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# DNA-methyltransferase1 (DNMT1) binding to CpG rich GABAergic and BDNF promoters is increased in the brain of schizophrenia and bipolar disorder patients

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## ABSTRACT

The down regulation of glutamic acid decarboxylase67 (GAD1), reelin (RELN), and BDNF expression in brain of schizophrenia (SZ) and bipolar (BP) disorder patients is associated with overexpression of DNA methyltransferase1 (DNMT1) and ten–eleven translocase methylcytosine dioxygenase1 (TET1). DNMT1 and TET1 belong to families of enzymes that methylate and hydroxymethylate cytosines located proximal to and within cytosine phosphodiester guanine (CpG) islands of many gene promoters, respectively. Altered promoter methylation may be one mechanism underlying the down-regulation of GABAergic and glutamatergic gene expression. However, recent reports suggest that both DNMT1 and TET1 directly bind to unmethylated CpG rich promoters through their respective Zinc Finger (ZF-CXXC) domains. We report here, that the binding of DNMT1 to GABAergic (GAD1, RELN) and glutamatergic (BDNF-IX) promoters is increased in SZ and BP disorder patients and this increase does not necessarily correlate with enrichment in promoter methylation. The increased DNMT1 binding to these promoter regions is detected in the cortex but not in the cerebellum of SZ and BP disorder patients, suggesting a brain region and neuron specific dependent mechanism. Increased binding of DNMT1 positively correlates with increased expression of DNMT1 and with increased binding of MBD2. In contrast, the binding of TET1 to RELN, GAD1 and BDNF-IX promoters failed to change. These data are consistent with the hypothesis that the down-regulation of specific GABAergic and glutamatergic genes in SZ and BP disorder patients may be mediated, at least in part, by a brain region specific and neuronal-activity dependent DNMT1 action that is likely independent of its DNA methylation activity.

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## 1. Introduction

When post-mortem brains of schizophrenia (SZ) and bipolar disorder (BP) patients are compared with those of non-psychiatric subjects (NPS), GABAergic and glutamatergic neuropathologies are detected in the hippocampus and cortex (Akbarian et al., 1995; Benes et al., 1992, 2007; Benes and Beretta, 2001; Fatemi et al., 2000; Ikegame et al., 2013; Impagnatiello et al., 1998; Guidotti et al., 2000; Lewis et al., 2005; Mill et al., 2008; Weickert et al., 2003). These neuropathologies are characterized by a decrease in the expression of glutamic acid decarboxylase67 (GAD1), reelin (RELN), nicotinic acetylcholine receptors, glutamate receptors, and tyrosine kinase receptors in GABAergic neurons (for a review see Guidotti et al., 2011; Grayson and Guidotti, 2013), and brain derived neurotrophic factor (BDNF) and vesicular glutamate transporter 1 (VGLUT1) in glutamatergic neurons (Weickert et al., 2003; Mill et al., 2008; Ray et al., 2014).

Population, family, and twin studies indicate that SZ and BP are highly heritable, neuropsychiatric diagnoses. Single alleles conferring increased risk have been identified but only account for small proportion of observed phenotypic variants (Sullivan et al., 2008; Li, 2010; Richards et al., 2012). Hence, it appears that these disorders are the consequence of synergistic interactions of multiple susceptibility genes with environmental neuroepigenetic factors (Costa et al., 2002; Ptak and Petronis, 2008). In support of a role for aberrant epigenetic mechanisms in the pathogenesis of altered GABAergic and glutamatergic gene regulation in SZ and BP disorders, we have recently reported that the down-regulation of GAD1, RELN, and BDNF-IX expression in brains of SZ and BP patients is associated with increased expression of DNA methyltransferase1 (DNMT1) and Tet-methylcytosine dioxygenase1 (TET1), and additional downstream alterations in the DNA demethylation pathway associated with various target genes (for a review see Grayson and Guidotti, 2013).

DNMTs and TETs each represent a distinct family of enzymes that methylate and hydroxymethylate the five position of cytosines, respectively, when present in shores of so-called CpG islands (CpGIs). Studies suggest that changes in methylation and/or hydroxymethylation, when

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associated with promoter domains, modulate transcription by altering local chromatin organization and nucleosome positioning (for a review see Guidotti et al., 2011; Grayson and Guidotti, 2013). In SZ post-mortem brain, altered DNA methylation and hydroxymethylation marks at the promoters of RELN (Abdolmaleky et al., 2005; Grayson et al., 2005), GAD1 (Dong et al., 2012), COMT (Abdolmaleky et al., 2011), BDNF (Mill et al., 2008; Gavin et al., 2012; Ikegame et al., 2013), and glucocorticoid receptors (NR3C1, Zhang et al., 2013) have been reported. Genome-wide promoter methylation analyses of post-mortem cortical DNA showed altered methylation associated with some 817 promoters including nitric oxide synthase (*NOS1*), v-akt thymoma viral oncogene homologue-1 (*AKT1*), *DNMT1*, dystrobrevin binding protein 1 (*DTNBP1*), protein phosphatase 3, catalytic subunit gamma isozyme (*PPP3CC*), and SRY (sex determining region Y) box 10 (*SOX10*) (Wockner et al., 2014).

