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GAD1 mRNA expression and DNA methylation in prefrontal cortex of subjects with schizophrenia

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**INTRODUCTION**

Cortical dysfunction in schizophrenia includes changes in GABAergic mRNAs, including decreased expression of *GAD1*, encoding the 67 kDa glutamate decarboxylase (*GAD67*) GABA synthesis enzyme. The underlying molecular mechanisms remain unclear. Alterations in DNA methylation as an epigenetic regulator of gene expression are thought to play a role but this hypothesis is difficult to test because no techniques are available to extract DNA from *GAD1* expressing neurons efficiently from human postmortem brain. Here, we present an alternative approach that is based on immunoprecipitation of mononucleosomes with anti-methyl-histone antibodies differentiating between sites of potential gene expression as opposed to repressive or silenced chromatin. Methylation patterns of CpG dinucleotides at the *GAD1* proximal promoter and intron 2 were determined for each of the two chromatin fractions separately, using a case-control design for 14 schizophrenia subjects affected by a decrease in prefrontal *GAD1* mRNA levels. In controls, the methylation frequencies at CpG dinucleotides, while overall higher in repressive as compared to open chromatin, did not exceed 5% at the proximal *GAD1* promoter and 30% within intron 2. Subjects with schizophrenia showed a significant, on average 8-fold deficit in repressive chromatin-associated DNA methylation at the promoter. These results suggest that chromatin remodeling mechanisms are involved in dysregulated GABAergic gene expression in schizophrenia.

**RESULTS**

To find out if H3K4me3 at the *Gad1* locus defines open chromatin and gene expression, and to examine potential effects of antipsychotic drug (APD) treatment on chromatin remodeling, we monitored open (H3K4me3) and repressive (H3K27me3; Hampsay and Reinberg 2003; Sims et al. 2003) chromatin marks that defines open chromatin at sites of active transcription [14,15]. Therefore, we separated open and repressive chromatin from human prefrontal cortex with site-specific anti-methyl-histone specific antibodies, followed by *GAD1* DNA methylation studies for each of the two chromatin fractions separately.

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forebrain (Fig. 1A, see also Methods). In this assay, neuronal differentiation is induced by withdrawal of fibroblast growth factor 2 (FGF2) and addition of sodium valproate (VA) to the cell culture medium [16]. In comparison to undifferentiated precursor cells (“+FGF2” in Fig. 1A, B), neurons (“-FGF2/+VA” in Fig. 1A, B) showed, on average, a 160-fold fold increase in Gad1 mRNA levels by qRT-PCR, and this associated with a 30-fold increase in H3K4me3 levels at the proximal Gad1 promoter (Fig. 1B). These changes were consistent in 3/3 experiments. In contrast to these dramatic increases in Gad1 mRNA and H3K4me3, levels of H3K27me3—the repressive mark—showed a two-fold decrease upon neural differentiation (Fig. 1B). Furthermore, cultured neurons treated with the antipsychotic, clozapine (“-FGF2+VA+Clz” in Fig. 1B), did not show consistent changes in Gad1 mRNA, or Gad1-associated H3K4me3 and H3K27me3. From these experiments, we draw two conclusions: First, the tagging of Gad1 nucleosomes with H3K4me3-a histone mark previously associated with open chromatin and actual or potential gene expression in non-neuronal tissues and cell lines ([6]—indeed reflects neuronal gene expression activity at that locus. Second, levels of open (H3K4me3) and repressive (H3K27me3) histone methylation at the Gad1 locus are not affected by treatment with the antipsychotic drug, clozapine.

Next, we separated open (H3K4me3) and repressive (H3K27me3) chromatin from postmortem human prefrontal cortex prepared by micrococcal nuclease-based digestion prior to immunoprecipitation as previously described [17,18]. Then, we monitored Gad1 mCpG methylation in subclones of PCR products amplified from the immunoprecipitated DNA after bisulfite conversion. Altogether 70 primer pairs within 8kb of Gad1 proximal promoter and 5’ end sequences were tested; 67 primers pairs produced amplicons that lacked sequence specificity (data not shown). This was not surprising given that bisulfite-conversion reduces the genetic code to a 3 letter code in the absence of methylation. The design of suitable PCR primers is further limited by the chromat prepation technique that produces mononucleosomes with less than 148 bp of genomic DNA. The remaining 3 primer pairs (Suppl. Table S1) covered altogether 12 Cpg’s positioned between −1120 to +3400 bp from the Gad1 transcription start site (Fig. 2A). Methylation frequencies in repressive chromatin immunoprecipitated with anti-H3K27me3 antibody were higher at 10/12 Cpg dinucleotides, in comparison to open chromatin fractionated with anti-H3K4me3 antibody (Binomial test, p < 0.01) (Fig. 2A upper panel). Two Cpg’s located within 200–250 bp upstream of Gad1 transcription start site remained unmethylated both in open and repressive chromatin, and DNA methylation levels were overall much lower at the promoter in comparison to intron 2 (Fig. 2A upper panel). Next, we monitored Gad1 Cpg methylation levels in subjects diagnosed with schizophrenia and their matched controls. Levels of Gad1 DNA methylation in open chromatin (H3K4me3) were strikingly similar between schizophrenia subjects and controls, with extremely low levels at the promoter (<0.5%) (Fig. 2A lower panel, and Fig. 2B) and a higher methylation frequency (approximately 15%) within intron 2 (Fig. 2A lower panel, and Fig. 2C). Unexpectedly, however, Gad1 DNA methylation in repressive chromatin (H3K27me3) of schizophrenia subjects was significantly different from control subjects: CpG

