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Epigenetic Determinants of Altered Gene Expression in Schizophrenia: a Dissertation

Hsien-Sung Huang

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EPIGENETIC DETERMINANTS OF ALTERED GENE EXPRESSION IN SCHIZOPHRENIA

A Dissertation Presented

By

Hsien-Sung Huang

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 9, 2008

Neuroscience
EPIGENETIC DETERMINANTS OF ALTERED GENE EXPRESSION IN SCHIZOPHRENIA

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May 9, 2008
Dedicated to my parents

Bi-Long Huang and Hsiu-Sa Huang Kao
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Firstly, I would like to thank my mentor, Schahram Akbarian, for giving me this opportunity to work in his lab. He taught me a lot and I really learned a lot from him. He also recruited talented people and good fundings into his lab. This provides us excellent environment for doing research. I have to say that I’m really evolved during my years in Schahram lab. In other words, there are changes of histone methylation at good gene promoters in my prefrontal cortex. Secondly, I want to thank my chairman, Paul Gardner, for his help and support. Therefore, from my qualifier, each TRAC meeting to recent thesis defense can move so smoothly and on schedule. His humorous and witty character brought a lot of joys and laughter to my life. I would also like to thank David Weaver for his help and guidance when I started in UMMS. He is a very careful and critical scientist. That’s what a scientist should be. I want to thank Job Dekker for his positive inputs, suggestions and discussions in my each TRAC meeting. He always looked at my project in different angles and those help me a lot. I would also like to thank Steven Grossman for donating his time and talent to serve as a member of my committee. He always brought up the clinical views to my projects. I also thank Sabina Berretta for serving as an external member for my DEC. Thirdly, I would like to thank the technical support from Anouch Matevosian, Catheryne Whittle and Yin Guo. They are very professional and responsible. Without their helps, I think my project will definitely slow down. Finally, I want to thank fellow students in Schahram lab including Frederick (Al) Schroeder, Yan Jiang, Nikolaos Mellios, Caroline Connor and Rahul Bharadwaj for working and cooperating together. I especially want to thank Al for giving me non-scientific advice and Yan for providing me scientific advice. Those advices really help me a lot at certain critical time points of my life. I would also like to thank people who helped and guided me during my time in UMMS.
ABSTRACT

Schizophrenia is a neurodevelopmental disorder affecting 1% of the general population. Dysfunction of the prefrontal cortex (PFC) is associated with the etiology of schizophrenia. Moreover, a substantial deficit of GAD1 mRNA in schizophrenic PFC has been reported by different groups. However, the underlying molecular mechanisms are still unclear. Interestingly, epigenetic factors such as histone modifications and DNA methylation could be involved in the pathogenesis of schizophrenia during the maturation of the PFC. In my work, I identified potential epigenetic changes in schizophrenic PFC and developmental changes of epigenetic marks in normal human PFC. Furthermore, mouse and neuronal precursor cell models were used to confirm and investigate the molecular mechanisms of the epigenetic changes in human PFC.

My initial work examined whether chromatin immunoprecipitation can be applied to human postmortem brain. I used micrococcal nuclease (MNase)-digested chromatin instead of cross-linked and sonicated chromatin for further immunoprecipitation with specific anti-methyl histone antibodies. Surprisingly, the integrity of mono-nucleosomes was still maintained at least 30
hrs after death. Moreover, differences of histone methylation at different genomic loci were detectable and were preserved within a wide range of autolysis times and tissue pH values. Interestingly, MNase-treated chromatin is more efficient for subsequent immunoprecipitation than crosslinked and sonicated chromatin.

During the second part of my dissertation work, I profiled histone methylation at GABAergic gene loci during human prefrontal development. Moreover, a microarray analysis was used to screen which histone methyltransferase (HMT) could be involved in histone methylation during human prefrontal development. Mixed-lineage leukemia 1 (MLL1), an HMT for methylation at histone H3 lysine 4 (H3K4), appears to be the best candidate after interpreting microarray results. Indeed, decreased methylation of histone H3 lysine 4 at a subset of GABAergic gene loci occurred in Mll1 mutant mice. Interestingly, clozapine, but not haloperidol, increased levels of trimethyl H3K4 (H3K4me3) and Mll1 occupancy at the Gad1 promoter. I profiled histone methylation and gene expression for Gad1 in schizophrenics and their matched controls. Interestingly, there are deficits of Gad1 mRNA levels and Gad1 H3K4me3 in female schizophrenics. Furthermore, I was also interested in whether the changes of Gad1 chromatin structure could
contribute to cortical pathology in schizophrenics with GAD1 SNPs. Remarkably, homozygous risk alleles for schizophrenia at the 5’ end of the GAD1 gene are associated with a deficit of GAD1 mRNA levels together with decreased GAD1 H3K4me3 and increased GAD1 H3K27me3 in schizophrenics.

Finally, I shifted focus on whether DNA methylation at the GAD1 promoter could contribute to a deficit of GAD1 mRNA in schizophrenia. However, no reproducible techniques are available for extracting genomic DNA specifically from GABAergic neurons in human brain. Therefore, I used an alternative approach that is based on immunoprecipitation of mononucleosomes with anti-methyl-histone antibodies differentiating between sites of active and silenced gene expression. The methylation frequencies of CpG dinucleotides at the GAD1 proximal promoter and intron 2 were determined from two chromatin fractions (H3K4me3 and H3K27me3) separately. Consistently, the proximal promoter region of GAD1 is more resistant to methylation in comparison to intron 2 of GAD1 in either open or repressive chromatin fractions. Interestingly, overall higher levels of DNA methylation were seen in repressive chromatin than in open chromatin. Surprisingly, schizophrenic subjects showed a significant decrease of DNA
methylation at the GAD1 proximal promoter from repressive chromatin.

Taken together, my work has advanced our knowledge of epigenetic mechanisms in human prefrontal development and the potential link to the etiology of schizophrenia. It could eventually provide a new approach for the treatment of schizophrenia, especially in the regulation of methylation at histone H3 lysine 4.
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ABBREVIATIONS

APD antipsychotic drug
BA Brodmann area
CBP CREB binding protein
CLZ clozapine
DSM-IV diagnostic and statistical manual of mental disorders IV
FGF2 fibroblast growth factor 2
GABA gamma-aminobutyric acid
GAD1 glutamic acid decarboxylase 1
GAD2 glutamic acid decarboxylase 2
GRIN2B glutamate receptor, ionotopic, N-methyl D-aspartate 2B
H3 histone 3
H3K4me3 trimethyl histone H3 lysine 4
H3K27me3 trimethyl histone H3 lysine 27
HAL haloperidol
HBB hemoglobin, beta
HDM histone demethylase
HMT histone methyltransferase
iBRAF inhibitor of BRAF35; HMG20A
MLL1 mixed-lineage leukemia 1
MNase micrococcal nuclease
NChIP native chromatin immunoprecipitation
NPY neuropeptide Y
PB phenyl butyrate
PFC prefrontal cortex
PMI postmortem interval
RIN RNA integrity number
SNP single nucleotide polymorphism
SST somatostatin
TAR1 telomere-associated repeat 1
TSS transcription start site
VA valproic acid
WM working memory
XSon crosslinking and sonication
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Parts of this dissertation have appeared in the following publications:


CHAPTER I: INTRODUCTION

Schizophrenia

Schizophrenia is a mental disorder which is characterized by positive and negative symptoms (Lecrubier et al., 2007). Positive symptoms are abnormal perceptions and thoughts, which include auditory hallucinations, delusions and disorganized thinking. In contrast, negative symptoms refer to loss or decrease of normal functions. For instance, patients could experience apathy, blunted affect, impaired attention and poor speech. Schizophrenia was originally described by Emile Kraepelin in 1887 (Engstrom and Weber, 2005) as dementia praecox or “dementia as early onset” and was given its current name by Eugen Bleuler in 1911 (Stotz-Ingenlath, 2000). However, this disease is widely believed to have accompanied humankind’s whole history. Importantly, Kraepelin’s and Bleuler’s pioneering works and observations have contributed to the current criteria for diagnosing schizophrenia (Andreasen and Carpenter, 1993). These criteria are specified in the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV; (Andreasen and Carpenter, 1993)), which is generally used by mental health professionals as a reliable and consistent tool for diagnosing mental disorders.
Based on the DSM-IV, schizophrenia is classified into 5 different subtypes: disorganized, catatonic, paranoid, undifferentiated and residual types. Each subtype consists of a different combination of positive and/or negative symptoms (Jablensky, 2006).