These alterations are the product of a dynamic balance between DNA methylation and demethylation. In fact, the regulation of both hyper- and hypo-methylated genomic DNA is under the control of complex networks of methylating, hydroxymethylating and demethylating enzymes and proteins. For example, 5-methylcytosine (5mC) at specific promoters can be oxidized forming 5-hydroxymethylcytosine (5hmC) by members of the TET family of proteins in mammalian brains (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). In addition, 5hmC is further oxidized by TET family members forming 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) (Ito et al., 2011; Yu et al., 2012; Cadet and Wagner, 2013). Both 5fC and 5caC are specifically recognized by thymine deglycosylase (TDG) producing abasic sites which are replaced by base excision repair (BER) enzymes forming unmodified cytosine (He et al., 2011; Maiti and Drohat, 2011; Hashimoto et al., 2012; Shen et al., 2014). The sequential deamination and repair of 5hmC by activation-induced cytidine deaminase (AID)/apolipoprotein B editing complex (APOBEC) and BER enzymes has been proposed (Guo et al., 2011), although AID/APOBEC enzymes do not appear to use double-stranded 5hmC-containing DNA as a substrate (Wu and Zhang, 2011; Shen et al., 2014). The growth and arrest and DNA damage inducible (GADD45) proteins have been implicated in the targeting of gene-specific DNA demethylation to specific genes in response to neuronal activity (Ma et al., 2009). While DNA demethylation is critical during neurodevelopment, the extent and frequency of active demethylation and the pathways utilized in adult brain are incompletely understood.

Although increases in promoter methylation/hypermethylation catalyzed by the overexpression of DNMT1 or TET1, respectively, may be one mechanism underlying the downregulation of GABAergic, glutamatergic and other gene targets in SZ and BP patient brain, the inhibitory action of DNMT1 and TET1 on gene expression could be the consequence of an interaction between the ZF-CXXC (zinc finger-CXXC) domains of DNMT1 and TET1 binding CpG dinucleotides as recognition sites (Long et al., 2013). The ZF-CXXC domain is a short (35–42 amino acids) polypeptide stretch found in numerous Zn-finger proteins that bind non-methylated CpGs at CpG islands (Long et al., 2013). In addition to DNMT1 and TET1, the domain is present in several additional chromatin modifiers, such as histone lysine demethylases (KDM2A and 2B), histone H3K4 methyltransferase (MLL1), methyl-binding domain protein 1 (MBD1) and the CXXC finger protein 1 (CFP1), that couple various DNA and histone modifications to CpG islands. For example, TET1 acts as a maintenance DNA demethylase that does not decrease methylation levels per se, but specifically prevents aberrant gene-specific methylation spreading into CpG islands in differentiated cells (Williams et al., 2011; Jin et al., 2014). Moreover, DNMT1 and TET1 target additional chromatin-modifying activities, including methyl CpG binding protein 2 (MeCP2) and methyl binding domain protein 2 (MBD2) to CpG rich promoter regions at selected genes through protein interacting domains. The ability of DNMT1 and TET1 to bind to candidate risk genes in post-mortem brain of SZ patients or to form complexes with other chromatin remodeling proteins such as MBD2 has not, until now, been systemically studied.

## 2. Methods and materials

### 2.1. Demographic characteristics

We obtained fresh-frozen PFC (BA9) and cerebellar tissue from the Harvard Brain Tissue Resource Center, McLean Hospital (Belmont, MA, USA). All samples were obtained from family-referred, community-based cases, and none were referred by a medical examiner's office. The demographics associated with each patient population are presented in Table 1. The methods, of tissue harvest, preparation and storage have been described in detail elsewhere (Veldic et al., 2004, 2005, 2007). As shown in Table 1 we find no significant diagnostic differences in post-mortem interval, pH, or age. The psychiatric diagnoses were established by two senior psychiatrists based on clinical and family histories and according to criteria in the Diagnostic and Statistical Manual of Mental Disorders IV. From the available neuropathology reports there were no signs of infarction, hemorrhage or inflammatory lesions. In addition, all control cases were free of neurological disorders, seizures, mental retardation, dementia, and metabolic disorders based on medical records.

