	TCA	50–225	6
Sertraline	SSRI	50–100	3
Escitalopram	SSRI	10–20	3

the endogenous reference genes *glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (BACT)*. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined and the point of early log phase of product accumulation is defined by assigning a fluorescence threshold above background expressed as the threshold cycle number or Ct. Differences in threshold cycle number were used to quantify the relative amount of PCR target contained within each tube. Relative expression of different gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2^{-DDCt}) for statistical analysis (Livak and Schmittgen, 2001). The DCt value of each sample was calculated as the Ct of the target gene minus the Ct of GAPDH, and then the DDCt value was obtained as the difference between the DCt of the sample and the DCt of the calibrator. According to this formula, the normalization ratio of the calibrator in each run is 1. The calibrator in each sample run was the same RNA extracted from a single healthy control and stored at −80 °C. After PCR, a dissociation curve (melting curve) was constructed in the range of 60– 95 °C (Lyon, 2001) to evaluate the specificity of the amplification products.

The primers used for PCR amplification designed using Primer 3 are the following:

BDNF:
Forward 5′-AAGAAGCAAACATCCGAGG-3′,
Reverse 5′-AAGGCACTTGACTACTGAGC-3′
GAPDH:
Forward 5′-GATTCCACCCATGGCAAATTC-3′
Reverse 5′-TGGGATTTCCATTGATGACAAG-3′
BACT:
Forward 5′-TGACCCAGATCATGTTTGAG-3′
Reverse 5′-TTAATGTCACGCACGATTTCC-3′

BDNF Gene Promoter Methylation

Genomic DNA was extracted by the salting-out method as described previously (Arosio *et al*, 2010). First, DNA underwent bisulfite modification to convert unmethylated cytosine residues to uracil, using the CpGenome DNA Modification Kit (Chemicon International, Purchase, NY), according to the manufacturer's instructions. Methylation analysis was performed by fluorescence-based real-time PCR using MS Opticon 2 Light Cycler Instrument (Roche, Germany). BDNF sequence amplified contained 17 CpG sites, and was located within the promoter region of the gene (see Figure 1 for sequence details). PCR was also performed for non-CpG-containing region of myoD, which served as control gene. Bisulfite-modified CpGenome universal unmethylated DNA (Chemicon International, Temecula CA, USA) was used as negative control. The percentage of methylation was calculated by the 2^{-DDCt} method (Livak and Schmittgen, 2001), where DDCt = (Ct, Target-Ct, myoD) sample−(Ct, Target-Ct, myoD) fully methylated DNA, multiplying by 100. For relative quantification, standard curves were generated separately for each gene and myoD from serial dilutions of bisulfite-modified CpGenome universal methylated DNA (Chemicon International). To confirm our result, we also used in selected DNA bisulfite-converted samples primers for the unmethylated DNA sequence calculating the % of methylation, as reported previously (Lu *et al*, 2008). These latter data are not shown to avoid redundancy.

The primers for bisulfite-converted DNA are herein reported:

M_BDNF:
Forward 5′-GTAGTTTTCGTAGGATGAGGAAGC-3′,
Reverse: 5′-AATATAAATTAACAACCCCGATACG-3′;
Product size 163 bp
U_BDNF:
Forward 5′-GTAGTTTTTGTAGGATGAGGAAGTG-3′,
Reverse: 5′-TATAAATTAACAACCCCAATACACA-3′
Product size 161 bp
myoD:
Forward 5′-TGATTAATTTAGATTGGGTTTAGAGAAGGA-3′,
Reverse 5′-CCAACTCCAAATCCCCTCTCTAT-3′
Product size: 162 bp

It is known that BDNF has an unique genomic structure with multiple promoters generating mRNAs containing different non-coding exons spliced upstream of a common coding exon (Martinowich *et al*, 2003). This complex transcriptional regulation leads to the creation of at least

ctcaaagttcatc ctttcgcctccaagttactttccgccaacacgtgacctcttcgcttcccagcttgcgcagccctggtggggaagcgcgggcgctaaccgc

-588

agggcttcctgggagagccccctccgaggttcgccaccccagccctggctcccgccgcgctcaccccggcacccggcggcgggg*gcagcccccgcaggat*