Age at onset of schizophrenia varies widely. Onset age also differs by gender. Men begin to develop schizophrenia from their late teens to early 20s, whereas women start to experience schizophrenia from their late 20s to early 30s (Hafner et al., 1998; Lieberman et al., 2001). Regardless of gender or culture, schizophrenia has a prevalence rate of 1%. The prevalence is lower in children, with onset by age 12, occurring only about 1 in 40,000. Importantly, childhood-onset schizophrenia is biologically and clinically continuous with adult-onset schizophrenia (Nicolson and Rapoport, 1999). Moreover, older adults infrequently develop schizophrenia, i.e., late-onset schizophrenia (onset after age 40) and very-late-onset schizophrenia-like psychosis (onset after age 60) are rare (Howard et al., 2000). These results suggest that the pathogenesis of schizophrenia involves developmental and degenerative processes.

The causes of schizophrenia are still unclear. No single cause can explain
this disorder, although genetic factors could be involved. For example, individuals
with schizophrenic parents or siblings are almost 10 times more likely to develop
schizophrenia. Indeed, at least 17 schizophrenia susceptibility genes have been
identified (Straub and Weinberger, 2006).

However, genetic factors don’t tell the whole story. For instance,
monozygotic twins have only a 50% concordance rate for schizophrenia, and
dizygotic twins have a 15% concordance rate (Lichtenstein et al., 2006). These
facts indicate that genetic factors are important, but are not the only key players in
the pathogenesis of schizophrenia. Therefore, the development of this disease
could be influenced by environmental factors during the development and
maturation of the brain.

The development of schizophrenia has been associated with several
environmental factors: malnutrition in utero (Kroll, 2007), birth trauma (Cannon et
al., 2002), viral infection in pregnancy (Fatemi et al., 2008), living in urban areas
(Fatemi et al., 2008), or even birth in winter or spring time (Davies et al., 2003).
Another favored theory is that this disorder could be influenced by abnormalities
in brain structure (Lawrie et al., 2008), neuronal circuitry, or neurotransmitters
(Benes, 2000). As to brain structure, schizophrenics’ brains have been shown to contain enlarged ventricles (Morgan et al., 2007) or more markedly asymmetric hemispheres (Mintz et al., 1982). Moreover, schizophrenic prefrontal cortex has shown dysfunctional activity (Johnson et al., 2006). Interestingly, this disease might be influenced by an imbalance of neurotransmitters such as dopamine, glutamate, serotonin and gamma-aminobutyric acid (GABA). These neurotransmitters could be involved in the connectivity of neuronal circuits which are related to the symptoms of schizophrenia (Carlsson, 2006).

The major treatment for schizophrenia includes antipsychotic medications. The rationale for using these drugs is to adjust the imbalance of neurotransmitters in schizophrenia (Stone and Pilowsky, 2007). Currently used antipsychotics fall into two categories: typical antipsychotics (or neuroleptics) and atypical antipsychotics. Typical antipsychotics, including haloperidol (Haldol), chlorpromazine (Thorazine), and fluphenazine (Prolixin), have been used to treat schizophrenic patients since the mid-1950s. Neuroleptics block dopamine $D_2$-like receptors and reduce positive symptoms, but have extrapyramidal side effects such as reducing the ability to initiate movement and causing the patient to remain
motionless (Bishnoi et al., 2007).

Atypical antipsychotics, which include quetiapine (Seroquel), risperidone (Risperdal), olanzapine (Zyprexa) and clozapine (Clozaril), have been prescribed to schizophrenic patients since the 1990s (Crilly, 2007). These drugs block a variety of neurotransmitter receptors, thus decreasing positive and negative symptoms. Unfortunately, clozapine has a very severe side effect, agranulocytosis (a severe decrease of blood granulocytes). When medications are discontinued in patients with schizophrenia after 1 year, they have a high relapse rate (69%) (Ayuso-Gutierrez and del Rio Vega, 1997). Moreover, schizophrenic patients are prone to be depressed and have an increased risk of committing suicide. In fact, around 10% of schizophrenic patients die by committing suicide (Pompili et al., 2007). Therefore, antidepressants are also administered to patients with schizophrenia.

Research on schizophrenia has been greatly facilitated by use of animal models. Mice displaying schizophrenia-associated phenotypes have been generated by manipulating schizophrenia susceptibility genes, including Disc1 (Hikida et al., 2007; Li et al., 2007; Pletnikov et al., 2008), Reelin (Brigman et al.,
2006; Pillai and Mahadik, 2008), Stop (Brenner et al., 2007; Powell et al., 2007), TA1 (Wolinsky et al., 2007), Synapsin II (Dyck et al., 2007), PLC-beta 1 (Koh et al., 2008), calcineurin (Miyakawa et al., 2003), Fgfr1 (Klejbor et al., 2006), neuregulin 1 (Karl et al., 2007), Nurr1 (Rojas et al., 2007), and GluR1 (Wiedholz et al., 2007). These genetically engineered mice have proven to be useful tools for studying the treatment and neuropathology of schizophrenia.

Other rodent models used to mimic schizophrenia symptoms have been generated either by drug treatment or by tissue lesion. For instance, rodents treated with drugs targeting neurotransmitter receptors or DNA methylation exhibit symptoms that bear similarities to aspects of schizophrenia. These drugs include amphetamine (dopamine-mimetic), phencyclidine (N-methyl D-aspartate [NMDA] antagonist), ketamine (NMDA antagonist), picrotoxin (GABA-A receptor antagonist)(Berretta and Benes, 2006), lysergic acid diethylamide (LSD, serotonin receptor agonist), and L-methionine (DNA methylation). An example of a model induced by tissue lesion is the rat with hippocampal-lesions (Chambers et al., 1996). So far, no animal model generated by any of these methods shows the full spectrum of schizophrenia symptoms.
Prefrontal cortex

The etiology of schizophrenia has been linked by several lines of evidence to alterations in the neural circuitry of the prefrontal cortex (PFC) (Mirnics et al., 2000; Hashimoto et al., 2008) and to disturbances in specific forms of memory associated with the PFC, particularly working memory (WM) (Lewis and Lieberman, 2000). The PFC is located at the most anterior area of the frontal lobe, which along with the parietal, temporal and occipital lobes, forms the human neocortex. The key function of the PFC is involved in complex cognition by using WM to hold information for later processing through either enhanced spiking activity (Miller et al., 1996) or synaptic facilitation (Mongillo et al., 2008).

In human, the PFC comprises 29% of the neocortex, whereas in macaques and cats the PFC occupies only 11.5 and 3.5% of the cortex, respectively (Fuster, 2002). These differences in the PFC reflect an evolutionary expansion that is related to the evolution in cognitive function of these species. The PFC develops and matures relatively later than other areas of cortex as shown by the PFC's late axon myelination, a marker of late neuronal development (Fuster, 2002). Even within the PFC, different areas develop in a different order. For instance, the
ventromedial PFC develops earlier than the lateral PFC (Fuster, 2002). This developmental difference has been associated in neuropsychological studies with lower functions (emotional and instinctual behaviors) in the ventromedial PFC and with higher executive functions (temporal organization of actions in the domains of behavior, cognition and language) in the lateral PFC (Fuster, 2002).