### 2.2. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

QPCR was carried out using the Applied Biosystems Real-Time PCR System with a SYBR green master mix (Fermentas, Glen Burnie, MD). Total RNA was isolated from brain samples using TRIzol reagent (Life Technologies, Grand Island, NY), which was further purified using the Qiagen RNeasy kit (Qiagen, Valencia, CA). Primer sequences for the genes analyzed are shown (Table S1 in Supplemental information). Each sample was run in duplicate and repeated twice. For normalizing mRNA expression, several housekeeping genes: Enolase 2 (*ENO2*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and beta-actin (*ACTB*) were chosen as internal controls. For each control, we measured the gene stability ranking using the NormFinder algorithm (Dong et al., 2012). This protocol allows for the identification of the housekeeping gene best suited for normalization. Because each of the genes studied yielded similar results when normalized to either *ENO2*, *GAPDH*, or *ACTB*, and because *ACTB* had the highest housekeeping gene stability (NormFinder), we normalized our data to *ACTB*.

### 2.3. Western blot analysis

For protein quantification we conducted measurements as described in detail elsewhere (Dong et al., 2012). Anti-DNMT1 monoclonal antibodies (0.5 µg/ml, Imagenex, San Diego, CA), or anti-TET1 monoclonal antibodies (Zymo Research, Irvine, CA), or MBD2 polyclonal antibody (Millipore, Billerica, MA) were used to detect the corresponding proteins. The levels of these proteins in NPS, BP or SZ were normalized to β-actin protein levels.

### 2.4. Chromatin immunoprecipitation assays

We performed chromatin immunoprecipitation (ChIP) based on protocols previously described (Dong et al., 2012). The percentages of immunoprecipitated DNA were calculated using the following: % (IP / total input) =  $2^{(Ct(10\% \text{ input}) - 3.32) - Ct(IP)} \times 100\%$ . ChIP grade anti-DNMT1 (Imagenex, San Diego, CA), anti-TET1 monoclonal antibody (Zymo Research, Irvine, CA, USA) and anti-MBD2 (Millipore, Billerica, MA) were used to precipitate cross-linked chromatin. The primer sequences for RELN, GAD1, GAD2, BDNF, and GAPDH are summarized in Table S1, Supplemental materials.

**Table 1**  
Demographic characteristics of the study groups.<sup>a</sup>

|                                       | NPS (n = 32) | Patient cohort |             | Statistical analysis        |
|---------------------------------------|--------------|----------------|-------------|-----------------------------|
|                                       |              | BP (n = 20)    | SZ (n = 22) |                             |
| M/F ratio                             | 20/12        | 5/15           | 15/7        | $\chi^2_2 = 9.49, p = .009$ |
| Age, y                                | 59.2 ± 21.4  | 59.6 ± 14.8    | 61.0 ± 11.7 | $F_{2,73} = .06, p = .94^b$ |
| Postmortem interval, h                | 21.8 ± 3.6   | 22.2 ± 5.1     | 23.9 ± 7.3  | $F_{2,73} = .45, p = .64^b$ |
| Brain pH                              | 6.4 ± 0.3    | 6.6 ± 0.3      | 6.5 ± 0.3   | $F_{2,73} = .47, p = .63^b$ |
| Cause of death                        |              |                |             |                             |
| Cardiopulmonary                       | 29           | 6              | 9           | $\chi^2_6 = 66.6, p = .000$ |
| Others                                | 3            | 14             | 13          | $\chi^2_6 = 61.2, p = .000$ |
| Antipsychotic drug use <sup>c,d</sup> | 0            | 17             | 18          | $\chi^2_6 = 85.4, p = .000$ |
| Antidepressant <sup>c,e</sup>         | 0            | 1              | 0           |                             |
| Mood stabilizers <sup>c,f</sup>       | 0            | 12             | 5           | $\chi^2_6 = 48.4, p = .000$ |
| Abuse or dependence                   |              |                |             |                             |
| Alcohol                               | 3            | 8              | 5           | $\chi^2_6 = 93.2, p = .000$ |
| Cocaine                               | 2            | 3              | 2           | $\chi^2_6 = 87.2, p = .000$ |
| Tobacco                               | 11           | 7              | 6           | $\chi^2_6 = 87.1, p = .000$ |

<sup>a</sup> Values are expressed as mean ± SD.

<sup>b</sup> One-way analysis of variance among patient cohorts.

<sup>c</sup> Present to the time of death.

<sup>d</sup> Includes the following (typical neuroleptics): haloperidol, fluphenazine, thioridazine, perphenazine, trifluoperazine, chlorpromazine and molindone; (atypical neuroleptics): clozapine, olanzapine, aripiprazole, risperidone and quetiapine.