*gaggaag**cg***gctc**cg**ggggaagcagcac**cg**agcagcag**cg**gag**cg**cccaacctgc**cg**cctcccctcc**cg**tccctgccttggcc**cgcg**gctc**cg**ctctc**cg**

cggct**cg**ccttc**cg**acctagctg***cgcaccgggggctgttaactcacatt***tgggaagccataacccattagagcaaacgcagtcataacttcattcaactcag

ccgctcgagagctcggcttacacaggttcctgtgggcaactagtggctcggcttggtgcctctcgcctagtcatcagtacctaagaggaaaagggaaagttg

ttgggctggttcgcgcttcgacgcatgcagatgttcccaaggacaagtcacttactcgccccctcccccagtccccatttgatcatcactcacgacctcatc

ggctggagacccttagtcatgatgggggaggggaggggcacgaacttttctaagaagtttccttttttacccagagagtcacagtgagtcggtcacgtaa

+1

acagcgaggttagtcgtcgccgttgccgccccccaccccctccctgctgcgcttttctggtattATTATTAAAGCGGTAGTCTGCCGGCGCTGA

TAAGCAACAAGTTCCCCAGCGGTCTTCCCGCCCTAGCCTGACAAGGCGAAGGTTTTCTTACCTGGCGACAGGGAAATC

TCCCGAGCCGAATTCAGCTTCGCCGGAGCCCCAGGTGTGA

Figure 1 Sequence of the human brain-derived neurotrophic factor (BDNF) exon I promoter (chr11: 27 743 605–27 744 379). Amplicon is highlighted with the 17 cytosine guanine dinucleotide (CpG) sites present (also bolded) and primer sequences (methylated) underlined. The transcriptional start site (+1) is indicated and the predicted CpG island is shown (criteria used: island size >100, % GC >50.0, obs/exp >0.60).

three pre-pro-BDNF isoforms. We decided to focus our attention on the BDNF exon I promoter as it was described as a brain-specific inducible promoter (Timmuskb, 1993).

Statistical Analysis

All results are expressed as mean ± SEM. Statistical differences of BDNF gene expression and DNA methylation changes at BDNF promoter of BD patients *vs* control subjects were determined by analysis of variance (ANOVA) followed by Dunnett's test, using Prism version 5 (GraphPad Software, San Diego, CA). The *P*-values <0.05 were considered to be statistically significant.

RESULTS

BDNF gene expression resulted to be significantly decreased in BD II subjects (0.53 ± 0.11; $P<0.05$ Dunnett's *post hoc* test; ANOVA: $P=0.0291$; $F=4.344$) but not in BD I patients (1.13 ± 0.19) compared with controls (1 ± 0.2; Figure 2).

An increase of DNA methylation at BDNF gene promoter was observed in BD II patients (but not in BD I) compared with controls (CONT: $24.0 \pm 2.1\%$; BD I: $20.4 \pm 1.7\%$; BD II: $33.3 \pm 3.5\%$ $P<0.05$ Dunnett's *post hoc* test; ANOVA: $P=0.0205$; $F=4.040$; Figure 3). A significant negative correlation was also observed between BDNF gene expression and percentage change in DNA methylation at BDNF promoter in BD II subjects ($P<0.01$, Spearman's $r=-0.7343$, Figure 4).

