

# Disease- and age-related changes in histone acetylation at gene promoters in psychiatric disorders

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Increasing evidence suggests that epigenetic factors have critical roles in gene regulation in neuropsychiatric disorders and in aging, both of which are typically associated with a wide range of gene expression abnormalities. Here, we have used chromatin immunoprecipitation-qPCR to measure levels of acetylated histone H3 at lysines 9/14 (ac-H3K9K14), two epigenetic marks associated with transcriptionally active chromatin, at the promoter regions of eight schizophrenia-related genes in  $n = 82$  postmortem prefrontal cortical samples from normal subjects and those with schizophrenia and bipolar disorder. We find that promoter-associated ac-H3K9K14 levels are correlated with gene expression levels, as measured by real-time qPCR for several genes, including, glutamic acid decarboxylase 1 (*GAD1*), 5-hydroxytryptamine receptor 2C (*HTR2C*), translocase of outer mitochondrial membrane 70 homolog A (*TOMM70A*) and protein phosphatase 1E (*PPM1E*). Ac-H3K9K14 levels of several of the genes tested were significantly negatively associated with age in normal subjects and those with bipolar disorder, but not in subjects with schizophrenia, whereby low levels of histone acetylation were observed in early age and throughout aging. Consistent with this observation, significant hypoacetylation of H3K9K14 was detected in young subjects with schizophrenia when compared with age-matched controls. Our results demonstrate that gene expression changes associated with psychiatric disease and aging result from epigenetic mechanisms involving histone acetylation. We further find that treatment with a histone deacetylase (HDAC) inhibitor alters the expression of several candidate genes for schizophrenia in mouse brain. These findings may have therapeutic implications for the clinical use of HDAC inhibitors in psychiatric disorders.

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## Introduction

Epigenetic mechanisms of gene regulation involve both DNA methylation and posttranslational modifications of histone proteins.<sup>1</sup> Although it is known that DNA methylation of cytosine residues at CpG dinucleotide sites results in gene silencing, the effects of posttranslational modifications on histone proteins are more complex.<sup>2</sup> Histone tails are subjected to many kinds of chemical modifications, such as methylation, acetylation, phosphorylation, ubiquitination and ribosylation,<sup>3</sup> which can lead to diverse effects on chromatin structure and gene activity. For example, acetylation of lysine residues usually correlates with chromatin accessibility and transcriptional activation, whereby lysine methylation has either activating or repressive effects on gene regulation.<sup>3</sup>

During the last several years, there has been an increased interest in the epigenetic origins of psychiatric diseases.<sup>4–7</sup> Of the diverse epigenetic machinery associated with gene regulation, DNA methylation has been the most widely studied in the context of psychiatric disorders. Altered methylation status of CpG sites has been found within the regulatory regions of several candidate genes in subjects with schizophrenia, including *HTR1A*,<sup>8</sup> *HTR2A*,<sup>9</sup> glutamic acid decarboxylase 1 (*GAD1*),<sup>10,11</sup> *REELIN*,<sup>12,13</sup> *COMT*,<sup>14</sup> *DRD2*<sup>15</sup> and *SOX10*.<sup>16</sup> More recently, epigenome-wide profiling has revealed large scale changes in DNA-methylation associated

with major psychosis, some of which involve genes associated with neuronal development as well as genes involved with glutamatergic and GABAergic neurotransmission.<sup>17</sup>

To date, much less is known about alterations in histone modifications in schizophrenia. Previous studies have quantified global levels of histone phosphorylation, acetylation and methylation, at different lysine (K), serine (S) and arginine (R) residues of histones H3 and H4. Overall, no significant differences in the levels of these histone marks were found in the prefrontal cortex of individuals with schizophrenia compared with normal control subjects,<sup>18</sup> however, higher methylation levels of histone H3 at R17 were detected within a subset of affected patients.<sup>18</sup> More recently, decreases in trimethylated H3 at K4 were found specifically at the *GAD1* locus in the prefrontal cortex of patients with schizophrenia compared with control subjects, in correlation with reduced *GAD1* mRNA levels.<sup>11</sup> These data suggest that changes in histone modifications at specific genomic loci, rather than on a global scale, may be occurring in schizophrenia, and identification of such loci may unveil the role of epigenetic regulation of gene expression in schizophrenia.

Given the widespread changes in gene expression that have been associated with psychiatric disorders,<sup>19,20</sup> we investigated the contribution of histone acetylation at specific gene promoters to gene expression regulation in schizophrenia and bipolar disorder. Previous studies have shown that acetylation

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levels of histone H3 at K9 (ac-H3K9) and at K14 (ac-H3K14) are highest at the predicted transcriptional start sites of active genes and are positively correlated with one another, as well as with transcriptional activity across a range of yeast genes.<sup>21</sup> Therefore, we measured histone acetylation at K9 and K14 at the proximal promoter regions of eight selected genes representing diverse functionalities that have been implicated in the pathophysiology of schizophrenia.

We find that histone acetylation at K9 and K14 is associated with gene expression levels for eight schizophrenia-related genes, and that histone acetylation patterns at specific loci show distinct disease and age-related effects in normal subjects and those with schizophrenia and/or bipolar disorder. Importantly, understanding the role of histone acetylation in schizophrenia and bipolar disorder may have relevant therapeutic implications, whereby the use of histone deacetylase (HDAC) inhibitors may be clinically beneficial by means of restoring abnormal histone acetylation patterns and accompanying gene expression deficits in schizophrenia and with aging in normal subjects.