The human prefrontal cortex has several sub-regions: ventromedial, dorsomedial (BA 9), ventrolateral, dorsolateral (BA 46), rostral (BA10) and orbitofrontal (BA 11 & 12) areas (Blumberg et al., 1999; Frey and Petrides, 2000; Grimm et al., 2006). The functions of these sub-regions have been determined by lesion and imaging approaches. The ventromedial PFC is involved during decision-making in choosing between rewarding and punitive outcomes (Oya et al., 2005). The dorsomedial prefrontal cortex has been implicated in decision-making and planning (Manes et al., 2002). The ventral and dorsal lateral prefrontal cortices together contribute to working memory (Muller and Knight, 2006), the rostral PFC is linked to prospective memory (Okuda et al., 2007), and the orbitofrontal PFC has been associated with encoding information and discrimination learning (Frey and Petrides, 2000; Chase et al., 2007).
The PFC is connected by neural circuits to other areas of the neocortex and to subcortical regions, and deficits in these circuits have been associated with neuropsychiatric diseases. For example, the PFC receives afferent input from the mediodorsal thalamic nuclei and, to a lesser extent, from the anterior thalamic nuclei (Lewis and Lieberman, 2000). On the other hand, the PFC sends efferent output to basal ganglia and the midbrain (substantia nigra/ventral tegmental area [SN/VTA]) (Beckstead, 1979). Therefore, the PFC indirectly regulates the release of dopamine. The human PFC is divided into 6 layers with two major types of neurons: excitatory pyramidal neurons and inhibitory GABAergic neurons (Lewis and Lieberman, 2000).

**GABAergic neurons**

GABA, an inhibitory neurotransmitter, is released by GABAergic neurons, which make up 25% of cortical neurons. The axons of GABAergic neurons project and act locally on pyramidal neurons, and their dendrites are smooth (Wonders and Anderson, 2006). GABAergic neurons mature in mice after birth. Different types of GABAergic neurons are regulated both before and after birth in distinct
temporal and spatial ways (Davila et al., 2005). Most cortical GABAergic neurons in rodents derive originally from the ganglionic eminence of the ventral forebrain (Wonders and Anderson, 2006), but, in the human neocortex, only 35% of GABAergic neurons originate in the ganglionic eminence (Wonders and Anderson, 2006). The other 65% of human cortical GABAergic neurons are from the ventricular and subventricular zones of the dorsal forebrain (Letinic et al., 2002).

Regulation of GABAergic neurons is activity-dependent and reversible. For instance, GABAergic neuronal markers (GABA, GAD, tachykinin and GABA_A receptors) decrease on the denervated side of the monkey visual cortex after monocular deprivation (Hendry and Jones, 1988; Benson et al., 1994; Hendry et al., 1994). Interestingly enough, GABAergic neurons also have excitatory effects during embryonic and neonatal stages due to the early expression of Na^+-K^+-2Cl^- co-transporters (NKCCs) and delayed expression of the K^+-Cl^- co-transporter (KCC2) in immature neurons (Ben-Ari, 2002; Liu et al., 2006). Remarkably, GABAergic excitation promotes neuronal differentiation and morphological maturation (Tozuka et al., 2005; Cancedda et al., 2007).
GABAergic neurons can be subdivided by their morphology, electrophysiology, transcriptional factors or neurochemical components (Flames and Marin, 2005; Wonders and Anderson, 2006). One class of chemical components comprises calcium-binding proteins such as parvalbumin (PV), calretinin (CR) or calbindin (CB). Another class of chemical components consists of neuropeptides such as neuropeptide Y (NPY), somatostatin (SST), vasoactive intestinal peptide (VIP) or cholecystokinin (CCK). Based on their chemical components, GABAergic neurons are classified into 3 distinct populations: PV, SST/CB and CR/VIP. PV- or SST-positive GABAergic neurons originate in the medial ganglionic eminence, whereas, CR-positive GABAergic neurons are originally from the dorsal caudate ganglionic eminence in mice (Flames and Marin, 2005; Wonders and Anderson, 2006).

Classification of GABAergic neurons by electrophysiology is according to their different firing patterns, e.g., fast-spiking, burst-spiking, or regular-spiking (Flames and Marin, 2005). Another classification of GABAergic neurons depends on morphology, e.g., small basket, large basket, nest basket, axo-axonic (chandelier cell), ivy (Fuentealba et al., 2008), small bipolar and small multipolar
(Martinotti cell) cells. Fast-spiking GABAergic neurons contain PV and exist as chandelier cells. Burst-spiking GABAergic neurons express SST/CB and exist as Martinotti cells. Moreover, regular-spiking GABAergic neurons have CR/VIP and appear as small bipolar cells (Flames and Marin, 2005).

GABAergic neurons contain the above neurochemical components in different relative abundance. For instance, half of GABAergic neurons contain PV, whereas only 30% are positive for SST/CB and 15% express CR/VIP (Wonders and Anderson, 2006). Moreover, different types of GABAergic neurons have different postsynaptic targets. PV-containing GABAergic neurons primarily innervate neuronal soma, initial segments of axons and proximal dendrites. CR/VIP-containing GABAergic neurons selectively innervate proximal dendrites. Furthermore, SST/CB-containing GABAergic neurons selectively innervate distal dendrites, soma and proximal dendrites (Wonders and Anderson, 2006). This rich pattern of innervation begins in utero and it is not completed until late adolescence (Di Cristo, 2007). Therefore, the maturation and formation of GABAergic synapses is a prolonged process. GABAergic neurons have also recently been classified by the transcriptional factors they express, thus reflecting
recent advances in understanding the transcriptional regulation of specification and differentiation of GABAergic neurons (Wonders and Anderson, 2006).

GABA is synthesized from glutamate by the enzyme, glutamic acid decarboxylase 1 (GAD1 or GAD67), which is highly expressed in the brain. Unlike GAD1, GAD2 (GAD65), another isoform of GAD, is highly expressed in both brain and pancreas (Chessler and Lernmark, 2000). Interestingly, GAD25 and GAD44, two spliced forms of GAD1, appear predominantly in embryonic brains, whereas GAD1 and GAD2 dominate in adult brains (Varju et al., 2002). Moreover, Gad1-knockout mice show decreased GABA levels and severe cleft palate, resulting in death during the first morning after birth (Asada et al., 1997). However, Gad2-knockout mice display normal GABA levels although they are susceptible to seizures (Asada et al., 1996). GAD1 mRNA levels have been shown to be robustly decreased in postmortem schizophrenic prefrontal cortices (Akbarian and Huang, 2006). Furthermore, schizophrenia has been associated with single nucleotide polymorphisms (SNPs) at the promoter and 5’ end of the GAD1 gene (Lappalainen et al., 2004; Addington et al., 2005; Lundorf et al., 2005; Straub et al., 2007b; Zhao et al., 2007; Du et al., 2008). These lines of evidence show the
importance of GAD1 in the pathogenesis of schizophrenia.

Epigenetic factors in schizophrenia

Hereditary information can be passed down through not only genetic factors but also epigenetic factors, which include post-translational, covalent modifications at the N-terminal tails of histone proteins and DNA methylation. Therefore, epigenetic modifications do not change the DNA sequence. Epigenetic mechanisms could contribute to a variety of cellular and molecular processes such as gene transcription, imprinting, chromosome X inactivation and heterochromatin formation (Peterson and Laniel, 2004; Hoffman and Hu, 2006).

Histone modification. Chromatin consists of DNA and proteins that are packed into higher-level DNA structures, chromosomes. The basic unit of chromatin structure is the nucleosome, which consists of one histone octamer wrapped 1.75 times with 146-bp DNA (Peterson and Laniel, 2004). Histones not only pack chromosomal DNA to fit into the nucleus, but they also increase the accessibility of nucleosomal DNA and internucleosomal contacts by post-translational modifications at the N-terminal tails of histones themselves. The
latter mechanism reflects the status of gene activity, e.g., gene expression or repression (see below).