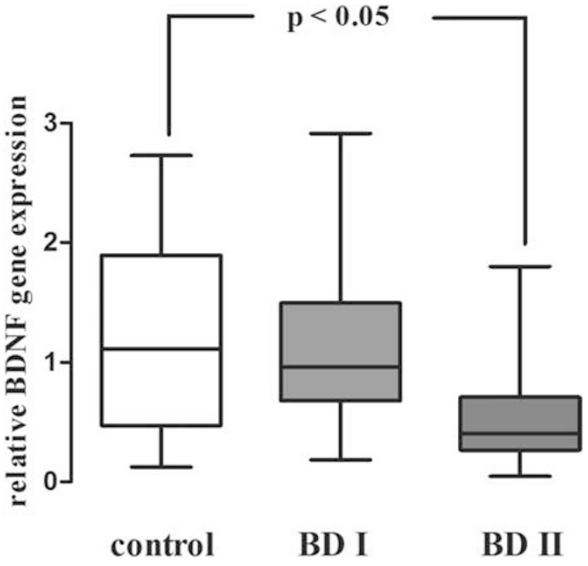

Figure 2 Levels of brain-derived neurotrophic factor (BDNF) mRNA in peripheral blood mononuclear cells from patients diagnosed with bipolar disorders type I (BD I; $n=16$) and BD type 2 (BD II; $n=16$). Box plots with whiskers from minimum to maximum represent 2^{-DDCt} values calculated by the Delta-Delta Ct (DDCt) method. Means of mRNA levels are expressed relative to control subjects ($n=14$).

When data were stratified on the basis of the different pharmacological treatments received by patients, those taking antidepressant drugs had a higher level of DNA methylation at BDNF promoter compared with antidepres-

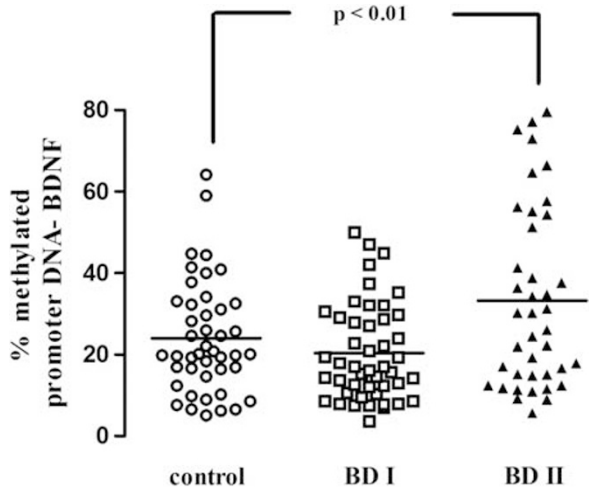

Figure 3 Amount of methylated DNA in the promoter region of brain-derived neurotrophic factor (BDNF) in controls, patients diagnosed with bipolar disorders type I (BD I; $n=16$) and BD type 2 (BD II; $n=16$). Scatter dot plots with mean values are shown.

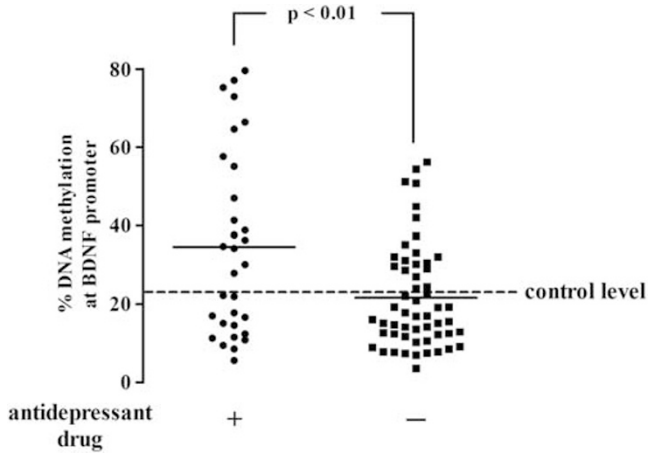

Figure 5 Amount of methylated DNA in the promoter region of brain-derived neurotrophic factor (BDNF) in peripheral blood mononuclear cells from patients diagnosed with bipolar disorders type I (BD I) + BDs type 2 (BD II) in therapy with (+) or without antidepressant drug (−). Scatter dot plots with mean values are shown.

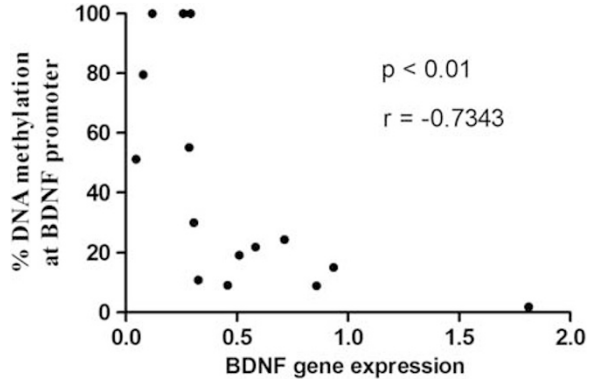

Figure 4 Correlation between brain-derived neurotrophic factor (BDNF) gene expression and percentage change in DNA methylation at BDNF promoter in bipolar disorders type 2 (BD II) subjects. Data are compared by Spearman's rank correlation coefficient ($P<0.01$, $r=-0.7343$).