## Materials and methods

**Samples.** This study utilizes postmortem human brain samples ( $n=82$  in total) from two different brain banks: The Harvard Brain Tissue Resource Center (HBT) and the Victorian Brain Bank Network (VBBN) at the Mental Health Research Institute. For the VBBN samples, approval was obtained from both the Ethics Committee of the Victorian Institute of Forensic Medicine and the Mental Health Research and Ethics Committee of Melbourne Health. Cases were split into two groups. The first group consists of brains from the HBT collection: the prefrontal cortex (Brodmann area 10) from  $n=50$  subjects ( $n=18$  normal subjects,  $n=16$  subjects with schizophrenia and  $n=16$  subjects with bipolar disorder). The second group consisted of young subjects from the VBBN collection: the prefrontal cortex (Brodmann area 46) from  $n=16$  subjects ( $n=8$  control,  $n=8$  subjects with schizophrenia; 18–36 years of age) and old subjects from the HBT collection: the prefrontal cortex (Brodmann area 10) from  $n=16$  subjects ( $n=8$  control and  $n=8$  subjects with schizophrenia; 55–92 years of age). Demographic data for individual subjects are shown in Supplementary Table 1. Ascertainment and diagnosis of all subjects were based on the diagnostic and statistical manual of mental disorders (DSM-IV) criteria (American Psychiatric Association 1994). In the case of the VBBN collection, an additional validated instrument, the Diagnostic Instrument for Brain Studies, was used.<sup>22</sup> None of the subjects had a record of treatment with valproic acid, an HDAC inhibitor. For the VBBN subjects, cadavers had been refrigerated within 5 h from death to ensure slowing of any autolysis of the CNS tissue; the recorded postmortem intervals (PMIs) include refrigeration times. For these samples, tissue integrity was assessed by pH, which is now recognized as a better measure of tissue preservation than PMI,<sup>23</sup> and all samples with  $\text{pH}<6.1$  were excluded. For all samples, RNA quality was assessed by Bioanalyzer tracings or gel electrophoresis and spectrophotometric measurements, which showed no evidence for degradation products or protein contamination.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP)-PCR was performed on postmortem human brain samples using an adaptation of a method previously described in detail.<sup>24</sup> Briefly, ~60–100 mg of the prefrontal cortex from human postmortem brain was fixed with 1% of formaldehyde for 15 min at room temperature then homogenized to isolate nuclei. DNA was sonicated in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1 × protease inhibitors cocktail (Roche, Germany)) to ~0.2–0.8 kb in size of DNA fragments. 100  $\mu\text{l}$  of precleared nuclear lysate was diluted with dilution buffer (1% Triton × 100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1 × protease inhibitors cocktail), and incubated with 3  $\mu\text{g}$  of histone ac-H3K9K14 (Upstate, Billerica, MA, USA), 3  $\mu\text{g}$  rabbit control IgG (Cell Signaling Technology, Danvers, MA, USA) or total histone H3 (Abcam, Cambridge, MA, USA) antibodies overnight at 4 °C. 60  $\mu\text{l}$  of Protein A Agarose beads (Millipore, CA, USA) were added and incubated for 2 h to capture the immune complexes. The protein–DNA complexes were washed and eluted in elution buffer (1% SDS and 0.1 M  $\text{NaHCO}_3$ ) at 65 °C for 20 min. The proteins were digested by proteinase K, and the cross-linking reaction was reversed at 65 °C overnight. DNA was purified with phenol/chloroform and ethanol precipitation, and analyzed by real-time PCR analysis.

**Gene expression analysis.** Real-time qPCR analysis was performed using the ABI PRISMs 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) on the recovered DNA from the ChIP experiments using primers directed against the proximal promoter regions of schizophrenia-related genes (Supplementary Table 2), or on cDNA prepared from the same samples using the primers designed in the exonic regions of selected genes (Supplementary Table 2) as described previously.<sup>25,26</sup> The proximal promoter region (~1 kb upstream from transcription start site) of each gene was obtained from UCSC browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>). Primers were designed to generate amplicons of 80–150 nucleotides with similar melting temperatures (64 °C) using Invitrogen's Primer Designer and their specificity for binding to the desired sequences was searched against the NCBI database. We analyzed the ChIP-qPCR data using the Percent Input Method (Invitrogen, Carlsbad, CA, USA). Briefly, the amplification efficiency (AE) of the qPCR reaction for each primer pair and sample was determined by the input DNA using the formula  $\text{AE} = 10^{-(1/\text{slope})}$ . The threshold cycle (Ct) value of Input, which is 1% of the immunoprecipitation (IP) reaction was adjusted to 100% by subtracting 6.644 cycles ( $\log_2$  of 100), and then the percent input was calculated by the formula  $100 \times \text{AE}^{(\text{adjusted input Ct} - \text{IP Ct})}$ . For gene expression, the amount of cDNA in each sample was calculated using SDS2.1 software (Applied Biosystems, Foster City, CA, USA) by the comparative Ct method and expressed as  $2^{\text{exp}(\text{Ct})}$  using beta-2-microglobulin (*B2M*) as an internal control.