Histone octamers contain histone H3-H4 tetramers flanked by H2A-H2B dimers. Histone H1 helps nucleosomes form higher-level chromatin structures by stabilizing already formed compact DNA fibers. Unstructured N-terminal tails of histones can be modified by methylation, acetylation, phosphorylation, ubiquitination, and sumoylation. Each type of modification reflects a distinct status of gene expression. For instance, acetylation and phosphorylation at specific residues are linked to gene activation, whereas methylation and ubiquitination are associated with either gene activation or repression, and sumoylation is linked to gene repression (Berger, 2007). Interestingly, different forms of methylated (mono-, di-, tri-) histones at lysine residues are involved in different states of gene activity, thus adding another layer of complexity to interpreting the epigenetic code.

Histone methylation is stable relative to the reversibility and dynamics of histone acetylation and phosphorylation (Bui et al., 2006; Bui et al., 2007; Adegbola et al., 2008). Histones can be methylated at either lysine or arginine
residues. Interestingly, lysine residues can be mono-, di- or tri-methylated, but arginine residues can only be mono- or di-methylated (symmetrically or asymmetrically in the latter case) (Bannister et al., 2002). Histones are methylated by histone methyltransferases (HMTs) specific for histone lysine residues (HKMTs; (Rea et al., 2000) or histone arginine residues (HRMTs; (Wang et al., 2001).

Histone methyltransferases for lysine residues (HKMTs) have been discovered. For example, HKMTs consist of the SET domain-containing protein family and Dot1/DOT1L proteins. The SET domain-containing protein family contains SET1, SET2, SUV39, EZH, SMYD, the PRDM family and others (Volkel and Angrand, 2007). In contrast to HKMTs, only one family of histone arginine methyltransferase (HRMT) has been found so far, the PRMT protein family (Zhang and Reinberg, 2001). Methylation at the histone tails can be removed by histone demethylases (HDMs). Similar to HMTs, HDMs are specific for histone lysine and arginine residues. For example, lysine-specific HDMs are LSD1 and the JmjC domain-containing protein family (Klose and Zhang, 2007), while an arginine-specific HDM is JMJD6 (Chang et al., 2007).

The methylation of different lysines reflects the status of gene activity. For
instance, methylations at histone H3 lysine 4 (H3K4), lysine 36 (H3K36) and lysine 79 (H3K79) are associated with gene activation (Volkel and Angrand, 2007). On the other hand, methylations at histone H3 lysine 9 (H3K9) and lysine 27 (H3K27) and at histone H4 lysine 20 (H4K20) are linked to gene repression. Interestingly enough, methylated lysines can be recognized by certain modulator protein domains, e.g., the chromodomain, WD40 repeat, Tutor domain, MBT domain and PHD finger domains (Volkel and Angrand, 2007). The downstream mechanisms for recognizing modulators are still unclear. Furthermore, histone methylations can be analyzed on a genomic scale by chromatin immunoprecipitation (ChIP) combined with DNA microarrays (ChIP-ChIP), ChIP combined with serial analysis of gene expression (ChIP-SAGE), or ChIP combined with high-throughput sequencing (ChIP-Seq) (Schones and Zhao, 2008).

The roles of histone lysine methylation have recently started to be investigated in neurons. For example, histone demethylases may be involved in neuronal differentiation (Jepsen et al., 2007), survival and dendritic development (Iwase et al., 2007). Enhanced levels of H3K4me3 and recruitment of a histone
methyltransferase, MLL1, accompany inhibitor of BRAF35 (iBRAF)-induced neuronal differentiation (Wynder et al., 2005) and decreased levels of H3K27me3 at Dlx5 promoter are associated with neuronal differentiation (Jepsen et al., 2007). Hyper- trimethylation of H3K9 is correlated with striatal neuron atrophy in CREB binding protein (CBP) (+/-) mice (Lee et al., 2008). In addition, histone methylation is also linked to neuron-specific imprinting for Igf2r, and Grb10 (Vu et al., 2004; Yamasaki et al., 2005; Yamasaki-Ishizaki et al., 2007). Moreover, levels of H3K4me2 and H3K4me3 at the promoters of glutamate receptor genes are correlated with corresponding mRNA levels in the human cerebellar cortex (Stadler et al., 2005).

Dysfunction of histone lysine methylation has also been implicated in psychiatric and neurological disorders. Regarding psychiatric diseases, X-linked mental retardation has been associated with histone demethylases such as SMCX and PHF8 (Iwase et al., 2007; Shi and Whetstine, 2007; Tahiliani et al., 2007). Interestingly, SMCX is also linked to autism spectrum disorder (Adegbola et al., 2008). Remarkably, in schizophrenic PFC, down-regulated metabolic gene expression was linked to high levels of methylation at H3R17 (Akbarian et al.,
2005). Moreover, in a mouse model of depression, chronic defeat-stressed mice, the gene for brain-derived neurotrophic factor (Bdnf) was regulated by increased dimethylation of histone H3 lysine 27 (H3K27me2) (Tsankova et al., 2006).

As to neurological diseases, the brains of humans and transgenic mice with Huntington’s disease (HD) showed increased levels of trimethylated H3K9 (H3K9me3) and increased expression of SETDB1, a histone methyltransferase for H3K9 (Ryu et al., 2006). Interestingly, traumatically injured brains of immature rats showed decreased histone H3 methylation in the hippocampal CA3 region (Gao et al., 2006). Remarkably, brains from humans and transgenic mice with Friedreich ataxia (FRDA) were found to have increased di- and tri-methylation of H3K9 at the FXN/Fxn gene (Al-Mahdawi et al., 2008). Moreover, an aggressive brain tumor, glioblastoma multiforme, has been associated with decreased levels of JHDM1B, a histone demethylase for H3K4me3 at rRNA. This evidence implicated the potential association of H3K4me3 mark with brain diseases (Frescas et al., 2007).

**DNA methylation.** Mammalian DNA is methylated at cytosines within CpG dinucleotides that occur as CpG islands, which are regions containing a high
frequency (at least 55%) of CpG dinucleotides within 500-bp DNA sequences (Takai and Jones, 2002). CpG islands are enriched in the promoter regions and coding exons of the human genome (Saxonov et al., 2006), although CpG islands in promoter regions are more resistant to methylation than those in coding exons. Hypermethylation in promoters is associated with gene silencing, which is initiated by promoter-associated methyl-CpG-binding proteins (MBDs), which recruit transcriptional repressors such as histone deacetylases to silence gene transcription. An example of this function is provided by the X-linked methyl-CpG-binding-protein-2 (MECP2), which is mutated in 76% of patients with classic Rett syndrome (Van den Veyver and Zoghbi, 2000). Hypermethylation at the promoters of tumor suppressor genes is also well known to be linked to cancer (Esteller, 2007). Furthermore, methylation in coding regions has been proposed to prevent internal initiation of transcription by recruiting histone deacetylases (Weber and Schubeler, 2007). DNA methylation doesn’t occur in all eukaryotes. No DNA methylation has been found in yeasts (Saccharomyces cerevisiae) or worms (Caenorhabditis elegans). Low levels of DNA methylation have been observed in insects (Drosophila melanogaster) and substantial levels
of methylation have been detected in honey bees (*Apis mellifera*) (Weber and Schubeler, 2007).

DNA methylation is catalyzed by 4 DNA methyltransferases (DNMTs): DNMT1, DNMT2, DNMT3a and DNMT3b (Hermann et al., 2004). DNMT1 is responsible for maintaining DNA methylation, whereas DNMT3a and 3b are responsible for *de novo* DNA methylation. Surprisingly, DNMT2 does not methylate DNA but does methylate cytosine 38 in the anticodon loop of tRNA (Goll et al., 2006). DNMT1, 3a, and 3b are crucial for development because knockout for any of these DNMTs is lethal for mice (Okano et al., 1999; Chen et al., 2007). In contrast, DNMT2-knockout mice are viable and fertile (Hermann et al., 2003). Genome-wide mapping of DNA methylation first requires detecting DNA methylation by one of three methods (Zilberman and Henikoff, 2007) (1) bisulfite conversion, (2) methylation sensitive restriction enzymes or (3) affinity purification of methylated DNA (methyl-binding proteins or anti-methylcytosine antibody) to separate methylated from un-methylated DNA. Once detected, methylated DNA can be analyzed by high-throughput sequencing or DNA microarray (Zilberman and Henikoff, 2007). Interestingly, DNA methylation has been implicated in the
etiology of schizophrenia and bipolar disorder (Veldic et al., 2004; Costa et al., 2007; Mill et al., 2008).