Figure 1. Histone methylation changes at the Gad1 promoter in a neuronal differentiation assay. (A) (a–j) Digitized images showing (a–c) undifferentiated neural precursors grown in FGF2 (fibroblast growth factor 2)-containing medium and (d–j) neurons differentiated in medium without FGF2 but with VA (sodium valproate); notice that precursors (a), but not neurons (d) are defined by nestin immunoreactivity, while both type of cells express robust H3K4me3 immunoreactivity (c,f); (g,i) representative examples of neuronal marker (g, NeuN and i, GABA) immunoreactivity. All images taken at 20×10 magnification. (B) (top) levels of Gad1 mRNA (y-axis, log scale), expressed relative to 18S rRNA and (middle and bottom) chip-to-input ratios (y-axis, log scale) of site-specific histone methylation (H3K4me3 and H3K27me3) in the nucleosomes positioned −374 to −273 bp of rat Gad1 promoter. Data expressed as mean ± S.E.M., with N = 3 for each of the three different culture conditions. Notice robust increase of Gad1 mRNA and H3K4me3 levels in differentiated cultures (“-FGF2+VA”), in comparison to undifferentiated cells (+FGF2); that treatment with the antipsychotic clozapine did not affect histone methylation and Gad1 gene expression in cultured neurons. VA = Valproic acid, Clz = Clozapine.

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methylation frequencies were on average 3.5% in the control cohort but only 0.4% in the disease cohort (Fig. 2A lower panel, and Fig. 2B). This DNA methylation deficit in repressive GAD1 chromatin of schizophrenia subject affected 5/8 CpG nucleotides (Fig. 2A lower panel) and was significant (Wilcoxon Signed Ranks Test, p = 0.018). In contrast, the CpG methylation frequencies at intron 2 were very similar in cases and controls (Fig. 2A, lower panel, and Fig. 2C) and were approximately 25% in both cohorts; these differences were not significant. Therefore, the deficit in prefrontal GAD1 mRNA levels in this cohort of schizophrenia subjects (Fig. 2, see also Methods) is associated with a selective decrease in DNA methylation in repressive GAD1 chromatin at the site of the proximal gene promoter.

Among various GAD1 single nucleotide polymorphisms (SNP’s), two are positioned within 2kb of the transcription start site (rs3749034 and rs2270335). These two SNP’s are included in a GAD1 haplotype that confers genetic risk for childhood-onset schizophrenia and accelerated loss of frontal gray matter [5]. In order to rule out that GAD1 genotypes were different in the cases and controls of the present study, we determined allele frequencies...
for the two SNP's. In both cohorts, allele frequencies were identical, which is expected given their close proximity (<1.5 kb) [Schizophrenia subjects, allele (1/1) 57%, (1/2) 29%, (2/2) 14%; controls (1/1) 79%, (1/2) 14%, (2/2) 7%]. Notably, the case and control cohort showed no significant difference in the number of subjects bi-allelic for the common allele (1/1), which defines the at risk haplotype [5] (Pearson chi-square $X^2 = 0.47$, df = 1, $p = 0.5$). Furthermore, overall allele frequencies were not significantly different between the two cohorts (Fisher's Exact Test).

**DISCUSSION**

To our knowledge, the present study is the first to assess DNA methylation in human brain separately for open and repressive chromatin. In the open chromatin fractions of the present study, which were defined by trimethylation of a specific histone lysine residue (H3K4) [14,15], **GAD1** DNA methylation was overall much lower, in comparison to repressive chromatin that is defined by trimethylation of another lysine residue, H3K27 [15]. However, even in repressive/silenced chromatin, only a fraction of **GAD1** CpG dinucleotides (<30% for intron 2, and <5% at the promoter) are subjected to DNA methylation in human prefrontal cortex.