<sup>e</sup> Includes: the following: trazodone, bupropion and venlafaxine.

<sup>f</sup> Includes: lithium and valproic acid.

## 2.5. Statistical analysis

Data were analyzed using the Boxplot of PASW v.18 software (SPSS, Chicago, IL, USA) to identify outliers, which were excluded for further statistical analysis. One-way ANOVA and analysis of covariance with Bonferroni *post-hoc* comparisons were performed for determining significance of mRNA content; binding of DNMT1, TET1 and MBD2 at selected gene promoters; DNMT1, TET1 and MBD2 protein content; and medication use, age, gender, post-mortem interval, substance use and alcohol abuse, between the three groups (NPS, BP and SZ), using PASW v.18 software. In all samples, p-values were two-tailed, and comparisons were considered to be statistically significant at  $p < 0.05$  level using Bonferroni's multiple comparison correction.

Relationships between DNMT1 mRNA and DNMT1 ChIP at the GAD1, RELN, or BDNF-IX promoters were analyzed with Pearson correlations and scatter plots.

## 3. Results

### 3.1. The expression (mRNA and protein levels) of DNMT1 and TET1 is increased in PFC of SZ and BP disorder patients

In previous studies, we showed that DNMT1 and TET 1 mRNAs and proteins are increased in the neocortex of SZ and BP disorder patients (Veldic et al., 2004; Ruzicka et al., 2007; Dong et al., 2012). Using a completely new cohort of post-mortem brain tissue, patients diagnosed with SZ (n = 22), BP disorder (n = 20), and a group of non-psychiatric subjects (NPS), (n = 32), we confirmed previous reports showing

that DNMT1 mRNA (overall ANOVA,  $F_{2,71}; p = 0.002$ , followed by Bonferroni comparison:  $p = 0.003$  SZ vs NPS;  $p = 0.031$  BP vs NPS) and TET1 mRNA (overall ANOVA,  $p = 0.01$ , followed by Bonferroni comparison:  $p = 0.023$  SZ vs NPS;  $p = 0.042$  BP vs NPS) are increased by 30–40% in the PFC of these patients (Table 2). DNMT1 and TET1 protein levels are also increased in SZ and BP patients (Table 2).

### 3.2. Higher DNMT1 but not TET1 binding at GAD1, RELN, and BDNF IX promoters in PFC of SZ and BP disorder patients

We and others (Guidotti et al., 2000; Abdolmaleky et al., 2005; Grayson et al., 2005; Dong et al., 2012; Gavin et al., 2012; Ikegame et al., 2013) have shown that GAD1, RELN, and BDNF-IX CpG rich promoter regions exhibit hypermethylation or hydroxymethylation in the neocortex of SZ and BP disorder patients. Moreover, the expression of these genes is also reduced.

Using ChIP assays with specific antibodies (see Methods and materials section), we demonstrated that the down-regulation of GAD1, RELN, and BDNF-IX expression is accompanied by an increased binding of DNMT1 and TET1 to the promoter domains of these genes. In Fig. 1, we show that there is a significant increase in DNMT1 binding to GAD1 (overall ANOVA,  $F_{2,67}, p < 0.0001$ , followed by Bonferroni comparison:  $p < 0.0001$ , SZ vs NPS;  $p < 0.0001$ , BP vs NPS), RELN (overall ANOVA,  $p < 0.0001$ , followed by Bonferroni comparison:  $p < 0.0001$ ; SZ vs NPS;  $p = 0.001$ , BP vs NPS), and BDNF-IX (overall ANOVA,  $p = 0.0001$ , followed by Bonferroni comparison:  $p = 0.04$ , SZ vs NPS;  $p < 0.001$  BP vs NPS) promoters in the PFC of SZ and BP disorder patients. In contrast, DNMT1 binding to GAD2 and GAPDH promoters

**Table 2**  
Expression of DNMT1 and TET1 in the PFC (Brodmann area 9) of schizophrenia (SZ) and bipolar disorder (BP) patients.

| Gene  | Parameters | Subject cohort |                            |                            | Overall ANOVA |      |         |
|-------|------------|----------------|----------------------------|----------------------------|---------------|------|---------|
|       |            | NPS (32)       | SZ (22)                    | BP (20)                    | F             | d.f. | p-Value |
| DNMT1 | mRNA       | 0.062 ± 0.000  | 0.086 ± 0.007 <sup>a</sup> | 0.082 ± 0.001 <sup>a</sup> | 6.965         | 2    | 0.002   |
|       | Protein    | 0.019 ± 0.004  | 0.039 ± 0.005 <sup>a</sup> | 0.030 ± 0.006              | 5.098         | 2    | 0.010   |
| TET1  | mRNA       | 0.065 ± 0.001  | 0.094 ± 0.013 <sup>a</sup> | 0.093 ± 0.001 <sup>a</sup> | 4.969         | 2    | 0.010   |
|       | Protein    | 0.28 ± 0.02    | 0.50 ± 0.07 <sup>a</sup>   | 0.48 ± 0.05 <sup>a</sup>   | 5.035         | 2    | 0.011   |

mRNA = the values were normalized to  $\beta$ -actin; protein = the values were normalized by  $\beta$ -actin.