Goals and rationales

The molecular mechanisms underlying decreased GAD1 mRNA in schizophrenic PFC are still unclear. Moreover, schizophrenia is a neurodevelopmental disorder whose etiology has been implicated in interacting with environmental factors (Thapar et al., 2007). Therefore, my thesis work focused on determining which epigenetic determinants could play a role in the deficit of GAD1 mRNA in the PFC of schizophrenic brains. Understanding the roles of epigenetic factors in the pathogenesis of schizophrenia will advance knowledge about the development and treatment of schizophrenia.
CHAPTER II: CHROMATIN IMMUNOPRECIPITATION IN POSTMORTEM BRAIN

The work presented in this chapter is reproduced from a study by Huang et al., published in *J Neurosci Methods* (Huang et al., 2006).

This work was conducted under the direction of Dr. Schahram Akbarian, and it is with gratitude to him and the other authors that I reproduce these data for the purposes of this dissertation. My contributions to this work consisted of conducting all native chromatin immunoprecipitation, crosslinked chromatin immunoprecipitation, RT-PCR running, human and mouse primer design and tests, mouse brain preparation and data analysis. Anouch Matevossian contributed by assisting in the mouse primer tests and RT-PCR. Yan Jiang contributed by taking photos for Fig. 1-1. Dr. Akbarian contributed by making a cartoon for Fig. 1-2. The manuscript was primarily prepared by Dr. Akbarian while I contributed to the Materials and Methods, Figures and Legends, and provided feedback on other sections.
Abstract

Histone modifications such as methylation could be involved in regulation of gene activity. However, modulation of gene status through histone methylation at different genomic loci is still unclear in human brains. Moreover, there has been no feasible method for chromatin immunoprecipitation applied to human postmortem brains so far. Two major concerns for this approach are autolysis time and tissue pH.

We used micrococcal nuclease (MNase) – treated chromatin immunoprecipitation (NChIP) to profile histone methylation in human prefrontal cortex. NChIP was applied to mouse cerebral cortex in parallel with formaldehyde-crosslinking and sonication chromatin immunoprecipitation (XChIP). Interestingly, the integrity of mono-nucleosomes is still maintained 30 hrs after death. Furthermore, the differences of histone methylations at different genomic loci are detectable and stably preserved across a wide range of autolysis time and tissue pH. Surprisingly, MNase-treated chromatin was at least 10-fold more sensitive than crosslinked and sonicated chromatin in immunoprecipitation. Therefore, this approach is feasible for profiling of histone methylation in
postmortem brain.

Introduction

The dynamic change of chromatin structure is associated with regulation of gene activity. Moreover, methylation at N-terminal tails of histones reflects the chromatin structure. Interestingly, histone modifications could be involved in the etiology of complex diseases such as neuropsychiatric diseases (Tsankova et al., 2007). However, little is known about epigenetic mechanisms of histone methylation in human brain. Furthermore, there is no feasible method available for chromatin immunoprecipitation in human postmortem brain. Recently, we showed “proof of principle” that methylation at histone lysine 4 is linked to developmental regulation and region-specific expression patterns of glutamate receptor genes in human cerebellum and prefrontal cortex (Stadler et al., 2005).

The objectives of this study are to figure out (1) if the integrity of the mono-nucleosome, the basic unit of chromatin, in brain tissues is still maintained after a long period of time after death (2) whether the two major confounding factors, autolysis time and tissue pH, could degrade histone methylation at
different genomic loci and (3) if MNase-treated chromatin is more efficient in immunoprecipitation than crosslinked and sonicated chromatin in postmortem brain. Here, we showed that nucleosomal DNA is still attached to histone core after 30 hrs of autolysis time in postmortem brain. Histone methylations at different genomic loci are not affected by a wide range of autolysis time and tissue pH. Remarkably, the immunoprecipitation of chromatin is more efficient in the MNase-treated condition than in the crosslinked and sonicated condition. This method should be applied to study epigenetic mechanisms of gene regulation in normal and diseased human brains.

Results

Nucleosomal organization of genomic DNA is preserved in postmortem brain

Nucleosome-bound genomic DNA is organized as an array of nucleosomes packaged into higher order structures for compartmentalization and compaction (Woodcock and Dimitrov, 2001). Previous studies on freshly prepared neurons had described a heterochromatic shell that separates the nucleolus from its
surroundings within the nucleus (Akhmanova et al., 2000). In tissue sections stained with the nucleophilic dye, DAPI, this shell and other condensed chromatin is visualized as intensely-labeled foci. Notably, neuronal nuclei in histological sections from postmortem cerebral cortex maintain DAPI-intense foci, including the shell that surrounds the nucleolus (Fig. 1-1). Therefore, higher order chromatin structures appear to be preserved, at least partially, in postmortem brain. However, it is likely that autolysis after death leads to progressive disorganization of higher order chromatin and, ultimately, disintegration of nucleosomal arrays. Hypothetically, chromatin degradation in postmortem brain could be viewed as three progressive stages; in stage 1, higher order chromatin structures and nucleosomal arrays remain intact (Fig. 1-2A); in stage 2, there is chromatin fragmentation but nucleosomes are maintained and genomic DNA remains bound to core histones (Fig. 1-2B). In stage 3, DNA and core histones become detached and disintegrate (Fig. 1-2C). Importantly, the mapping of histone modification patterns at defined genomic sequences only requires that DNA:histone interactions, i.e. the nucleosomes as the fundamental unit of chromatin, are preserved in cadaver brain. To examine this, we treated homogenates from
cerebral cortex with micrococcal nuclease (MNase), a DNase that selectively spares DNA bound to the nucleosome core particle. After MNase treatment and gel electrophoresis, a robust, approximately 150-bp sized DNA fragment became visible both for human and mouse cerebral cortex subjected to autolysis for up to 33 hours (Fig. 1-3A). As a control, genomic chromatin was stripped of protein prior to MNase treatment, and no DNA was detected (Fig. 1-3A). Furthermore, mouse cerebral cortex samples were immediately frozen after death and compared to autolytic samples from the same animal. There was no change in the intensity of the 150-bp fragment even after extended autolysis up to 33 hours (Fig. 1-3B). We conclude that the bulk of nucleosome-bound genomic DNA remains attached to the core histones at least for 30 hours after death.

Chromatin immunoprecipitation in human postmortem brain tissue reveals robust differences in histone methylation levels at various genomic loci

Recently, we reported that, in the human brain, glutamate receptor gene promoters show differences in levels of histone methylation (Stadler et al., 2005). To confirm that histone methylation is detectable at regulatory sequences of
genes other than those encoding glutamate receptors, we quantified 5'-regulatory sequences from four unrelated, single-copy genes (Table 1-2) by real-time PCR (Fig. 1-4A, B). Human samples were prepared by MN-digestion, followed by immunoprecipitation with anti-trimethyl-histone H3-lysine 4 (anti-H3K4me3) and anti-trimethyl-histone-H3-lysine 27 (anti-H3K27me3) antibody. The H3K4me3 modification defines open chromatin at sites of actual or potential transcription, while the H3K27me3 modification defines silenced or condensed chromatin (Peterson and Laniel, 2004). We observed that the open chromatin-associated modification, H3K4me3, was comparatively high at the promoter of a housekeeping gene, β2-microglobulin (B2M), that shows stable expression in human postmortem brain (Mimmack et al., 2004) (Fig. 1-4C). In contrast, levels of the closed chromatin-associated modification, H3K27me3, were highest at the proximal promoter of GAD1, which encodes the 67kDa form of glutamic acid decarboxylase, which is expressed in GABAergic neurons that comprise 20% of cortical neurons (Hendry et al., 1987) (Fig. 1-4D). Furthermore, we found that the locus control region of the β-globin locus, located 60kb upstream of the β-globin gene (HBB), shows the lowest levels of histone
methylation (Fig. 1-4C, D). This observation is in good agreement with a recent study on various mouse and human cell lines showing lower levels of histone methylation at genomic sites further removed from proximal promoter sequences (Bernstein et al., 2005). We conclude that histone methylation levels in human postmortem brain show robust differences across various genomic sites, similar to recent observations made in freshly prepared extracts from dividing cells (Bernstein et al., 2005).

