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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

E. Dong [a], W.B. Ruzicka [b,c], D.R. Grayson [a], A. Guidotti [a,*]

[a] Department of Psychiatry, College of Medicine, University of Illinois at Chicago, United States
[b] Program in Structural and Molecular Neuroscience, McLean Hospital, Belmont, MA, United States
[c] Department of Psychiatry, Harvard Medical School, Boston, MA, United States

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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.

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

* Corresponding author at: 1601 W. Taylor St., Chicago, IL 60612, United States. Tel.: +1 312 413 4594.
E-mail address: aguidotti@psych.uic.edu (A. Guidotti).

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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 (*DTNBP10*, 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: $\%\ (\text{IP} / \text{total input}) = 2^{(\text{Ct}(10\%\ \text{input}) - 3.32) - \text{Ct}(\text{IP})} \times 100\%$. 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.

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