Note: 2 samples from NPS and 1 sample from SZ were excluded from statistical analysis because they were identified as outliers when analyzed using the Boxplot of PASW v.18 software (see Methods and materials section).

<sup>a</sup> Significant difference from NPS. One-way ANOVA followed by Bonferroni comparison.

was one to two orders of magnitude lower than the binding of DNMT1 and failed to increase in SZ and BP disorder patients (DNMT1 bound to GAD2: NPS =  $0.0047 \pm 0.0006$ ; BP =  $0.0021 \pm 0.0009$ ; SZ =  $0.0024 \pm 0.001$ ), (DNMT1 bound to GAPDH: NPS =  $0.012 \pm 0.007$ ; BP =  $0.018 \pm 0.015$ ; SZ =  $0.010 \pm 0.007$ ).

As shown in Fig. 2, mRNA levels corresponding to DNMT1 significantly and positively correlated with DNMT1 binding to the RELN and GAD1 promoters. The binding of DNMT1 to BDNF promoter IX also positively correlated with DNMT1 mRNA levels, but due to considerable variability in the measurements of BDNF-IX in the BP disorder group, this correlation was statistically significant only when the SZ and NPS groups were considered separately. Importantly, increased binding of DNMT1 to GAD1 and RELN was not observed in the cerebellum of SZ and BP disorder patients (GAD1: NPS =  $0.20 \pm 0.014$ , BP =  $0.215 \pm 0.060$ , SZ =  $0.231 \pm 0.030$ ; n = 7 per group), (RELN: NPS =  $0.068 \pm 0.012$ , BP =  $0.054 \pm 0.038$ , SZ =  $0.040 \pm 0.016$ ; n = 7 per group).

While there is a significant binding of TET1 to GAD1, RELN, and BDNF-IX promoter regions, the binding did not increase (Fig. 1) in psychiatric patients in spite of the significant increase in TET1 mRNA and protein in the neocortex of SZ and BP patients (Table 2). The lack of increased TET1 binding to GAD1, RELN, and BDNF-IX promoters in SZ

and BP disorder patients is not the consequence of methodological considerations because in a recent study using the same technique, we demonstrated an increase in TET1 binding to GAD1 and RELN in cerebellum of autistic spectrum disorder patients (Zhubi et al., 2014). It is interesting to note that binding of MBD2 (a reader of promoter methylation and/or a member of a co-repressor complex interacting with DNMT) to GAD1, RELN, and BDNF-IX in the same patients was increased (Fig. 1).

### 3.3. Potential confounding variables

To show that the observed results on the levels of DNMT1 and MBD2 binding to the GAD1, RELN, and BDNF-IX promoters cannot be attributed to potential confounding variables (age, brain pH, post-mortem interval), we analyzed the data with one-way ANOVA and analyses of covariance (ANCOVA). We found no significant differences in demographic variables among the three groups (SZ, BP and NPS) by ANOVA (Table 1), with the exception of gender (BP disorder group) in which females were twice as prevalent as males. Hence, we investigated whether gender might be a factor responsible for the changes observed in BP disorder patients. ANCOVA did not reveal a statistically significant correlation between gender and changes in DNMT1 ( $p = 0.117$ ), and TET1 ( $p = 0.544$ ) mRNA, between gender and DNMT1 binding to