Differences in the levels of microarray expression values, from our previously published microarray dataset,<sup>27</sup> were calculated by ANOVA, performed using the National Institutes of Aging Array Tools,<sup>28</sup> with the FDR controlled at a default setting of 0.1, according to Benjamini and Hochberg.<sup>29</sup>

**Statistics.** The demographic characteristics for each cohort were compared using Student's *t*-tests to verify matching for age, sex ratio, PMIs and tissue pH. The PMI between the two brain banks were significantly different ( $46.13 \pm 2.43$  vs  $21.3 \pm 1.74$  h;  $P < 0.0001$ ) (Supplementary Table 1), which could be due, in part, to different criteria for defining PMI (see above). Importantly, the PMIs did not show any significant difference when compared by cohort. The gene expression and ChIP-qPCR data values were analyzed for normal distribution using the Kolmogorov–Smirnov method, which confirmed that the data were normally distributed for all subjects. Given that the data were normally distributed, each data set was interrogated for outliers using the Grubbs' test, which resulted in the removal of ChIP-qPCR values from one of the old control subjects from the HBT collection. For assessment of disease effects of the qPCR and ChIP-qPCR data among the control, schizophrenia and bipolar disorder cohorts, significant differences were determined by one-way ANOVA and Student's unpaired *t*-tests (GraphPad 5.0; San Diego, CA, USA). The effects of demographic and brain collection parameters (age, sex, PMI and tissue pH) on the disease effect for all data were assessed by ANCOVA (XLSTAT software, Addinsoft, New York, NY, USA). From this analysis, age showed a significant contribution to data variation in gene expression and/or ChIP data for all genes tested (Supplementary Table 3). Tissue pH showed a significant effect on gene expression only for translocase of outer mitochondrial membrane 70 homolog A (*TOMM70A*) in the schizophrenia comparison and for *GAD1* in the bipolar disorder comparison, but no significant effects of pH on ChIP data were observed for any genes (Supplementary Table 3). Pearson's product moment correlation analysis was further performed for the ac-H3K9K14 levels (as percentage input) and the *B2M*-normalized expression values against the age of the subjects, and for ac-H3K9K14 levels against the gene expression values.

## Results

**Disease effects on gene expression.** We selected eight diverse "schizophrenia-related" genes (Table 1) for this study based on the following criteria: (1) genes showing

differential expression in schizophrenia and/or bipolar disorder from published microarray studies;<sup>30,31</sup> (2) genes showing CNS cell type-specific expression patterns based on comparison with previous transcriptome studies performed on isolated astrocytes, neurons and oligodendrocytes;<sup>32</sup> (3) genes representing different functions/pathways related to schizophrenia based on review of the literature.<sup>20,33–35</sup> Additionally, these selected genes are representative of different gene co-expression networks, based on our previous studies, which identified over 20 gene co-expression modules in the prefrontal cortex from subjects with schizophrenia and bipolar disorder.<sup>36</sup> We first tested for expression differences for five neuronally expressed genes, GABAergic neurotransmission: *GAD1*; mitochondrial function/import: *TOMM70A*; neurotransmitter receptor signaling: serotonin 5-hydroxytryptamine receptor 2C (*HTR2C*) and regulator of G protein signaling 4 (*RGS4*); signal transduction: protein phosphatase, Mg<sup>2+</sup>/Mn<sup>2+</sup> dependent, 1E (*PPM1E*) in the postmortem prefrontal cortex (Brodmann area 10) from a cohort of subjects with schizophrenia and bipolar disorder from the Harvard Tissue Resource Center (group 1; Supplementary Table 1). Real-time qPCR analysis revealed decreased expression of *HTR2C*, *TOMM70A*, *RGS4* and *PPM1E* in subjects with schizophrenia and bipolar disorder compared with matched controls, and a decrease expression in *GAD1* only in subjects with schizophrenia (Figure 1).

**Histone acetylation at gene promoters.** To test for correlations between gene expression activity and promoter histone acetylation, we performed ChIP-qPCR assays on cortical samples from these same subjects, using an antibody directed against ac-H3K9K14, followed by real-time qPCR analysis using primers directed against the proximal promoter regions of these genes. Linear regression analysis revealed that gene expression levels were correlated with promoter-associated ac-H3K9K14 levels for *GAD1*, *TOMM70A*, *HTR2C* and *PP1ME*, but not for *RGS4*, in all 50 subjects (psychiatric cases and controls) (Figure 2). Ac-H3K9K14 levels were also compared among all psychiatric cases and controls, and no significant differences were detected (data not shown).

**Table 1** Summary of genes selected for this study

Gene ID	Gene description	Cell type association <sup>a</sup>	Function	SCZ <sup>b</sup>	SCZ <sup>c</sup>	BP <sup>c</sup>
<i>GAD1</i>	Glutamic acid decarboxylase 1	Neuron	GABAergic neurotransmission	↓	↓	↓
<i>HTR2C</i>	5-hydroxytryptamine (serotonin) receptor 2C	Neuron	Neurotransmitter receptor signaling	↓	↑	↓
<i>RGS4</i>	Regulator of G-protein signaling 4	Neuron		↓	—	↓
<i>TOMM70A</i>	Translocase of outer mitochondrial membrane 70 homolog A	Neuron	Mitochondrial function/import	↓	↓	↓
<i>PPM1E</i>	Protein phosphatase, Mg <sup>2+</sup> /Mn <sup>2+</sup> dependent, 1E	Neuron	Signal transduction	↓	↓	↑
<i>MBP</i>	Myelin basic protein	Oligodendrocyte	Myelination-associated	↓	↓	↓
<i>UGT8</i>	UDP glycosyltransferase 8	Oligodendrocyte	White matter function	↓		↓
<i>H1FNT</i>	H1 histone family, member N	Ubiquitous	Chromatin-related	↓	NP	NP

