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Histone Methylation at H3K9; Evidence for a Restrictive Epigenome in Schizophrenia

Kayla A. Chase¹, David P. Gavin¹, Alessandro Guidotti¹, and Rajiv P. Sharma^{1,*}

¹The Psychiatric Institute, University of Illinois at Chicago, 1601 W. Taylor St., Chicago, IL 60612

Abstract

OBJECTIVE—Epigenetic changes are stable and long-lasting chromatin modifications that regulate genomewide and local gene activity. The addition of two methyl groups to the 9th lysine of histone 3 (H3K9me2) by histone methyltransferases (HMT) leads to a restrictive chromatin state, and thus reduced levels of gene transcription. Given the numerous reports of transcriptional down-regulation of candidate genes in schizophrenia, we tested the hypothesis that this illness can be characterized by a restrictive epigenome.

METHODS—We obtained parietal cortical samples from the Stanley Foundation Neuropathology Consortium and lymphocyte samples from the University of Illinois at Chicago (UIC). In both tissues we measured mRNA expression of HMTs GLP, SETDB1 and G9a via real-time RT-PCR and H3K9me2 levels via western blot. Clinical rating scales were obtained from the UIC cohort.

RESULTS—A diagnosis of schizophrenia is a significant predictor for increased GLP, SETDB1 mRNA expression and H3K9me2 levels in both postmortem brain and lymphocyte samples. G9a mRNA is significantly increased in the UIC lymphocyte samples as well. Increased HMT mRNA expression is associated with worsening of specific symptoms, longer durations of illness and a family history of schizophrenia.

CONCLUSIONS—These data support the hypothesis of a restrictive epigenome in schizophrenia, and may associate with symptoms that are notoriously treatment resistant. The histone methyltransferases measured here are potential future therapeutic targets for small molecule pharmacology, and better patient prognosis.

Keywords

Epigenetics; Schizophrenia; Postmortem Brain; Lymphocyte; Chromatin; Histone Methylation

*Corresponding author: Rajiv P. Sharma MD, Professor, Psychiatric Institute, 1601 West Taylor Street, Chicago, IL 60612, Tel.: (312) 413-4508; Fax: (312) 413-4503. rsharma@psych.uic.edu.

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Conflict of Interest

All authors declare that they have no conflict of interest.

Contributors

Kayla A. Chase (KAC), David P. Gavin (DPG), Alessandro Guidotti (AG), Rajiv P. Sharma (RPS).

Conception of study – KAC, DPG and RPS

Data collection – KAC and DPG

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1. Introduction

Schizophrenia is conceptualized as a disorder of gene transcription and regulation. Consequently, chromatin is the ideal scaffold to examine this manifested pathophysiology of schizophrenia, as it constitutes the interface between the underlying genetic code and its surrounding biochemical environment. Through post-transcriptional modifications of histone proteins, gene expression can be either transcriptionally active in a 'euchromatic' environment, temporarily quieted in 'facultative heterochromatin,' or completely silenced in 'constitutive heterochromatin' (Zhang and Reinberg 2001). Post-translational modifications to lysine 9 of the H3 protein (H3K9) are uniquely able to reflect these three levels of transcriptional regulation. H3K9 modifications located in the promoter regions of actively transcribed genes are often acetylated (H3K9acetyl). Conversely, quieted transcription in gene-rich areas of the genome are often associated with mono- or dimethyl H3K9 (H3K9me2), while completely silenced areas of the genome are associated with trimethylated H3K9 (H3K9me3). In particular, the formation of H3K9me2 is catalyzed by histone methyltransferases (HMTs), including Eu-HMTase2 (G9a), Eu-HMTase1 (GLP), and SETDB1 (Krishnan et al. 2011). The different degrees of lysine methylation are possible due to the cooperation of these HMTs, which are able to form large heteromeric complexes (Fritsch et al. 2010).