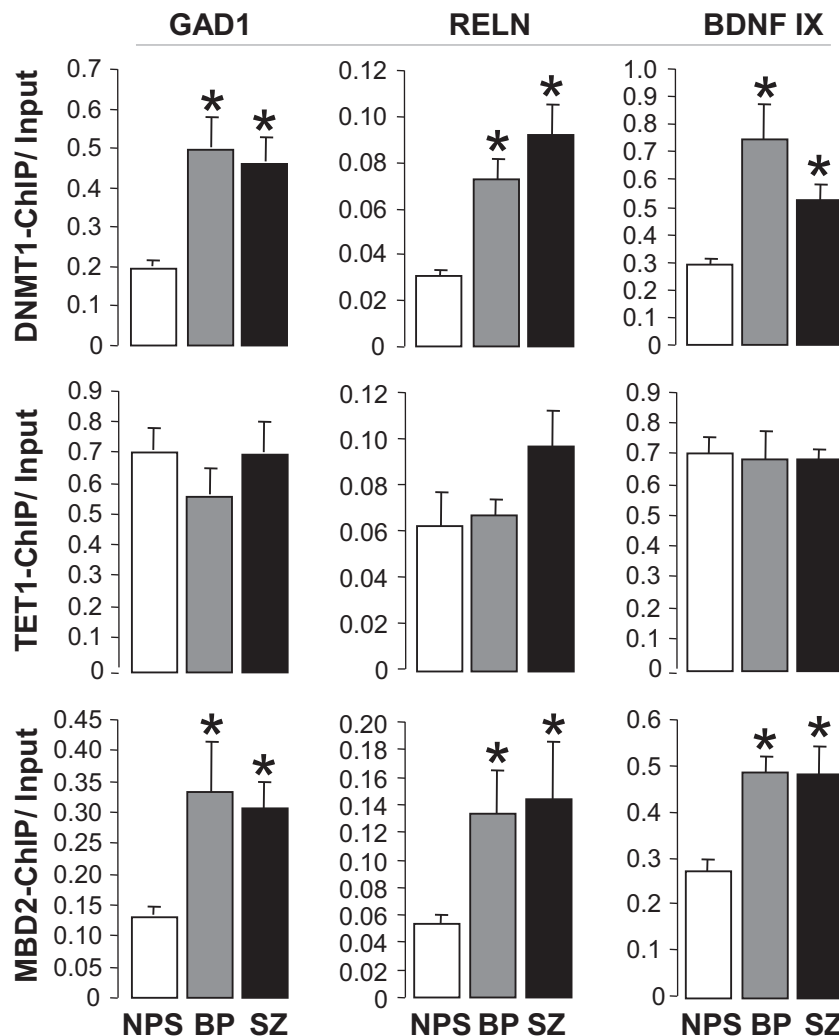


**Fig. 1.** DNMT1, TET1, and MBD2 binding to GAD1, RELN, and DBNF-IX promoters in PFC (BA9) of non-psychiatric subjects (NPS), schizophrenia (SZ), and bipolar (BP) disorder patients. Chromatin immune precipitation was performed as described in the [Methods and materials](#) section using the indicated antibodies. Top panels show DNMT1 ChIP results at the GAD1, RELN and BDNF IX promoters. Middle panels correspond to TET1 ChIP data and bottom panels show data obtained with MBD2 antibody. Denotes significant difference from NPS (\* $p < 0.05$ ). One-way ANOVA followed by Bonferroni comparison. Note: 1 sample from NPS and 1 sample from SZ were excluded from statistical analysis because they were identified as outliers when analyzed using the Boxplot of PASW v.18 software (see [Methods and materials](#) section).

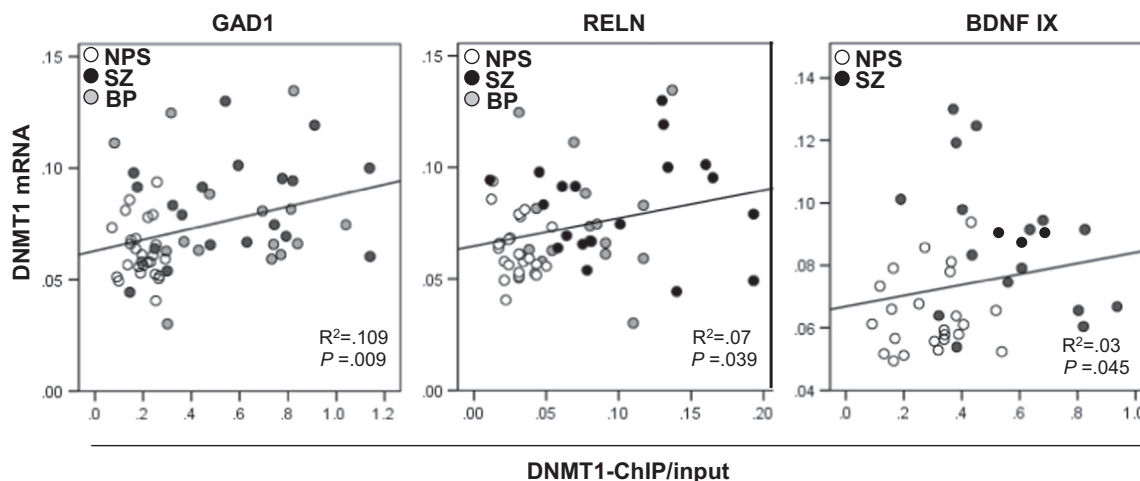


**Fig. 2.** Pearson correlation between DNMT1 mRNA expression and DNMT1 binding to GAD1, RELN, and BDNF-IX promoters. The panels (left, GAD1; middle, RELN; right, BDNF-IX) show the relationship between DNMT1 mRNA and DNMT1 ChIP binding at the respective promoters. Note: Two samples from NPS and two samples from SZ were excluded from statistical analysis because they were identified as outliers when analyzed using the Boxplot of PASW v.18 software (see [Methods and materials](#) section).