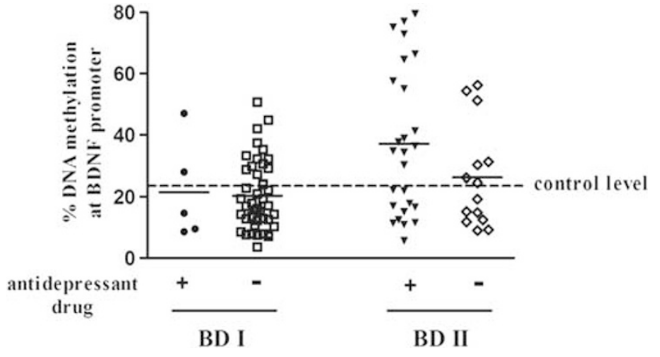

Figure 6 Amount of methylated DNA in the promoter region of brain-derived neurotrophic factor (BDNF) in peripheral blood mononuclear cells from patients diagnosed with bipolar disorders type I (BD I) or BD type 2 (BD II) in therapy with (+) or without antidepressant drug (−). Scatter dot plots with mean values are shown.

sant-free patients ($34.6\pm4.2\%$ *vs* $21.7\pm1.8\%$, $P=0.0015$, unpaired *t*-test; Figure 5). When patients were divided in BD I and BD II, it was still observed an increase in DNA methylation in BD II subjects under antidepressant drugs (Figure 6), even if not significantly due mainly to the reduced number of samples. Moreover, DNA methylation resulted to be significantly reduced in subjects under therapy with lithium ($20.1\pm3.8\%$; $P<0.05$ Dunnett's *post hoc* test) or valproate ($23.6\pm2.9\%$; $P<0.05$ Dunnett's *post-hoc* test) compared with treatment with other drugs ($35.6\pm4.6\%$; ANOVA: $P=0.0065$; $F=4.040$; Figure 7). Data analysis based on BDNF mRNA levels upon different therapies was not performed due to the smaller number of samples available for gene expression analysis when compared with DNA methylation studies.

Data stratification for BD I or BD II according to mood state did not produced significant changes for all the different types of episode (ie, euthymia, depression, mania, hypomania, or mixed; Figures 8a and b), even if a tendency to DNA methylation decrease it was observed in BD II in mania/hypomania/mixed status (Figure 8b). When BD subjects were grouped (BD I and BD II), DNA methylation resulted to be reduced in patients in mania/hypomania/mixed status ($19.1\pm2.2\%$; $P<0.05$ Newman–Keuls test) *vs* subjects in euthymia ($34.9\pm5.3\%$) and/or in depression ($33.4.\pm4.5\%$; ANOVA: $P=0.0176$; $F=4.236$; Figure 8c).

DISCUSSION

According to reported data, the degree of methylation of the BDNF promoter was found to be significantly increased in patients with BD II, but not BD I, as compared with controls, and it was negatively correlated with BDNF mRNA levels. Moreover, BDNF regulation seemed to be differentially affected by pharmacological treatments.

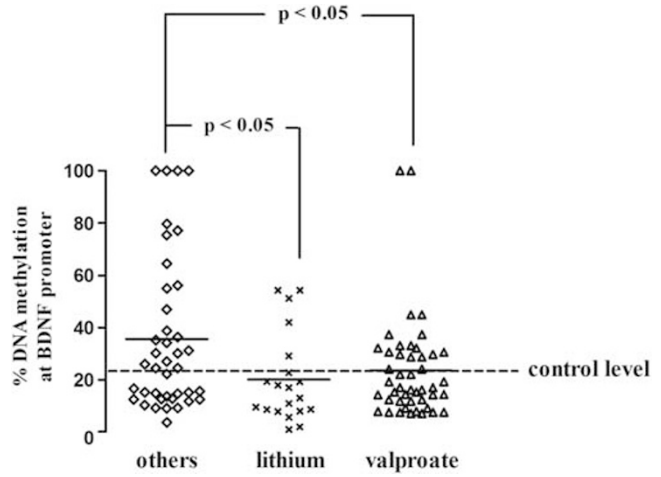

Figure 7 Amount of methylated DNA in the promoter region of brain-derived neurotrophic factor (BDNF) in patients diagnosed with BD (bipolar disorders type I (BD I; $n=49$) + BD type 2 (BD II; $n=45$)) treated with lithium, valproate or other drugs. Scatter dot plots with mean values are shown.