H3K9 methylation has not been extensively studied in the brain, and until recently the regulation and role of the enzymes responsible for its formation were not known. Postnatal, neuronal-specific GLP/G9a knockdown produces a significant decrease in global H3K9me2 levels and inappropriate gene expression, leading to deficits in learning, reduction in exploratory behaviors and motivation in mice (Schaefer et al. 2009; Shinkai and Tachibana 2011; Tachibana et al. 2005; Tzeng et al. 2007). In humans, deletions or loss-of-function mutations of G9a results in Kleefstra Syndrome, characterized by a severe learning disability and developmental delay (Nillesen et al. 2011; Kleefstra et al. 2005). In humans, increased SETDB1 mRNA expression and resultant elevated H3K9me3 levels have been documented in Huntington Disease (HD) (Ryu et al. 2006; Fox et al. 2004).

A hallmark of schizophrenia is aberrant gene regulation, with the vast majority of studies reporting a down-regulation of gene transcription, suggesting that the epigenome of patients with schizophrenia is restrictive (Akbarian et al. 1995; Guidotti et al. 2000; Fatemi et al. 2005; Impagnatiello et al. 1998; Jindal et al. 2010). Postmortem brain studies indicate a reduction of an open histone modification, H3K4me3, and elevated expression of the histone deacetylase HDAC1 mRNA expression (Cheung et al. 2010; Sharma et al. 2008). The use of peripheral blood mononuclear cells as a reflection of overall chromatin state or at particular gene promoters has been successfully implemented in clinical studies of subjects afflicted depression, alcoholism, and schizophrenia. Peripheral blood cell studies have indicated that schizophrenia is associated with an abnormally condensed chromatin structure; (Issidorides et al. 1975; Kosower et al. 1995) specifically increased restrictive H3K9me2 and reduced H3K9 acetylation (Gavin et al. 2009b). Additionally, H3K9 acetylation in schizophrenia patients is less responsive to in vivo treatment with HDAC inhibitors when compared to both patients with bipolar disorder and nonpsychiatric controls (Sharma et al. 2006; Gavin et al. 2008). Finally, a correlation exists between age of onset of psychiatric symptoms of schizophrenia and baseline levels of H3K9me2 (Gavin et al. 2009b). It is the hypothesis of this paper that schizophrenia can be characterized by a restrictive epigenome, which is observable in both brain and peripheral blood, and has specific and observable effects on psychopathology. We have focused on levels of H3K9me2, indicative of facultative heterochromatin, and the enzymes that catalyze this modification, in patients with schizophrenia to examine their role in this illness.

2. Methods

2.1. Patient Information

2.1.1. Postmortem Brain samples—The Stanley Foundation Neuropathology Consortium (SFNC) (Bethesda, USA) generously allowed use of fresh-frozen parietal cortex postmortem tissue for this study. Patient demographic and clinical characteristics, methods of tissue harvest, preparation, and storage, have been previously described in detail elsewhere (Torrey et al. 2000), and selected data are presented in Table 1.

2.1.2. Lymphocyte Samples—Subjects were recruited from the University of Illinois at Chicago medical center, after receiving approval from the UIC Institutional Review Board. All subjects provided written informed consent before participating in any study procedures. Healthy individuals with no Axis I disorder (as assessed by a SCID interview), and no known first-degree familial history of psychosis were recruited and matched to patient groups on age and sex. Patient demographic characteristics are presented in Table 2.

2.2. Lymphocyte Cell Model

A blood sample was obtained by sterile venipuncture and collected in 0.5M EDTA, pH 8.0. Blood was then diluted 1:1 with Hanks Balanced Salt Solution (HBSS without calcium; GIBCO). Diluted blood (blood + HBSS) was carefully layered over Ficoll-Paque®, and centrifuged at 1,800RPM for twenty minutes at room temperature. The resulting buffy interface was collected, diluted with HBSS, and pelleted at 2,000RPM for ten minutes at 10°C. (Jayaraman et al. 1999; Gavin et al. 2009a)

2.3. mRNA Extraction

Total RNA from all samples (postmortem brain and lymphocyte) were isolated using TRIZOL reagent (Life Technologies, Grand Island, NY). mRNA samples were further processed for DNA removal using Ambion DNase (Ambion, Austin, TX), followed by RNA purification using Qiagen RNeasy minikit (Qiagen, Valencia, CA), and RNA integrity (RIN) assessment using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