GAD1 ( $p = 0.151$ ), RELN ( $p = 0.953$ ), and BDNF-IX ( $p = 0.737$ ), or between gender and TET1 binding to GAD1 ( $p = 0.736$ ), RELN ( $p = 0.568$ ), and BDNF-IX ( $p = 0.935$ ) in the BP disorder group. When the effects of demographic variables such as cause of death, tobacco, alcohol, and cocaine abuse, entered as covariates, were examined on group differences of DNMT1 or MBD2 binding to target genes, we found that the observed differences among the three groups were still significant. In addition, similar analyses of history of antipsychotic medication, antidepressants, or treatment with mood stabilizers, entered as covariates, failed to influence group differences observed for DNMT1 and MBD2 binding to GAD1, RELN, and BDNF-IX.

Hence, our analyses of covariance suggest that the increased binding of DNMT1 at RELN, GAD1, BDNF-IX promoters in PFC of SZ and BP disorder patients could not be attributed to confounding demographic variables including the use of antidepressants, mood stabilizers, antipsychotics, or drugs of abuse. However, before excluding the existence of an interaction between the increase in DNMT1 binding and medications, we need to study a larger number of SZ and BP disorder patients who have not been treated with antipsychotic medications.

#### 4. Discussion

Studies in post-mortem brains of SZ and BP disorder patients (Dong et al., 2012; Gavin et al., 2012), and in experimental animal models of SZ (Matrisciano et al., 2012, 2013) show that altered DNA methylation and hydroxymethylation of promoter proximal regions of actively expressed genes is associated with reduced gene expression. Typically, enriched methylation or hydroxymethylation of CpG rich promoter regions of GAD1, RELN, and BDNF is accompanied by an increased expression of DNMT1 and TET1, enzymes that play an important role in the dynamic regulation of gene promoter methylation (for a review see Grayson and Guidotti, 2013). In this study, we confirm previous observations that the expression of DNMT1 and TET1 is increased in the PFC of SZ and BP disorder patients. Consistent with the increase in TET1, we previously reported that the levels of 5hmC are elevated in both total DNA and in the promoters of GAD1 and BDNF-IX of the inferior parietal lobule of SZ and BP disorder patients (Dong et al., 2012; Gavin et al., 2012). Moreover, the increase in 5hmC levels at GAD1 and BDNF-IX promoters correlates with reduced mRNA expression (Dong et al., 2012). The increase of DNMT1, particularly evident in PFC GABAergic neurons of SZ patients (Ruzicka et al., 2007), also correlates with reduced expression of GAD1 and RELN. However, studies showing the levels of 5mC at the RELN and GAD1 promoter regions, point to either an increase (Abdolmaleky et al., 2005; Grayson et al., 2005;

Lintas and Persico, 2010), no change (Mill et al., 2008; Huang et al., 2007) or no methylation (Tochigi et al., 2008).

We (Dong et al., 2012) and others (Huang and Akbarian, 2007) have reported that 5mC levels at the GAD1 promoter are not increased in PFC of SZ patients even though DNMT1 expression is increased in GABAergic neurons and the expression of GAD1 mRNA is decreased (Akbarian et al., 1995; Guidotti et al., 2000; Lewis et al., 2005). Hence, these results suggest that cytosine methylation might not be the only mechanism by which DNMT1 regulates gene expression. Here, we used chromatin immune precipitation to test the relationship between DNMT1 and TET1 levels and binding of these proteins to target gene promoters in SZ and BP disorder patients. Our studies revealed, for the first time, that the binding of DNMT1 at promoter regions of GAD1, RELN, and BDNF-IX is markedly increased in the PFC of SZ and BP disorder patients, compared to NPS.