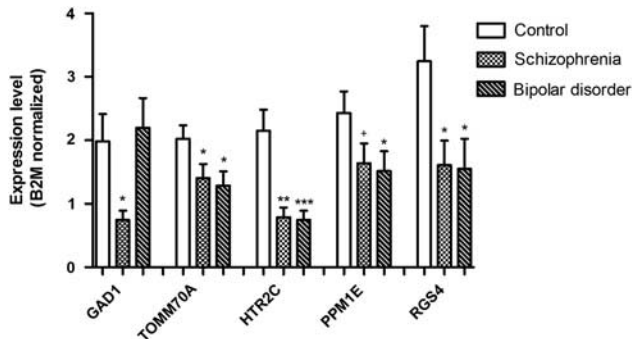
The arrows designate the direction of the significant gene expression change in schizophrenia (SCZ) or bipolar disorder (BP) from previous microarray studies. "—" designates no significant change in expression; NP, indicates not present in the dataset.

<sup>a</sup>Cell-type specific expression was determined by comparing with transcriptome datasets for astrocytes, neurons and oligodendrocytes, from Cahoy *et al.*<sup>32</sup>

<sup>b</sup>From Narayan *et al.*<sup>30</sup>

<sup>c</sup>From the Stanley Medical Research Database, <https://www.stanleygenomics.org/> and Kim and Webster.<sup>31</sup>

**Age effects on gene activity.** ANCOVA of the demographic and sample variables with the experimental data values revealed that age significantly contributed to the variation in gene expression and/or ac-H3K9K14 levels among disease cohorts for all genes tested. Therefore, we further highlighted the effects of age on ac-H3K9K14 levels by performing Pearson's linear correlation analyses. Promoter associated ac-H3K9K14 levels were significantly negatively associated with age for *GAD1*, *RGS4*, *PPM1E*,

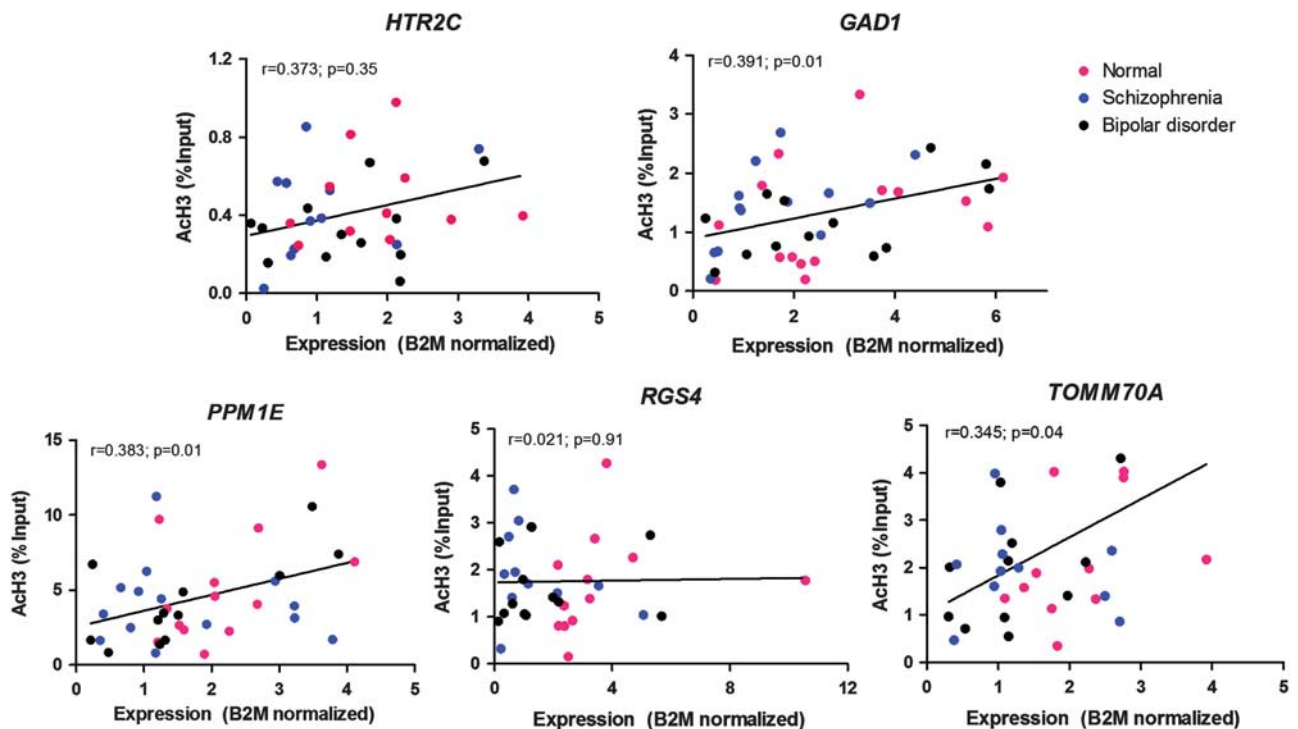


**Figure 1** Real-time PCR analysis for the indicated genes in subjects with schizophrenia, bipolar disorder and non-psychiatric controls. Real-time qPCR assays were performed as described in the materials and methods on postmortem Brodmann area (BA) 10 from subjects with schizophrenia, bipolar disorder and matched controls (group 1;  $n = 50$  total). Data shown are gene expression values normalized by the housekeeping gene, *B2M*. Asterisks denote significant differences in expression as determined by one-way ANOVA followed by Student's *t*-test: \* $P < 0.05$ ; +  $P < 0.08$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

*HTR2C* and *TOMM70A* in normal subjects (Table 2). Importantly, there was also an effect of age on levels of gene expression in normal subjects for all genes except *HTR2C* (Table 2). The same effects of age on histone acetylation and gene expression levels were observed for *GAD1*, *TOMM70A* and *PPM1E* in subjects with bipolar disorder (Table 2); however, in marked contrast, and with the exception of *GAD1*, there was no effect of age on histone acetylation levels in the prefrontal cortex from subjects with schizophrenia (Table 2).