2.4. Real Time RT-PCR Quantification

In all samples (postmortem brain and lymphocyte), total RNA was used to prepare cDNA via the Applied Biosystems High Capacity Archive Kit (#4368813). For detection and measurement of expression, Fermentas Maxima SYBR Green/ROX qPCR Master Mix (#K0222) was used. PCR mixtures were run on a Stratagene Mx3005P™ QPCR System, cycle threshold (C_T) value was used for relative quantification of target gene expression, and all values were normalized to three housekeeping genes, GAPDH, TFRC and β -Actin, using a geometric mean (Vandesompele et al. 2002). Primer design is described in detail elsewhere, (Chase and Sharma 2012) and primer sequences are listed in the supplemental table. For the RNA extracted from postmortem brain tissue, pH and RNA Integrity (RIN) scores were also examined. These values were used as an internal measure of RNA quality control to determine the reliability of gene expression data. These values demonstrated no significant interactions with any of the biological measures examined in this paper.

2.5. Immunoblotting

Postmortem brain samples were extracted using the Guanidinium thiocyanate/-phenol-chloroform extraction method (Life Technologies). An equal concentration of protein was boiled in Laemmli buffer and loaded onto 10–20% Tris-glycine gel (Invitrogen# EC61355) in 1× running buffer (0.125 M TRIS base, 0.95 M glycine, 0.5% SDS). Proteins were transferred to a Nitrocellulose membrane (0.45µm; Invitrogen# LC2001) in transfer buffer

(20% methanol, 0.05% SDS, glycine, TRIS base). The membrane was treated with H3K9me2 mouse monoclonal antibody, at a concentration of 1:1000 for 48h (Abcam #Ab1220), and later a secondary antibody (1:2000 concentration; two hours; Amersham Biosciences# NA934V). Membranes were further processed utilizing α -actin (1:4000; one hour) for loading/transfer control and a secondary antibody (1:2000; two hours; Amersham Biosciences# NA931V). All membranes were developed with chemiluminescence methodology using ECL Plus (Amersham Biosciences# W319851) after incubation with the secondary antibody.

2.6. Clinical Measures for Harvested Lymphocytes

Clinical measures included: age of illness onset, lifetime antipsychotic treatment and duration of illness, age of participant, family history of mental illness, and gender. Total lifetime antipsychotic use was carefully documented, and patients in their first episode of illness with less than 16 weeks of total lifetime antipsychotic treatment were considered as first episode psychosis subjects (Rosen et al. 2012). The Positive and Negative Syndrome Scale (PANSS) was administered to clinical participants from whom lymphocytes were collected. The PANSS is a clinician administered rating questionnaire assessing symptom severity in patients with schizophrenia, with ratings ranging from 1 (absent) to 7 (extreme). The PANSS is separated into three smaller modules; a positive scale, a negative scale and a general psychopathology scale, and symptom severity is assessed through summation of Likert scores (Kay et al. 1987).

2.7. Data Analysis

SPSS (version 15.0 for Windows) was used for all statistical analyses. Analysis of all mRNA data was conducted on C_T levels of the gene of interest, normalized to the geometric mean of three control genes. Data is presented as mean values $\pm$ standard error. Western blot analysis was conducted on optical density (OD) levels, and is presented as the ratio of H3K9me2 OD divided by α -actin OD $\pm$ standard error. An experimental replicate for postmortem brain was defined as $\sim$ 100mg of tissue from a single participant, while lymphocyte analyses were defined as a single blood draw from a participant. A probability level of $p < 0.05$ was the criterion to achieve statistical significance.