**Histone methylation levels in human cerebral cortex are maintained across wide ranges of autolysis time and tissue pH**

We wanted to find out if autolysis time and tissue pH, two commonly used indicators for the overall preservation of postmortem samples (Barton et al., 1993; Siew et al., 2004), affect results from chromatin immunoprecipitation assays. The differences in levels of methylated H3 (H3K4me3 and H3K27me3) between the various genomic loci were consistently observed in samples of a wide range of postmortem intervals (approximately 5-30 hours) and tissue pH (6.0 – 6.8) (Fig. 1-5A-D). For each gene promoter (GAD1 and GRIN2B), the association between
postmortem intervals or tissue pH with histone methylation levels were very weak, with Pearson’s coefficient of determination ($R^2$) ranging from $0.18 - 0.006$, while the $\beta$-globin locus ($HBB$) showed a moderate association with tissue pH ($R^2 = 0.43$) but not with postmortem interval ($R^2 = 0.016$). Together, these findings suggest that postmortem factors such as tissue pH and autolysis time are not major confounds for chromatin immunoprecipitation assays with anti-histone antibodies.

Comparing two alternative chromatin preparation techniques – enzymatic digestion versus crosslinking and sonication

Chromatin fragmentation is necessary in order to immunoprecipitate molecular markers at sites of individual genes or specific genomic loci. We wanted to compare two alternative chromatin fragmentation methods, MNase digestion and formaldehyde-crosslinking followed by sonication (XSon) (O’Neill and Turner, 2003; Das et al., 2004). Cortical gray matter from adult mice was frozen immediately after death or subjected to autolysis for a period of 20 hours, and then prepared either by MNase or XSon for subsequent immunoprecipitation
with the anti-H3K4me3 antibody. The immunoprecipitation efficiencies, expressed as ChIP-to-input ratios, were for four out of four genes tested at least one order of magnitude higher in MNase-treated chromatin extracts, in comparison to XSon (Fig. 1-6). This finding was observed both in freshly frozen (Fig. 1-6A) and autolytic (Fig. 1-6B) tissue, suggesting that MNase digestion is the superior technique for the purposes of immunoprecipitation with anti-histone antibodies. Notably, for each of the 4 genes, ChIP-to-input ratios of MNase-treated chromatin extracts were very similar between freshly frozen and autolytic samples (Fig. 1-6A, B). This result, obtained in mice, is in good agreement with our observation that in human brain, gene-specific histone methylation differences are not confounded by postmortem interval (Fig. 1-5B, D).

**Discussion**

The methodological study reported here demonstrates three key findings: (1) the structure of nucleosomes is still maintained in postmortem brain for at least 30 hrs after death, (2) differences in levels of histone methylation at different genomic loci are detectable in postmortem brain and are preserved within a wide range of
autolysis time and tissue pH and (3) chromatin digested with MNase is more effective for subsequent immunoprecipitation as compared to the conventional method of formaldehyde-crosslinking followed by sonication.

We observed that nucleosomal DNA is still attached to histones at least 30 hr after death in human and mouse postmortem brain. The autolysis time of most postmortem brains from human brain banks is within 30 hrs. Therefore, it is feasible to study histone modifications at different genomic loci from the available human postmortem brains. Another striking finding in this study is that histone lysine methylation at different genomic loci are maintained in postmortem brain even within a wide range of autolysis time and tissue pH. It has been shown that the turnover rate of histone methylation is similar to that of histone itself in mammalian cells (Borun et al., 1972; Byvoet et al., 1972; Thomas et al., 1972). Therefore, histone methylation will be an ideal histone mark in postmortem studies. Furthermore, histone modifications include not only methylation but also acetylation, phosphorylation, ubiquitination and sumoylation. It would be interesting to study the stability of those non-methylation modifications in postmortem brain.
Based on our results, we find that MNase-treated chromatin is much better for subsequent immunoprecipitation with anti-histone antibodies in comparison with crosslinked and sonicated chromatin. One study has shown that NChIP has higher signal-to-noise ratio in comparison to XChIP (O’Neill and Turner, 2003). Another advantage of NChIP is that most anti-histone antibodies are raised against native and unfixed antigens. MNase doesn’t destroy the native form of chromatin. Moreover, the amount of tissues we used in NChIP or XChIP is around 50 mg of cortical gray matter. We didn’t enrich nuclei from tissue because nuclear membranes could be fragmented in frozen and autolytic conditions. It could be possible that purified nuclei or larger amounts of materials will increase the efficiency of XChIP. However, human postmortem brains are generally preserved in frozen condition from human brain banks and limited amounts of postmortem brains are available for researchers. Therefore, the NChIP procedure described here is superior to XChIP in postmortem brain. Another reason why NChIP is more suitable for postmortem tissue is that NChIP reduces artificial crosslinking with unrelated chromatin fragments. On the other hand, it could cause false association in ChIP assays. For instance, the three-dimensional structure of
chromatin such as the juxtaposition and clustering of nucleosomes from different chromosomal regions (Dekker, 2003) could become disorganized after death.

As to the limitations of NChIP, it is more difficult to study protein of interest other than histones. The reason is that the basic requirement of NChIP is the stable interaction between the protein-of-interest and the genomic DNA in its native condition. Therefore, core histones are best choices for NChIP because of their strong interaction with nucleosomal DNA. Another concern for NChIP is that nucleosome spacing at defined genomic locus could differ in different samples. Moreover, the length of genomic DNA used in NChIP is around 146 bp in comparison with 500 to 1000 bp in XChIP. Fortunately, the patterns of histone methylation at orthologous loci are highly conserved between human and mouse even though many methylated sites don’t show sequence conservation (Bernstein et al., 2005). Therefore, changes in nucleosome spacing are unlikely to become a major confounding factor for NChIP. Furthermore, this potential confounding factor could be easily addressed by performing parallel immunoprecipitation experiments with two or more site- and residue-specific anti-histone antibodies.

It is important to note that until now, analysis of mRNA levels was the only
method available to measure gene expression activity in postmortem human and primate brain (Hasenkamp and Hemby, 2002; Pongrac et al., 2002). We predict that chromatin-based approaches, including histone methylation mapping, will become a valuable complement to mRNA profiling and could provide mechanistic insight into transcriptional regulation of specific genes in healthy and diseased human brain.

**Materials and Methods**

**Brain samples**

Tissue samples were dissected from Brodmann area 10 of postmortem prefrontal cortex of 16 normal adult subjects (Table 1-1), that were part of a brain collection at the University of California as previously described (Akbarian et al., 2005). From each of the frozen samples, 75-100 mg of cortical gray matter was trimmed off and collected into microfuge tubes on dry ice, then stored at –80°C until further processed. Brains of adult, 8-16 week old, mice with mixed genetic background were removed from the skull, and the cerebral cortex of the left hemisphere frozen immediately on dry ice while the cerebral cortex of the right
hemisphere was kept untreated at room temperature for up to 33 hours before freezing. For all subsequent steps, the right and left cerebral cortex were processed in parallel. All procedures were approved by the Institutional Review Board and Animal Care and Use Committee of the University of Massachusetts Medical School.