### Histone acetylation differences in young vs old subjects.

To further explore the age effect on histone acetylation, we measured ac-H3K9K14 levels at the promoter regions of three of the neuronal genes, *GAD1*, *TOMM70A* and *HTR2C*, plus two oligodendrocyte-expressing genes, myelin basic protein (*MBP*) and UDP glycosyltransferase 8 (*UGT8*), and a ubiquitously-expressed gene, H1 histone family, member N (*H1FNT*) in the postmortem prefrontal cortex from a second cohort of subjects (group 2; Supplementary Table 1). This cohort was comprised of young subjects (18–36 years of age) and old subjects (55–92 years of age) with schizophrenia and age-matched controls ( $n = 32$  in total). Consistent with the results from subjects in group 1 above, Pearson's correlation analysis of ac-H3K9K14 levels against age revealed strong negative correlation with age in normal subjects (Table 2; Figure 3), but not in subjects with schizophrenia, despite measuring levels in a cohort of subjects with a greater age range (18–91 years). Examining our previous microarray data



**Figure 2** Correlation between gene expression levels and acetylation of histone H3 at K9 and K14. ChIP-qPCR assays were performed on postmortem BA10 from the same subjects as in Figure 1, measuring ac-H3K9K14 levels at the promoter regions of the indicated genes (as designated by UniGene IDs). Rabbit IgG was used as a negative control for the pull-down. Histone acetylation is presented as percentage input DNA. Gene expression levels were determined by real-time qPCR, from Figure 1. Each point represents one subject. Pink, control subjects; blue, subjects with schizophrenia; black, subjects with bipolar disorder. Pearson's correlation ( $r$ ) values are indicated within each graph.

**Table 2** Correlation analysis (Pearson's *r* values) of ChIP-qPCR and gene expression data against age

Gene ID	ChIP-qPCR data						Gene Expression data:					
	Normal		Schizophrenia		Bipolar disorder		Normal		Schizophrenia		Bipolar disorder	
	Age, <i>r</i> -value	P-value	Age, <i>r</i> -value	P-value	Age, <i>r</i> -value	P-value	Age, <i>r</i> -value	P-value	Age, <i>r</i> -value	P-value	Age, <i>r</i> -value	P-value
<i>RGS4</i>	-0.658	0.020	-0.079	0.788	-0.241	0.406	-0.503	0.056	-0.307	0.285	-0.879	< 0.0001
<i>PPM1E</i>	-0.731	0.007	0.306	0.288	-0.681	0.007	-0.579	0.023	-0.350	0.221	-0.809	0.001
<i>GAD1-1</i>	-0.557	0.048	-0.623	0.017	-0.789	0.001	-0.527	0.043	-0.460	0.098	-0.737	0.003
<i>GAD1-2</i>	-0.581	0.023	-0.167	0.550	ND	ND	-0.504	0.005	0.128	0.500	ND	ND
<i>HTR2C-1</i>	-0.602	0.023	-0.008	0.978	-0.448	0.108	-0.078	0.781	-0.252	0.385	-0.812	0.000
<i>HTR2C-2</i>	-0.429	0.111	-0.245	0.361	ND	ND	-0.499	0.006	0.244	0.194	ND	ND
<i>TOMM70A-1</i>	-0.756	0.002	-0.132	0.652	-0.604	0.022	-0.491	0.063	-0.384	0.175	-0.829	0.000
<i>TOMM70A-2</i>	-0.261	0.341	0.266	0.320	ND	ND	-0.529	0.003	0.271	0.148	ND	ND
<i>MBP</i>	-0.682	0.005	0.416	0.109	ND	ND	-0.505	0.005	0.590	0.001	ND	ND
<i>UGT8</i>	-0.376	0.166	0.138	0.610	ND	ND	0.153	0.428	0.527	0.003	ND	ND
<i>H1FNT</i>	-0.480	0.082	-0.300	0.260	ND	ND	-0.053	0.786	0.252	0.179	ND	ND