Given that DNA methylation around the proximal promoter typically contributes to transcriptional repression, it was expected that subjects with schizophrenia show increased **GAD1** DNA methylation. Instead, we observed for the repressive chromatin fraction of schizophrenia subjects a significant decrease in CpG methylation at the proximal **GAD1** promoter. Further studies are necessary in order to determine if these changes are related to altered **GAD1** gene transcription. Notably, the schizophrenia subjects of the present study had lower **GAD1** mRNA levels in comparison to the matched control (see Methods and Fig. 3). One plausible explanation would be that in the affected cases, there is an increased proportion of **GAD1** nucleosomes tagged with the repressive mark, H3K27me3, but without concomitant methylation of the genomic DNA (Fig. 4). Therefore, repressive chromatin-associated histone methylation at the **GAD1** locus in schizophrenia appears to be, at least in part, independent from CpG methylation. The present study faces an important limitation because reliable PCR amplification across multiple subjects was achieved only for a dozen **GAD1** CpG's, and we cannot exclude an important role for any of the approximately 400 additional CpG sites that surround the first 5 kb of **GAD1** transcription start site. Therefore, our findings have to be viewed as preliminary. Furthermore, it will be of interest to find out in future studies whether the observed DNA methylation deficits in H3K27me3-tagged **GAD1** nucleosomes of schizophrenia subjects are specific for that gene, or part of a more widespread DNA methylation defect of the disorder.

**METHODS**

All procedures were approved by the institutional review board of the University of Massachusetts Medical School. Demographics, postmortem confounds and RNA data for the case and control cohorts are presented in Table 1. Each pair consisted of a subject...
with schizophrenia and a control matched for age, gender and autolysis time. The prefrontal cortex of the 14 schizophrenia subjects included in the present study showed, in comparison to the matched control, a decrease in GAD1 mRNA levels, as determined by qRT-PCR with 3 different sets of PCR primers and normalization to the housekeeping gene transcript, β2-microglobulin (B2M) (Table 1 and Fig. 3). In addition, for 3 of the matched pairs the decrease in GAD1 mRNA levels in the schizophrenia subject had been observed in a previous study that used in situ hybridization histochemistry [19]. The remaining 11 matched pairs were not included in that earlier study, but were collected by the same brain bank and subjected to the same diagnostic criteria and inclusion/exclusion criteria as previously described [19].

For DNA methylation studies, nucleosomes first were extracted from prefrontal cortex gray matter and then immunoprecipitated with two anti-methyl-histone antibodies [anti-histone H3-tri-methyl-lysine 4 (H3K4me3) and anti-H3-tri-methyl-lysine 27 (H3K27me3)] to separate open chromatin at sites of actual or potential transcription from repressive and silenced chromatin, exactly as described [17,18]. Salmon sperm as a blocking agent was omitted and instead all samples were first pre-cleared by sodium bisulfite conversion followed by purification and elution, procedure. DNA purified from immunoprecipitates was subjected to bisulfite conversion and sequencing. For each subject, DNA methylation levels were evaluated by two sample t-test, or in case of non-normal distribution, by Mann-Whitney test separately for open and repressive chromatin fractions. Furthermore, for each case and control, genotyping for selected GAD1 single nucleotide polymorphism was performed using matrix-assisted laser desorption/ionization mass spectrometry (Sequenom), in conjunction with SpectroDesign software for PCR and MassEXTEND primers.

**Cell cultures**
Neural stem cells were prepared from forebrain of E14.5 SASCO SD rat embryos (Charles River). Live cells were plated out at 1.2–1.4 x 10^5 cells per 100-mm poly-l-lysine coated dishes pre-coated with 1 μg/ml poly-l-ornithine (sigma) and 4 μg/ml fibronectin (R&D systems), and treated daily with 10 μg basic fibroblast growth factor 2 (FGF2-R&D systems). At DIV3, cells were passaged and plated out at 0.8–1.0 x 10^6 cells per pre-coated plate and expanded as above for a further 3–4 days (expansion approx 300%). Cells were passaged again and plated out at 1.2–1.4 x 10^6, and after 1–2 days FGF2 was removed, cells washed once with media and then resuspended in DMEM/M2/F12 media (Invitrogen) without FGF2 but with 0.5 mM sodium valproate and with or without 1 micromol clozapine, and harvested after 4 days.

**SUPPORTING INFORMATION**

**Table S1**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Age</th>
<th>PMI</th>
<th>Brain pH</th>
<th>RIN</th>
<th>GAD1 mRNA Exon 3–4</th>
<th>Exon 13–14</th>
<th>Exon 17</th>
<th>G</th>
<th>APD</th>
<th>No.</th>
<th>%</th>
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<tr>
<td>Mean ± S.E.M.</td>
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<tr>
<td>Cases</td>
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<td>13.6±2.4</td>
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<td>0.038±0.015</td>
<td>0.085±0.018</td>
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<td>86</td>
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<td>Controls</td>
<td>14</td>
<td>60.5±5.2</td>
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<td>5/9</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References

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DNA Methylation in Psychosis