Micrococcal nuclease digestion

The first step of the protocol is the enzyme-based digestion of chromatin fibers into mononucleosomes, using micrococcal nuclease as a DNAse that selectively spares nucleosome-bound genomic DNA. Each sample, 75mg (human) or 50 mg (mouse) of cerebral cortex, was homogenized in 375 (human) or 250 (mouse) µl ice-cold douncing buffer (DB) 10mM Tris.Cl [pH 7.5]/4mM MgCl₂/1mM CaCl₂, and then incubated with 5 U/ml of micrococcal nuclease (MNase) (Sigma-Aldrich, St. Louis, MO, USA) for 7 min at 37°C. The DNase activity was stopped by adding EDTA to 10mM, and then a 10-fold volume excess of hypotonic lysis buffer (0.2mM EDTA [pH 8.0] /0.1mM benzamidine /0.1mM phenylmethylsulfonyl fluoride (PMSF)/1.5mM 1,4-dithio-DL-threitol (DTT) was
added and samples were incubated on ice for 60 min, with a brief vortexing at 10 min intervals. The purpose of the hypotonic lysis buffer step is to extract the intact nucleosomes from cellular and nuclear debris of the postmortem tissue. Debris is then removed by centrifugation at 3000g. For each sample, 500 μl were used for input control and gel electrophoresis and the remaining nucleosome-laden supernatant was divided into 1.6-ml aliquots for immunoprecipitations.

**Cross-linking and sonication**

Additional samples from mouse cerebral cortex (50 mg) were crosslinked at room temperature for 10 min in 1% formaldehyde / phosphate buffered saline (PBS). Crosslinking was stopped by adding glycine to a final concentration of 0.125M. Samples were incubated at room temperature for 5 min, dounced and then pelleted by centrifugation, washed in PBS two times and then redisolved in 1 ml of 100 mM Tris/ 100 mM NaCl/30 mM MgCl₂ / 0.1% NP-40/ 0.1 mM PMSF, then dounced again, pelleted and dissolved in 300 μl lysis buffer containing 1% SDS/ 10 mM EDTA / 50 mM Tris.Cl [pH 8.0]) / 0.1 mM PMSF. Samples were then sonicated, by applying ten 30 sec pulses at power level 6 (Branson, Danbury CT),
then centrifuged at maximum speed. The supernatant was diluted with 3x vol FSB buffer (5 mM EDTA/ 20 mM Tris.Cl [pH 7.5] / 50 mM NaCl) and divided into aliquots to serve as input and to be used for immunoprecipitation.

**Chromatin immunoprecipitation**

To each 1.6-ml aliquot of supernatant, 160 µl of 10 x incubation buffer (IB, 50 mM EDTA, 200 mM Tris.Cl, 500 mM NaCl, [pH 7.5], were added, together with 3.5 µl of one of two site-specific antihistone antibodies: (i) anti-trimethyl-H3-lysine 4 (H3K4me3) or (ii) anti-trimethyl-H3-lysine 27 (H3K4me27). Both antibodies were polyclonal (rabbit) and purchased from Upstate Biotechnology, Lake Placid, NY, USA. The specificity of these antibodies in chromatin immunoprecipitation assays has been established (Stadler et al., 2005); H3K4me3 is a marker for open chromatin and active transcription, while H3K4me27 primarily defines inactive and silenced loci within euchromatic regions. Samples were incubated overnight at 4°C on a rotating wheel and then 90 µl of protein G agarose slurry (Upstate) was added to each sample. The agarose has been pre-incubated twice in 1.6 ml of 1 x buffer IB and once in 1x buffer IB / 100 g/ml sonicated salmon sperm DNA. After
addition of protein G agarose, samples were incubated for 60 min at 4°C, then pelleted by gentle centrifugation at 100g for 30 sec, the supernatant removed and the agarose together with the captured immune complexes resuspended in 1 ml low-salt immune complex wash buffer [2mM EDTA/20mM Tris.Cl [pH 8.0]/500 mM NaCl/0.1% sodium dodecyl sulfate (SDS)/1% Triton X-100], after 3 min on a rotator, pelleted again and resuspended in high-salt immune complex wash buffer, [2mM EDTA/20mM Tris.Cl [pH 8.0]/500 mM NaCl/1% sodium dodecyl sulfate (SDS)/1% Triton X-100], then pelleted and resuspended in LiCl immune complex wash buffer (250 mM LiCl/1mM EDTA/10mM Tris.Cl [pH 8.0]/ 1% IGEPAL-CA360/1% deoxycholic acid), after 3 min then washed twice in 1mL 1x TE (10 mM Tris.Cl [pH 8.0]/ 1mM EDTA). Captured immune complexes were eluted twice in 2x 250 μl elution buffer EB, freshly prepared on the day of the experiment [100mM NaCO₃/1% SDS]. After elution and repeated centrifugation at 200g (to remove the agarose), EDTA to 10mM and 40 mM Tris.Cl [pH 6.5] were added to the supernatant and samples were digested with 50 μg/ml proteinase K for at least 2.5 hrs at 50°C, then purified by phenol-chloroform extraction, ethanol-precipitated with glycogen as carrier and dissolved in 50 μl of sterile 4mM
Tris.Cl [pH 8.0]. Samples that had been crosslinked with formaldehyde were first incubated for 4 hours at 67°C to reverse crosslinks, then digested for 60 min with proteinase K prior to DNA extraction and purification.

**Quantification of immunoprecipitated DNA and selection of custom-made primers**

A DNA Engine Opticon 2 Light Cycler (MJ Research, Waltham, MA) was used for quantitative, real-time PCR with the DNA intercalating dye SYBR Green (Molecular Probes, Eugene, OR, USA), together with custom-made primers and a general PCR protocol, as described (Stadler et al., 2005). Primers were positioned within the first 1000 bp surrounding the transcription start sites of (a) *GAD1*, which encodes the 67kDa isoform of glutamic acid decarboxylase, (b) *GRIN2B*, the gene for the N-methyl-D-aspartate (NMDA) receptor subunit NR2B, (c) the housekeeping gene *B2M*, β2-microglobulin, and (d) a regulatory element of the β-globin locus (*Table 1-2*). Selected primer pairs had to fulfill 4 criteria; (i) product size within the range of 80-130 bp, in order to amplify from the 147 bp fragments of nucleosomal DNA, (ii) product specificity, as determined by
sequencing and single peak on SYBR Green-based melting curve; (iii) cycle thresholds consistently within the linear range of the amplification curve; (iv) an amplification efficiency within $\pm 25\%$ of the theoretical maximum $\varepsilon_{\text{max}} = 1/\ln2 = 1.443$. Each PCR product from the chromatin immunoprecipitates (ChIPs) was normalized to sample input, using the equation $V = (1+E)^{\Delta ct(\text{Input-ChIP})}$, with $E = \varepsilon_{\text{max}} / \varepsilon_{\text{primer pair}}$ and the exponent defined as the differences in cycle threshold between ChIP and input.