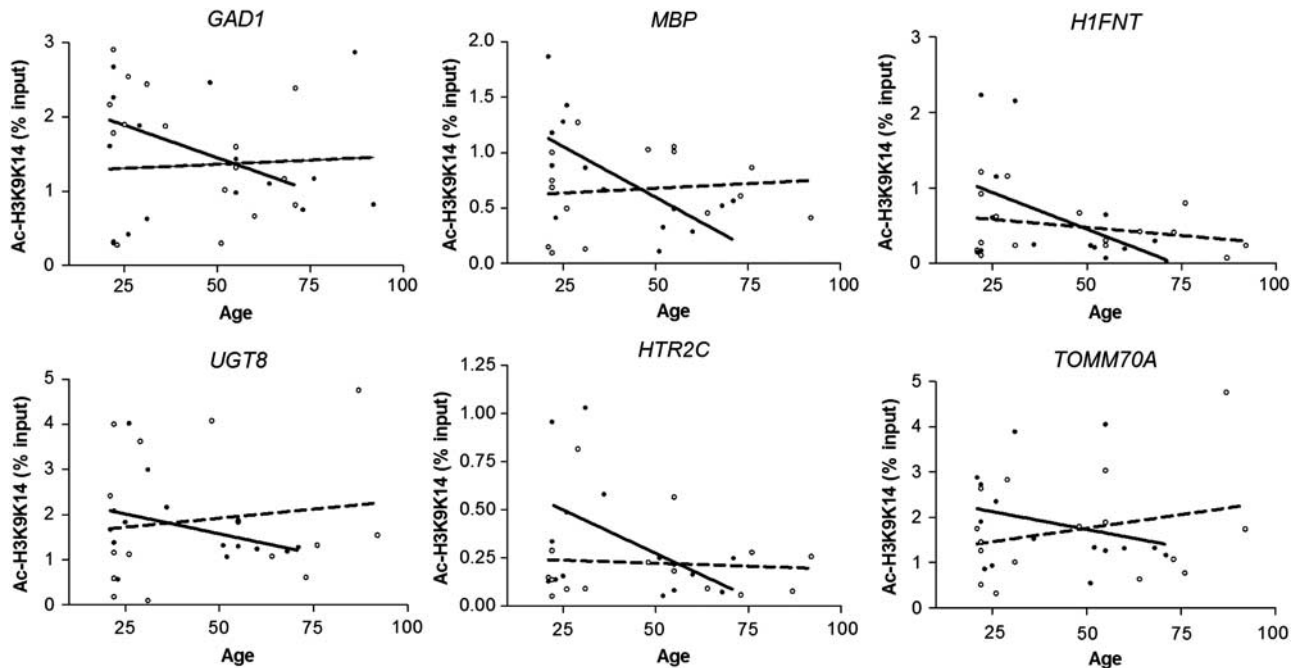
ND, not determined. Bold font indicates a statistically significant correlation. The values for *GAD1-2*, *HTR2C-2*, *TOMM70A-2*, *MBP*, *UGT8* and *H1FNT* are from subjects in group 2, with the gene expression data reflecting the microarray correlations shown in Supplementary Figure 1.

generated from the prefrontal cortex from case and control subjects ranging in age from 18–81 years (GEO accession #GSE21138),<sup>30,37</sup> which consisted of one-half of the same young subjects as used in this study, we similarly find that the expression levels of these genes decreases with age (Supplementary Figure 1).

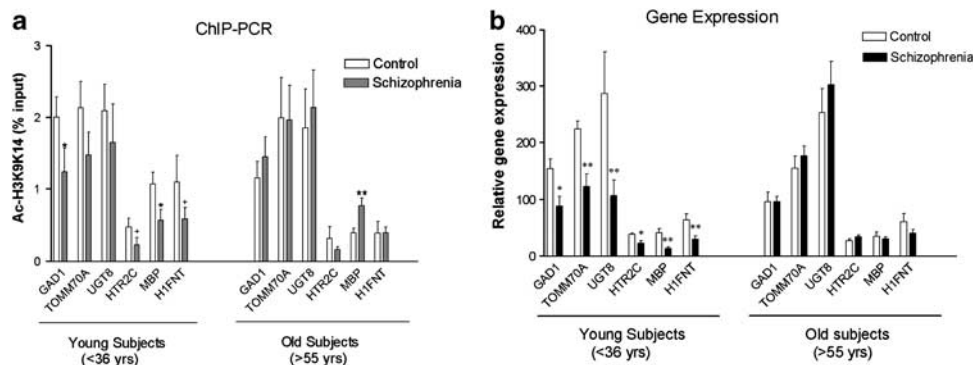
From the linear plots shown in Figure 3, it is apparent that for several genes, ac-H3K9K14 levels do not decrease with advanced age because levels are low in subjects with schizophrenia at an early age, and remain low throughout aging. Hence, we performed group-wise comparisons of the ChIP-qPCR data according to age. This analysis revealed hypoacetylation of H3K9K14, the promoter regions of *GAD1*, *UGT8*, *HTR2C* and *H1FNT* in young subjects with schizophrenia compared with matched controls (Figure 4A). In contrast, only *HTR2C* showed a decrease in ac-H3K9K14 levels in old subjects compared with matched controls, although this did not reach significance ( $P=0.071$ ) (Figure 4A). Interestingly, ac-H3K9K14 levels at the *MBP* promoter were significantly increased in old subjects compared with matched controls (Figure 4A).

Again, we examined whether the expression of these genes from our previous microarray studies (GEO accession #GSE21138), which were performed on the prefrontal cortex from one-half of the same young subjects,<sup>30</sup> was associated with ac-H3K9K14 levels. We also examined gene expression from subjects at late stage, although they were different than those used for ChIP-qPCR in the current study. Consistent with the observed hypoacetylation of H3K9K14 in young subjects with schizophrenia, we find that the expression of *GAD1*, *TOMM70A*, *HTR2C*, *MBP*, *UGT8* and *H1FNT* are decreased in young-aged subjects compared with age-matched controls (Figure 4B). Old-aged subjects with schizophrenia compared with their age-matched controls showed no significant changes in expression of these genes, consistent with the lack of difference in ac-H3K9K14 levels in older subjects (Figure 4B).