3. Results

3.1. mRNA Levels of HMT Gene Expression

We performed a multiple linear regression with each HMT gene of interest as the dependent variable. For postmortem brain tissue we examined sex, age, pH, RIN and diagnosis, whereas for lymphocytes we examined sex, age, and diagnosis as explanatory variables. In these two cohorts, we found that a diagnosis of schizophrenia is a significant predictor for GLP mRNA expression in both postmortem brain samples ($\beta = 0.44$, $F(1,24) = 5.80$, $p < 0.05$), and in lymphocytes ($\beta = -0.41$, $F(1,40) = 7.91$, $p < 0.01$), indicating that patients with schizophrenia demonstrated increased levels compared to nonpsychiatric controls (Fig. 1a). Similarly, a diagnosis of schizophrenia is also a significant predictor for increased SETDB1 mRNA levels in both postmortem brain samples ($\beta = 0.39$, $F(1, 24) = 4.33$, $p < 0.05$), and in lymphocytes ($\beta = 0.37$, $F(1,40) = 6.19$, $p < 0.05$; Fig. 1b). A diagnosis of schizophrenia is not a significant predictor for elevated G9a mRNA levels in postmortem brain samples ($\beta = 0.22$, $F(1,24) = 1.22$, $p = ns$), but is for lymphocytes ($\beta = -0.317$, $F(1,40) = 4.46$, $p < 0.05$; Fig. 1c). Interestingly, in both postmortem tissue ($r = 0.79$, $p < 0.001$) and lymphocytes ($r = 0.54$, $p < 0.001$), GLP and SETDB1 mRNA expression are positively correlated (data not shown).

To establish whether there exist differences in HMT mRNA among schizophrenic patients taking psychotropic medication, and those who were not, we performed a second multiple

linear regression analysis on each individual cohort. The overall or type-specific use of antipsychotic, antidepressant or mood stabilizing medication are not significant predictors of HMT mRNA levels in either the postmortem or the lymphocyte cohorts.

3.2. H3K9me2 levels in the Postmortem Brain

In a previously published study we documented elevated global H3K9me2 levels in lymphocytes obtained from schizophrenia patients compared to nonpsychiatric controls (Gavin et al. 2009b). In the current study we attempted to discern whether this abnormality in a restrictive histone modification is present in brain tissue from the SFNC cohort as well. We performed a multiple linear regression with H3K9me2 levels as the dependent variable, with sex, age, and diagnosis as explanatory variables. We found that diagnosis of schizophrenia is a significant predictor of H3K9me2 levels extracted from postmortem brain tissue ($r = -0.40$, $F(1,24) = 4.58$, $p < 0.05$; Fig. 2). GLP ($r = 0.65$, $p < 0.001$) and SETDB1 ($r = 0.44$, $p < 0.05$) are positively correlated with H3K9me2 levels, as discovered through a Pearson Correlation (data not shown).

3.3. Clinical Correlates with Lymphocyte HMT mRNA Levels

Lymphocyte levels of G9a mRNA demonstrated a positive correlation with the PANSS negative subscale total ($r = 0.61$, $p < 0.05$; Fig. 3a), GLP mRNA is positively correlated with the PANSS general subscale total, ($r = 0.64$, $p < 0.01$; Fig. 3b), and SETDB1 mRNA is more highly expressed in patients with longer durations of illness compared to both normal controls and patients in the ‘first episode psychosis’ group (ANOVA, $F(2,30) = 3.66$, $p < 0.01$; Fig. 3c). Patients with a family history of schizophrenia also had significantly increased levels of lymphocyte SETDB1 mRNA ($t_{18} = 2.52$, $p < 0.05$; Fig. 3d).

4. Discussion

The current paper demonstrates an increase in GLP and SETDB1 mRNA in both postmortem parietal cortex and lymphocyte samples from patients with schizophrenia, as well as an increase in G9a mRNA in lymphocytes. G9a and GLP are responsible for the bulk of H3K9me2 modifications across the genome (Shinkai and Tachibana 2011; Tachibana et al. 2005), and SETDB1 is the only euchromatic HMT to specifically di- and tri-methylate H3K9 (Zee et al. 2010; Wang et al. 2003), but all three of these HMTs are able to form large heteromeric complexes, thus allowing for the sequential degrees of lysine methylation (Fritsch et al. 2010). Further, we demonstrate that the ultimate outcome of their catalytic activity, H3K9me2, is significantly increased in patients with schizophrenia as compared to nonpsychiatric controls. Moreover, GLP and SETDB1 mRNA are positively correlated with H3K9me2 levels. These findings add gravity to our previous demonstration of increased H3K9me2 levels in lymphocytes from schizophrenic patients (Gavin et al. 2009b).