We also observed a positive correlation between DNMT1 mRNA expression and binding of DNMT1 to the GAD1 and RELN promoters in the PFC but not in the cerebellum of the same patients. The differential responsiveness of genes in GABAergic neurons of PFC and cerebellum did not come as a surprise. In fact, GABAergic neurons in PFC are inhibitory interneurons, whereas GABAergic Purkinje neurons in cerebellum are principal neurons projecting from cerebellar cortex to the deep cerebellar nuclei. Furthermore, RELN, which is expressed in GABAergic interneurons in the PFC, is expressed in excitatory granular neurons in the cerebellar cortex. Hence, the data suggest that the binding of DNMT1 to GABAergic promoters may be a neuron-specific and perhaps neuronal activity dependent mechanism. Because the levels of DNMT1 mRNA positively correlate with binding of DNMT1 to the GAD1 and RELN promoters in the absence of any clear alterations in promoter methylation, it suggests that DNMT1 might regulate the expression of GABAergic genes using a mechanism which is, at least in part, independent from cytosine methylation.

At present we do not have data to suggest the precise mechanism by which DNMT1 binding to GAD1, RELN, and BDNF-IX promoters is increased in SZ and BP disorder patients. While we presume that DNMT1 binds DNA directly, it may also bind indirectly to promoters. That is, DNMT1 may interact with MBD2, or other ancillary chromatin remodeling proteins, and form a chromatin repressor complex which facilitates transitions between active and inactive chromatin states.

One possibility that should be considered is that DNMT1 and TET1 each encodes ZF-CXXC zinc finger domains as part of their primary structure which contains eight conserved cysteine residues that bind two zinc ions. The CXXC protein domain, like the methyl binding domain (MBD), binds specifically to CpGs, but in the unmethylated state (Lee et al., 2001). In addition to DNMT1 and TET1, CXXC domains

are present in a variety of chromatin-associated proteins, including CFP1, MLL1 and 2, KDM2A and 2B, and TET3 (Long et al., 2013). These independently folded structures facilitate binding of the associated proteins with high affinity to clusters of unmethylated CpGs and are thought to target these proteins to non-methylated CpG islands (Long et al., 2013; Rose and Klose, 2014). The CXXC domains of DNMT1 and TET1 are very similar with the exception that the TET1 domain lacks the conserved motif KFGG (Lys-Phe-Gly-Gly). Recent in vitro binding data show that the CXXC domain of TET1 prefers binding unmethylated over methylated CpGs by ~3:1 (Zhang et al., 2010). In contrast, DNMT1 binds strongly to unmethylated and less so to methylated CpG dinucleotides with a preference for unmethylated dinucleotides of 48 to 1 (Zhang et al., 2010). Another study, using transient expression assays, shows that the CXXC domain of TET1 fails to bind DNA and is dispensable for catalytic activity (Frauer et al., 2011). The above studies support the concept that while the CXXC domain of DNMT1 is a functionally independent binding motif, the corresponding domain of TET1 likely requires additional regions of the protein for full biological function. Moreover, the presence of a CXXC domain may not be sufficient per se to confer DNA binding in isolation. Available data indicate that both DNMTs and TET proteins have the ability to modify the methylation status of CpG dinucleotides and to act as transcriptional repressors but while DNMT1 acts as a context-dependent transcriptional repressor in addition to its normal cytosine methylating activity, TET1 may function primarily as an active 5-methylcytosine hydroxylase.

The increased binding of DNMT1 to SZ and BP disorder risk genes appears to be specific because the promoters of genes whose expression is not altered, such as GAD2 and GAPDH, fail to show increased DNMT1 binding. Most importantly, we found that in contrast to DNMT1, TET1 chromatin immune precipitation assays fail to show a positive correlation between TET1 mRNA expression and promoter binding. In conclusion, the data are consistent with the hypothesis that the reduced expression of genes observed in SZ and BP disorder patients may be mediated, at least in part, by region specific variations in DNMT1 action that is likely independent of promoter methylation mechanisms.

An important point implicit in this study is the observation that SZ and BP disorders, although distinct clinical entities, often share common epigenetic features. In both disorders, patients exhibit reduced levels of GAD1, RELN, BDNF, and NMDA receptor expression. Similarly, in both SZ and BP disorders, the expression of DNMTs and TETS are increased and 5mC and 5hmC are enriched at several GABAergic and glutamatergic target genes (reviewed in Grayson and Guidotti, 2013). Here we show that the binding of DNMT1 to GAD1, RELN and BDNF is equally increased in PFC of SZ and BP disorder patients suggesting that the two disorders share common pathophysiological epigenetic features.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.schres.2014.10.030>.

## Author disclosure

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## Contributors

E. Dong performed the experiments presented and was primarily responsible for data analysis. Dr. Ruzicka was also involved with data analysis. Drs. Guidotti and Grayson were involved with experimental design, data analysis and manuscript preparation. All authors approved the final manuscript.

## Conflict of interest

The authors declare no conflict of interest.

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