**HDAC inhibitors and schizophrenia candidate genes.** The role of histone acetylation on gene regulation is especially pertinent because of the therapeutic potential of HDAC inhibitors, which have gained considerable attention as a relevant therapeutic option for many neurological disorders<sup>38,39</sup> including psychiatric disorders.<sup>5,40</sup> Our previous studies have focused on novel, HDAC1/3-selective HDAC inhibitors, including HDACi **4b**.<sup>25,41</sup> To gain insight into the potential usefulness of novel selective HDAC inhibitors, such as HDACi **4b**, we screened our previously published microarray data from HDACi **4b**-treated mouse brain<sup>25</sup>(GEO accession #GSE26317) for schizophrenia candidate genes as determined from the SZGene database (www.szgene.org). We found that HDACi **4b** treatment altered the expression of several candidate genes for schizophrenia; from the top 45 candidate genes listed on the SZGene database, 17 genes, including *RGS4* and *MBP*, two genes from this study, were found to be altered in the mouse brain by **4b** (Figure 5). This is a significant overrepresentation of **4b**-regulated candidate genes than would be expected by chance (Fisher's exact test;  $P=0.02$ ). For most of these genes, which have been shown to



**Figure 3** Ac-H3K9K14 levels as a function of age in control subjects and those with schizophrenia. ChIP-qPCR assays were performed on postmortem BA46 from control subjects (closed circles, solid line) and those with schizophrenia (open circles, dashed line) representing a wide age range (group 2;  $n = 32$  subjects in total). Pearson's ( $r$ ) values are shown in Table 2.



**Figure 4** Histone H3K9K14 is hypoacetylated at the promoter regions of genes in young subjects with schizophrenia and associated with decreased gene expression levels. (a) ChIP-qPCR assays were performed on young and old subjects with schizophrenia and age-matched controls (group 2;  $n = 32$  subjects in total), measuring ac-H3K9K14 levels at the promoter regions of the indicated genes (UniGene IDs). Asterisks denote significant differences in ac-H3K9K14 levels, as determined by Student's  $t$ -test: \*,  $P < 0.05$ , +,  $P < 0.08$ . (b) Significant differences in microarray expression values were determined by ANOVA as described in Materials and methods; \*,  $P < 0.05$ , \*\*,  $P < 0.01$ .

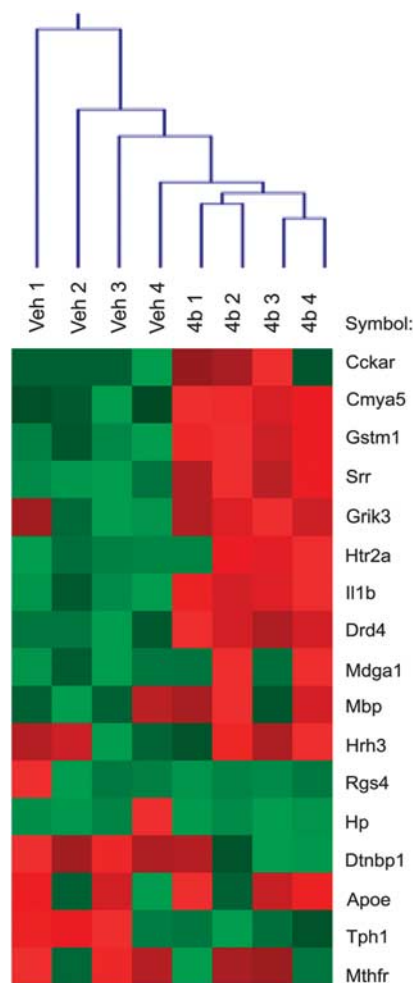
be decreased in expression in schizophrenia, HDACi **4b** caused an elevation of gene expression (Figure 5).

## Discussion

In this study, we measured gene expression and promoter-associated histone ac-H3K9K14 levels in human postmortem cortex for eight genes representing diverse functions associated with schizophrenia in order to assess the role of epigenetic mechanisms on gene activity. In particular, we included assessment of *GAD1*, which encodes the 67-kDa glutamate decarboxylase GABA synthesis enzyme. Deficits in the expression of *GAD1* are considered to be among the most

frequently replicated findings in schizophrenia postmortem brain<sup>42,43</sup>(reviewed in ref. 44). The major findings from this study are: (1) histone ac-H3K9K14 levels are correlated with gene expression levels for several schizophrenia-related genes, including *GAD1*; (2) age is strongly negatively associated with promoter-associated histone acetylation levels in normal subjects and those with bipolar disorder, but not schizophrenia and (3) histone H3K9K14 levels are hypoacetylated at the promoter regions of important genes in young subjects with schizophrenia.

Epigenetic mechanisms of gene regulation involve both DNA methylation and an array of posttranslational modifications of histone proteins.<sup>1</sup> Although DNA methylation has been more



**Figure 5** Heatmap depiction of the 17 schizophrenia candidate genes found to be significantly regulated by HDACi **4b** treatment in the mouse brain. Official UniGene symbols are shown for each gene. Each colored pixel represents an individual gene expression value. Relative decreases in gene expression are indicated by green and increases in expression by red. Two-dimensional hierarchical clustering of the samples is shown along the top.

widely studied in the context of psychiatric disorders, in this study we focused on histone acetylation at two specific lysine residues, K9 and K14. We demonstrated correlation of ac-H3K9K14 levels with expression levels of selected genes in the postmortem human prefrontal cortex. These findings are consistent with previous studies showing that acetylation of histone H3 at K9 and K14 are positively correlated with one another and associated with transcriptional activity across a majority of yeast genes.<sup>21</sup> Epigenetic studies in yeast have also found that ac-H3K9 and ac-H3K14 levels are correlated with levels of trimethylated H3K4 (H3K4me3), another epigenetic mark associated with active gene transcription and abundant at the transcription start sites of genes. Genome-wide maps of histone H3K4me3 have been previously identified in the human prefrontal cortex<sup>45</sup> and these data are freely available on the UCSC web browser (<http://genome.ucsc.edu>). Again, consistent with the findings from yeast, we found that the promoter loci bearing ac-H3K9K14

marks for *GAD1*, *RGS4*, *HTR2C*, *PPM1E* and *UGT8* also harbor H3K4me3 marks in the human prefrontal cortex. An example of this overlap is shown for *UGT8* in Supplementary Figure 2.