Our investigations into the role of H3K9me2 in schizophrenia pathophysiology, as opposed to other H3K9 modifications, were motivated by the hypothesis that initial inactivation of gene promoter activity at various schizophrenia candidate genes can result in gradual entrenchment of the heterochromatin state as a result of disease chronicity and disuse (Sharma et al. 2012). Areas of H3K9me2 can then act as a platform for additional restrictive adaptors, thus resulting in the spreading of heterochromatin across previously unmodified gene rich areas. As such, the gene altering effects of medications are unable to overcome this restrictive burden, leading to repeated medication failures (Sharma et al. 2012). Support for this hypothesis has been previously demonstrated, (Sharma et al. 2008; Benes et al. 2007) including the finding that schizophrenia patients clinically treated for four weeks with the HDAC inhibitor, valproic acid, displayed no increase in peripheral blood cell acetylated histones 3 or 4 as compared to bipolar patients (Sharma et al. 2006). Here, we find an

increase in both H3K9me2 levels and the enzymes which catalyze this modification, providing additional evidence supporting an increased heterochromatin state in schizophrenia.

The major role of the parietal cortex is to integrate and evaluate sensory information (Andersen & Buneo, 2003; Cohen & Andersen, 2002). It is one of the last areas of the human brain to fully mature, (Geschwind, 1965) thus early life environmental insults could have a profound effect. Disordered thought, a common symptom in schizophrenia, is most likely explainable through disruption of this system (Torrey, 2007). Patients with schizophrenia report either acute (McGhie & Chapman, 1961) or blunted (Freedman, 1974) sensitivity to sensory stimuli, and demonstrate overall impairment of sensory integration (Manschreck & Ames, 1984; Torrey, 1980). Similar patterns of transcriptional regulation are observed across the cortex, consequently, results from the parietal cortex likely reflect patterns of gene transcription in other brain regions (Hawrylycz et al., 2012).

Due to its heterogeneity, examining schizophrenia as a binary measurement of illness when examining biological relevancy can be limiting (Arango et al. 2000; Buchanan and Carpenter 1994). Through utilizing the PANSS, biological underpinnings that do not demarcate cleanly with diagnostic categories, can be correlated directly with specific symptomatology. Correlations between methyltransferase enzymes and clinical symptomatology indicate that these restrictive enzymes could contribute to specific facets of the illness, particularly negative and general symptoms, which are particularly resistant to improvement. Increased severity of negative symptoms are correlated with poorer disease prognosis, (Wieselgren et al. 1996) and are not alleviated through our current regimen of psychotropics.

Additionally, SETDB1 mRNA levels are also correlated with other markers of a worse disease prognosis, including a more chronic form of the illness, and a history of schizophrenia in the family. Pharmacological targeting of increased levels of SETDB1 improves motor performance and extends survival in HD mice, indicating the promise of treatments that modulate gene silencing mechanisms in neuropsychiatric disorders (Ryu et al. 2006).

The main weakness of this current study was that clinical symptoms were correlated with mRNA extracted from peripheral tissue. Enzymes relating specifically to synaptic function were not examined, but rather overall mechanisms of epigenetic regulation that are not tissue specific. While postmortem investigations are able to serve as a useful snapshot at the time of death, the ability to measure and monitor histone marks over time as marker of disease progression, improvement, or as a predictor of pharmacological response are only possible using peripheral blood cells. A strong rationale for the use of blood chromatin 'levels' as a type of biosensor that registers the epigenetic milieu has been proposed elsewhere (Sharma 2012). Furthermore, previous studies have indicated the mRNA patterns of expression patterns in lymphocytes are capable of distinguishing between psychiatric diagnostic groups (Middleton et al. 2005).