An emerging field of interest in psychiatry is represented by the study of the potential role of epigenetics in the different mental diseases (Das Das *et al*, 1997–1998; Abdolmaleky *et al*, 2005; Grayson *et al*, 2005). As the main affected organ in mental disorders, the brain, is not easily accessible in living subjects, a key issue in biological psychiatry is the search of peripheral biomarkers (Iga *et al*, 2006; Gavin and Sharma, 2010) for differential diagnosis and, mostly, for prediction of treatment response.

It has already been proposed the use of PBMCs as an important tool for the understanding of DNA methylation status in psychiatric patients even if it has to be taken into account that genes expression evaluation in PBMCs may not be reflective of the expression of the same gene in the brain (Gavin and Sharma, 2010).

In this perspective, this study provides, in PBMCs from BD II patients, preliminary molecular evidence for a relationship between the disease and epigenetic alterations of BDNF, confirming its role as a potential biomarker in BD, as suggested previously (Kapczinski *et al*, 2008). In fact, decreases in BDNF have already been detected in different psychotic disorders (Buckley *et al*, 2007; Chen da *et al*, 2009; Rizos *et al*, 2008; Sen *et al*, 2008), including BD (Kapczinski *et al*, 2008). In addition, a decrease in BDNF expression has been observed in many animal models of stress (Smith *et al*, 1995; Nibuya *et al*, 1995; Roceri *et al*, 2002; Tsankova *et al*, 2007). Recent evidences suggested the central role of BDNF in both stress and BD (Kapczinski *et al*, 2008). Furthermore, a reduction of BDNF, both mRNA (Dwivedi *et al*, 2003; Pandey *et al*, 2008) and protein (Karege *et al*, 2005) levels, has been observed in postmortem brain of depressed and teenage suicide victims.

Consistent with the aforementioned data from both clinical and preclinical samples, the first relevant result of this study is the selective reduction of BDNF gene expression in PBMCs of BD II subjects. A further interesting finding is the increase of DNA methylation at BDNF gene promoter in BD II subjects linked with the reduction in gene expression. Of note, we observed changes selectively in BD