The second major finding from this study is that age is strongly negatively correlated with promoter-associated histone acetylation levels in normal subjects. Normal aging is known to be accompanied by genomic instability and changes in gene expression,<sup>46</sup> and evidence now suggests that epigenetic factors are a major cause of these age-related changes in mice and humans.<sup>47,48</sup> Most epigenetic studies of the aging brain have focused on DNA methylation where positive correlations between DNA methylation and chronological age have been demonstrated for selected genes, such as *GAD1*,<sup>49</sup> as well as genome wide.<sup>47–49</sup> However, information on how histone modifications change with age is more limited.<sup>50</sup> Here, we have shown that histone acetylation levels are negatively correlated with age at several gene promoters, including *GAD1*, *RGS4*, *HTR2C*, *PPM1E* and *MBP* and that the expression levels of these genes are similarly negatively correlated with age. The gene expression data are consistent with a previous study showing that the expression of several schizophrenia candidate genes, including *RGS4* and *GAD1*, decreases with age in the postmortem prefrontal cortex from normal individuals.<sup>51</sup> We also found that promoter-associated histone acetylation levels were significantly negatively correlated with age in subjects with bipolar disorder, but not schizophrenia, indicating disease-specific effects of epigenetic gene regulation. We further show that these effects are not unique to cell type-specific gene promoters, as acetylation changes were detected in both neuron- and glia-expressed genes.

The mechanism of the reduced site-specific acetylation with age is unclear; however, a few possibilities could be considered. Altered acetylation levels of histones could occur by changes in the activities of HDAC enzymes. For example, a decrease in HDAC activity has been observed in normal rat liver with increasing age.<sup>52</sup> Another possibility is that acetylated histones are replaced by newly synthesized unmodified ones. Although it has been shown that histone turnover in the brain is slow,<sup>53</sup> it could be potentially substantial with aging. It is also possible that some histone modifications decay with time at the promoters of genes that are not active in aged individuals. The lack of an age effect on histone acetylation observed in the brains of subjects with schizophrenia could be due to abnormalities in any of the above-mentioned mechanisms.

Thirdly, we demonstrated that histone H3 is hypoacetylated in young subjects with schizophrenia when compared with age-matched controls. Such hypoacetylation of histone proteins could be reversed by the actions of HDAC inhibitors, thereby improving the associated gene expression deficits. To date, 18 human HDAC subtypes have been identified, which can be divided into four main groups, classes I–IV.<sup>38</sup> Valproic acid, an inhibitor of class I HDACs,<sup>54</sup> has a long and established history of efficacy in the treatment of bipolar disorder. Reports have further shown that typical and atypical antipsychotics are more potent, more efficacious and less toxic if they are co-administered with valproic acid,<sup>55–57</sup> although, some studies did not report such benefit.<sup>58–60</sup>

Nonetheless, the beneficial effects of valproic acid that were observed in schizophrenia suggest that more potent and/or more selective HDAC inhibitors may represent a new opportunity for pharmacological interventions for this disorder. Consistent with this view, previous studies have shown that another class I HDAC inhibitor, MS-275, potently activates *GAD1* gene expression in NT2 cells accompanied by decreased *GAD1* promoter methylation,<sup>10</sup> and in the current study, we have shown that HDACi **4b** altered the levels of 17 schizophrenia candidate genes in the mouse brain (see Figure 5). Consistent with these findings, previous studies have demonstrated that inhibition of the class I HDACs, HDAC2 and HDAC3, enhances cognition and memory function in rodents.<sup>61,62</sup>

One final note is that the similarity between histone hypoacetylation observed with normal aging and in young subjects with schizophrenia is consistent with emerging data showing phenotypic overlap between normal aging and early-stage schizophrenia. Normal aging has been linked to alterations in white matter density and volume, gray matter volume decline, cognitive dysfunction, shortened telomeres, microglia activation and psychotic symptoms,<sup>63–66</sup> which also characterize schizophrenia at first episode or recent onset.<sup>67–71</sup> Furthermore, our own previous studies have demonstrated that normal human aging and early-stage schizophrenia share common molecular phenotypes.<sup>37</sup>

In summary, our data demonstrate that gene expression changes associated with psychiatric disease and aging result from epigenetic mechanisms of gene regulation involving histone acetylation. These findings provide a relevant basis for the use of HDAC inhibitors as therapeutic treatment for schizophrenia, particularly in young subjects (that is, <36 years of age), whereby the use of HDAC inhibitors may be therapeutically beneficial by means of restoring abnormal histone acetylation patterns and accompanying gene expression deficits in schizophrenia leading to improved clinical symptoms. Similarly, HDAC inhibitors may also be useful for treatment of age-related phenotypes, such as psychosis and cognitive decline, which are similar to those typically observed in subjects with schizophrenia.

### Conflict of interest

The authors declare no conflict of interest.

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