The present study hypothesized that schizophrenia may be due to abnormal regulation of fundamental epigenetic mechanisms, thus, we chose to measure overall levels of H3K9me2 opposed to specific gene promoters, based on the assumption that while the individual genes silenced in the brain and blood may not be the same, similar global pathogenic processes may be occurring in both tissues.

The results of this paper indicate that chromatin is more restrictive in patients with schizophrenia, and may be significantly contributing to disease pathology. If, through pharmacological interventions, a reduction in this histone hyper-restrictive insult in

schizophrenia can be relaxed, inducing a type of “genome softening,” then neuronal gene expression can be enhanced, thus allowing for increased plasticity and improved therapeutic response (Sharma 2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. mRNA expression in both postmortem parietal cortical samples from the Stanley Foundation Neuropathology Consortium (on the left) and lymphocyte samples from University of Illinois at Chicago (on the right) and a. GLP mRNA levels, b. G9a mRNA levels and c. SETDB1 mRNA levels are all shown as means for independent experiments $\pm$ SEM; $p=ns$ indicates there is no significance, while * $p<0.05$, ** $p<0.01$.

Postmortem H3K9me2 Levels

Representative Western Blot

Fig. 2. H3K9me2 levels are significantly increased parietal cortical samples from patients with schizophrenia when compared to nonpsychiatric controls. Below graph, a representative western blot image is shown. All data is shown as a ratio of optical density levels $\pm$ SEM; * $p < 0.05$.

Fig. 3.

Clinical Correlates with Lymphocyte HMT mRNA Levels a. A rise in G9a mRNA is significantly correlated with increasing PANSS negative subscale totals; $p<0.05$. b. GLP mRNA is significantly increased upon worsening of PANSS general subscale scores; $p<0.01$. c. Patients with chronic schizophrenia have significantly higher levels of SETDB1 mRNA compared to control subjects. Patients with first episode psychosis show no significant differences in SETDB1 mRNA levels compared to either chronic schizophrenic patients or normal controls; $p<0.01$. d. SETDB1 mRNA levels are significantly increased in patients with a family history of schizophrenia; $p<0.05$.

Table 1

Categorical Demographics Comparing Healthy Controls and Schizophrenia Cases in Postmortem Brain Samples

Demographic	Healthy Controls		Patients with Schizophrenia	
<u>Sex</u>				
	N (%)		N (%)	
Males	8 (62)		8 (62)	
Females	5 (38)		5 (38)	
Total	13		13	
	² =.000, df = 1, <i>p</i> = <i>ns</i>			
<u>Age (Mean±SD)</u>	48.1±10.7		44.5±13.1	
<u>pH</u>	6.3±.24		6.2±.23	
<u>RIN</u>	4.9±0.97		5.5±0.90	
<u>Medication</u>				
	Yes (%)	No/unknown (%)	Yes (%)	No/unknown (%)
Antipsychotics	0 (0)	0 (0)	11 (85)	2 (15)
Antidepressants	0 (0)	0 (0)	4 (30)	9 (70)
Mood Stabilizers	0 (0)	0 (0)	3 (23)	10 (77)

Table 2

Categorical Demographics Comparing Healthy Controls and Schizophrenia Cases in Lymphocyte Samples

Demographic	Healthy Controls		Patients with Schizophrenia	
<u>Sex</u>				
	N (%)		N (%)	
Males	14 (73)		16 (64)	
Females	5 (26)		9 (36)	
Total	19		25	
	² =.627, df = 1, <i>p</i> = <i>ns</i>			
<u>Age (Mean±SD)</u>	31.5±8.7		30.5±11.1	
<u>Medication</u>				
	Yes (%)	No/unknown (%)	Yes (%)	No/unknown (%)
Antipsychotics	0 (0)	0 (0)	18 (72)	7 (28)
Antidepressants	0 (0)	0 (0)	9 (36)	16 (64)
Mood Stabilizers	0 (0)	0 (0)	1 (4)	24 (96)