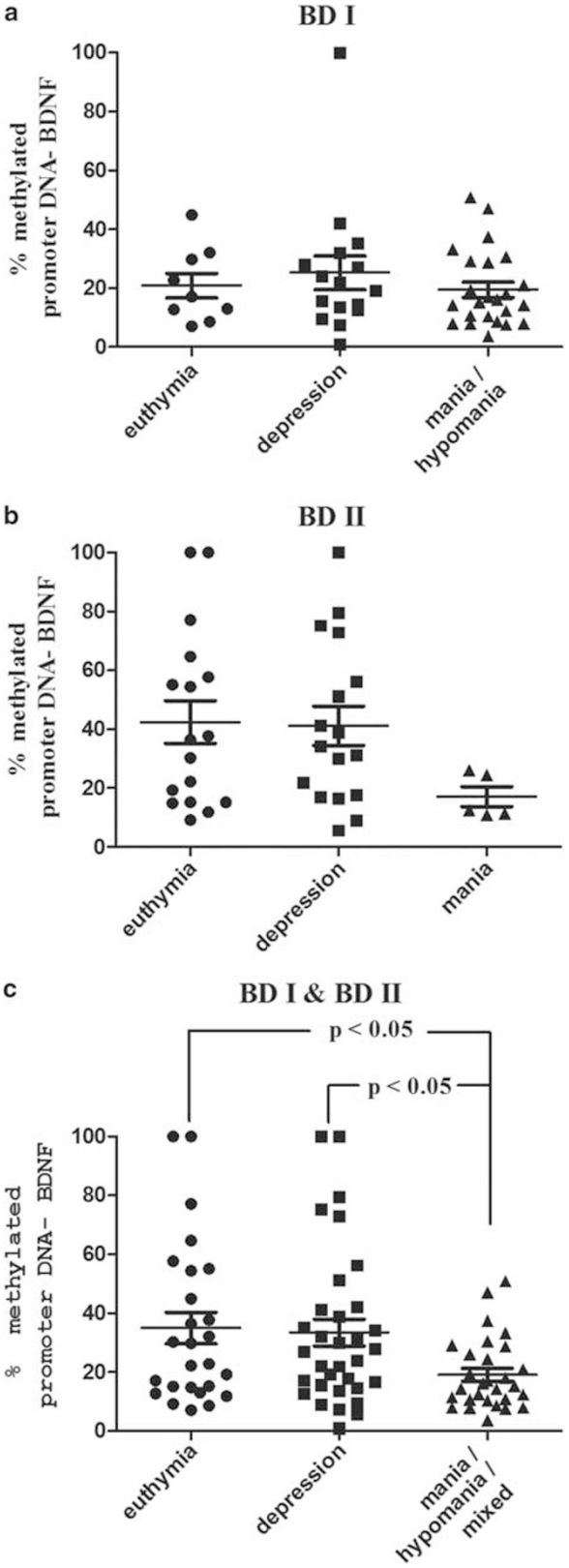

Figure 8 Amount of methylated DNA in the promoter region of brain-derived neurotrophic factor (BDNF) in patients diagnosed with (a) bipolar disorders type I (BD I; $n=49$); (b) BD type 2 (BD II; $n=45$) or (c) BD I and BD II according to their mood state.

type II subjects, in agreement with a recent report showing that BDNF levels resulted to be decreased in chronic or late stage individuals with BD compared with those at early stages of the illness (Kauer-Sant'Anna *et al*, 2009). In this perspective, a possible trait d'union between the two studies may be represented by the fact that, in our sample, BD II patients showed an overall longer duration of illness (ie, later stages of illness) compared with BD I.

To the best of our knowledge, this is one of the first studies showing that DNA methylation in human PBMCs may be a mechanism responsible for the abnormal regulation of the *BDNF* gene. Our observation are in agreement with a very recent study, which suggested the analysis of DNA methylation at the CpG island upstream of exon I of the *BDNF* gene as a valid biomarker for the diagnosis of major depression (Fuchikami *et al*, 2011). Before our and Fuchikami *et al* (2011) data, only an association between DNA methylation and BDNF genotype at a nonsynonymous SNP affecting exonic CpG sites has been observed (Mill *et al*, 2008). In another study, using a mouse model of depression, the reduction of BDNF expression in the hippocampus was mediated by repressive histone methylation (Tsankova *et al*, 2007). The same authors also showed that chronic treatment with imipramine increased histone acetylation normalizing BDNF expression (Tsankova *et al*, 2007). In addition, it has been reported that the mood stabilizers lithium and valproate increased BDNF expression in rat cortical neuronal cultured cells (Hashimoto *et al*, 2004; Yasuda *et al*, 2007; Hao *et al*, 2004) and protein levels in brain (Chuang, 2004; Fukumoto *et al*, 2001; Einat *et al*, 2003), overall suggesting that BDNF alterations can be reversed by the proper treatment. Previous studies have shown that valproate is able to alter DNA methylation (Fuks *et al*, 2000, 2003; Nan *et al*, 1998) inducing hypomethylation of specific genes (Candelaria *et al*, 2007; Dong *et al*, 2008; Garcia-Manero *et al*, 2006).

Our findings show that lithium and valproic acid, two of the most widely used mood-stabilizing compounds for treatment and prophylaxis of BD, are associated with reduced DNA methylation of BDNF promoter in BD subjects when compared with any other treatment. This information is of particular importance, providing new insight on the specific mechanism of action of these drugs for the treatment of BD. It also points out that BDNF regulation might be a key target for the effects of these mood stabilizers.

Increasing evidence has indicated an association between BDNF and the mechanisms of action of antidepressants and mood stabilizers (Castrén and Rantamäki, 2010; Coyle and Duman, 2003). In line with these results, a remarkable finding of our study is the high BDNF promoter DNA methylation level, associated, in turn, with a reduction of BDNF gene expression, in subjects receiving poly-therapy with antidepressant drugs (mostly BD II subjects) compared with those who were on mood stabilizers exclusively (predominantly BD I subjects). These evidence thus suggest that the difference in the BDNF gene regulation among BD subjects could depend on the different therapy. Our data may apparently be in contrast with previous data, suggesting that antidepressants are able to induce an upregulation of BDNF expression in the hippocampus of rats (Shirayama *et al*, 2002; Nibuya *et al*, 1995; Russo-Neustadt *et al*, 1999). However, it should be considered that the aforementioned reports mainly described drugs effects in animal models of depression, that may not exactly reflect mechanisms involved in humans.

Several clinical studies showed changes of plasma or serum BDNF levels before and after antidepressant treatments in major depressive disorder (MDD) patients, mainly reporting increases of BDNF levels after antidepressant treatment (Brunoni *et al*, 2008). To our knowledge, just in one study using postmortem brain samples, including both MDD and BD subjects, BDNF levels were found to be reduced in the hippocampus of subjects with MDD not treated with antidepressants, compared with either MDD taking antidepressants or BD patients (Chen *et al*, 2001).

Antidepressants have been traditionally used to treat depressive episodes of BD, but over the last several years, many reports pointed out the paucity of controlled data in terms of safety and efficacy in BD subjects (Ghaemi *et al*, 2003; Sachs *et al*, 2007), particularly in light of increased rates of mood switch, rapid cycling, and suicidal ideation, with current major guidelines actually discouraging (or recommending particular caution for) the use of these agents (Grunze *et al*, 2010). Our data are thus in agreement with the poor efficacy of antidepressant treatment in BD as BDNF gene regulation is not counteracted by their use.

It needs to be highlighted that other factors have been reported to modify DNA methylation patterns such as aging or environmental stimuli (eg, nutrition, life style, toxin, and exposure; Tost, 2010) and the contribution of one or more of these might have somehow conditioned present findings. In addition, type of current episode *vs* euthymia, the presence of psychiatric comorbidity and different levels of symptom severity might have exerted a variable influence over epigenetic patterns as well. In our experimental samples we did not observe epigenetic changes in BD I and BD II subjects according to their clinical status, even if DNA methylation resulted reduced when BD subjects were grouped in patients in mania/hypomania /mixed status.

In conclusion, present results provide a further piece of evidence for BDNF as a possible peripheral biological marker in BD, deserving further appreciation in terms of epigenetic investigation. It has already been suggested that the evaluation of BDNF levels may be a general biomarker of neuronal dysfunction (in BD in particular) and that a proper treatment may reverse possible alterations (Grande *et al*, 2010). The evaluation of BDNF methylation levels could be of relevance to predict an early response to different treatments.

Moreover, our findings are consistent with the epigenetic theory of major psychoses (Mill and Petronis, 2009), supporting the importance of DNA methylation alterations in the etiology of BD.

Our data regarding drugs effects on DNA demethylation at BDNF promoter outline also the possibility that common pharmacological interventions in BD—particularly antidepressants and mood stabilizers—may have profound differences over methylation patterns. Nevertheless, in order to further support BDNF role in the mood disorders treatment, these preliminary findings need to be replicated in a larger population and to be confirmed in the human brain.

ACKNOWLEDGEMENTS

This work was supported by grants from the Monzino Foundation, Programma Strategico RF 2007, conv. PS39, TERCAS (2009-2012) and PRIN (20077R93XF_004), Italian Ministry of Health.

DISCLOSURE

Dr Dell'Osso has been in the Speaker Bureau of Astra Zeneca, Bristol Myers Squibb, Janssen-Cilag, Eli Lilly, Pfizer, Glaxo Smith Kline, Lundbeck, Cyberonics and Italfarmaco. Prof Altamura is a consultant for Roche, Merck, Astra Zeneca, Bristol Myers Squibb, Janssen-Cilag and Lundbeck. The remaining authors declare no conflict of interest.

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