**Immunohistochemistry**

Fresh frozen tissue blocks (frontal lobe) were thawed at room temperature and then immersion-fixed in 4% phosphate-buffered paraformaldehyde for 20 hours, cryoprotected in 30% phosphate-buffered sucrose, frozen and cut in parallel series of 10-$\mu$m thick sections that were collected in ice-cold PBS for free-floating immunohistochemistry. Sections were washed, incubated overnight at $4^\circ\text{C}$ in 0.3% Triton X-100/0.1M sodium phosphate [pH 7.4]/1:500 final dilution of anti-histone antibody (anti-H3K4me2 or anti-H3K4me3, rabbit polyclonal, Upstate) / 1:500 final dilution of the SMI-32 antibody that binds to non-phosphorylated
neurofilament (mouse monoclonal, Sternberger Monoclonals/Covance, Berkeley, CA), washed, incubated for 60 min with FITC- and Texas-Red conjugated secondary antibodies (VectorLabs) in 1:200 final dilution, washed, slide-mounted, briefly air dried, counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and then coverslipped with Vectashield (VectorLabs). Sections were examined with a Zeiss Axiovert 200M microscope using a 63x, 1.4 N.A. oil-immersion objective, a Retiga-1300R cooled CCD camera (Qimaging) using Slidebook 4.0 software (Intelligent Imaging Innovations).
Figure 1-1. Architecture of postmortem brain nuclei. (A,B) Digitized images of a neuronal nucleus from human cerebral cortex (87 year old individual, 8.5 hrs autolysis time); (A) Labeling with nucleophilic dye, DAPI, reveals numerous heterochromatic foci, including a shell (arrows) surrounding the nucleolus (asterisk); (B) Immunohistochemical labeling with anti-methyl-histone H3-lysine4 antibody (FITC, green channel) and anti-neurofilament H (SMI-32) antibody (Texas Red). Note that histone immunoreactivity is confined to the nucleus and spares the nucleolus. Arrowhead in (A,B) marks non-neuronal nucleus. Images taken at 63x10 magnification.
**Figure 1-2. Hypothetical scheme for progressive loss of chromatin organization during tissue autolysis.** (A) shows freshly preserved nucleus, compartmentalized into loose, ("eu"-) and condensed ("hetero"-) chromatic regions, “Stage 1”; (B) nucleus from decomposing tissue, with the DNA still attached to core histones while higher order chromatin structures degenerate, “Stage 2”; (C) nucleus after advanced autolysis, with DNA now detached from histones and nucleosomes disintegrated, “Stage 3”.

Figure 1-3. Nucleosomal DNA is maintained in postmortem brain. (A) Images from ethidium bromide-stained 3% agarose gels showing prominent, approximately 150-bp DNA fragment in homogenates from cerebral cortex treated with micrococcal nuclease (MNase) and RNAse A. Left lane on each gel shows DNA ladder, arrowhead mark 500, 200 and 100 bp (top to bottom). H = human, M1, M2, M3 = mouse no. 1-3, PMI = postmortem interval (hours). Notice prominent 150 bp band in human sample (11% of 80mg tissue) after 30 hours of tissue autolysis, but no detectable sample in negative control (n), defined as a sample that was stripped of protein by chloroform/phenol extraction prior to MNase digestion. Notice also prominent 150 bp band in both freshly preserved (PMI = 0 hrs) and autolytic (PMI = 5 (M2) and 33 (M3)) mouse cerebral cortex (each lane represents 17% of a 50 mg tissue sample). The weakly labeled higher MW DNA fragments are likely to represent polynucleosomes. (B) Graph comparing quantitative amount of 150 bp DNA after MNase treatment in autolytic cortex (x-axis, postmortem interval in hours), relative to samples from the same animals that were immediately frozen after death (y-axis, optical density autolytic / fresh frozen cortex). N=2 animals/time point.
Figure 1-4. Histone methylation levels at defined genomic sequences in human brain tissue. (A,B) Graphs illustrate quantitative PCR for B2M promoter sequences. (A) SYBR-green based melting curve from immunoprecipitates with anti-H3K4me3 and anti-H3K27me3 antibodies, showing single peak for specific product, (B) Amplification curves of inputs (red circle), immunoprecipitates (blue circle) and negative controls (green circle), dotted line indicating cycle threshold. (C,D) summarized ChIP-to-input ratios (y-axis) for 3 different genomic loci (HBB, GAD1, GRIN2B) (x-axis) after immunoprecipitation with anti-H3K4me3 (C) or anti-H3K27me3 antibody (D). Data are normalized to housekeeping gene, B2M. N= 10 samples/gene, data shown as mean ± S.E.M.
Figure 1-5. Autolysis time and tissue pH are not major confounds for chromatin immunoprecipitates from human postmortem brain. Graphs showing levels of H3K4me3 (A,B) and H3K27me3 (C,D) at GAD1, GRIN2B and HBB genes, normalized to B2M, in relation to sample pH (A,C) or postmortem interval (B,D). Notice that differences in histone methylation levels between the 3 genes are similar across the 16 samples despite variable autolysis time and tissue pH. (Linear regression model, $R^2 = 0.334 \sim 0.001$)
Figure 1-6. Chromatin fragmentation by MNase treatment results in higher immunoprecipitation efficiencies. (A, B) Bar graphs show immunoprecipitation efficiencies, expressed as ChIP-to-input ratios (y-axis, log scale) of chromatin extracts prepared by MNase treatment (black bars) or crosslinking and sonication (XSon) (white bars); Samples are from mouse cerebral cortex, (A) immediately frozen after death, (B) collected after 20 hours of autolysis at room temperature. Data shown as mean ± S.E.M. (N=5 animals/group). Notice consistently higher ChIP-to-input ratios in MNase-treated samples, in comparison to XSon. Notice further that ChIP-to-input ratios are similar between MNase-treated samples from freshly preserved or autolytic cortex. * = P < 0.01 (Two sample t test)
(A) 

(B)
Table 1-1: Postmortem brain collection

<table>
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<th>N</th>
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<td>Mean ± SEM, hrs</td>
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</tr>
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<td>Species</td>
<td>Chromosomal location</td>
<td>Product location</td>
<td>Primer sequence</td>
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<tr>
<td>------</td>
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CHAPTER III: PREFRONTAL DSYFUNCTION IN SCHIZOPHRENIA INVOLVES MIXED-LINEAGE LEUKEMIA 1-REGULATED HISTONE METHYLATION AT GABAERGIC GENE PROMOTERS

This work presented here in this chapter is reproduced from a study by Huang et al., published in J Neurosci (Huang et al., 2007).

This work was conducted under the direction of Dr. Schahram Akbarian, and it is with gratitude to him and the other authors that I reproduce these data for the purposes of this dissertation. My contributions to this work consisted of drug injection, mouse brain preparations, rat cell culture, immunofluorescence staining, native chromatin immunoprecipitation, crosslinked and sonicated chromatin immunoprecipitation, mRNA extraction, RT-PCR running, human, mouse and rat primers design and tests, in situ hybridization and cell counting, custom oligo array design and screen, data analysis. Anouch Matevossian contributed by assisting in mouse and rat primer tests, RT-PCR, native chromatin immunoprecipitation and mRNA extraction. Catheryne Whittle contributed by assisting in establishing and maintaining rat differentiated neuron cultures. Yin Guo contributed by assisting in immunofluorescence and DAB staining for Fig.
2-4A and human postmortem brain preparation. Se Young Kim contributed by assisting with the western blot of Fig. 2-4E. Dr. Schumacher provided Mll1 mutant mice. Dr. Baker contributed by assisting with the statistical analysis. Dr. Claudia Schmauss provided drd2<sup>−/−</sup> drd3<sup>−/−</sup> mice. The manuscript was primarily prepared by Dr. Akbarian, while I contributed to the materials and methods, figures and legends, and provided feedback on other sections.
Abstract

Alterations in GABAergic mRNA expression play a key role in prefrontal dysfunction in schizophrenia and other neurodevelopmental diseases. Here we show that histone H3-lysine 4 methylation, a chromatin mark associated with the transcriptional process, progressively increased at GAD1 and other GABAergic gene promoters (GAD2, NPY, SST) in human prefrontal cortex (PFC) from prenatal to peripubertal ages and throughout adulthood. Alterations in schizophrenia included decreased GAD1 expression and H3K4-trimethylation, predominantly in females and in conjunction with a risk haplotype at the 5' end of GAD1. Heterozygosity for a truncated lacZ knock-in allele of mixed-lineage leukemia 1 (Mll1), a histone methyl-transferase expressed in GABAergic and other cortical neurons, resulted in decreased H3K4 methylation at GABAergic gene promoters. In contrast, Gad1 H3K4 (tri)methylation and Mll1 occupancy was increased in cerebral cortex of mice after treatment with the atypical antipsychotic, clozapine. These effects were not mimicked by haloperidol or genetic ablation of dopamine D2 and D3 receptors, suggesting that blockade of D2-like signaling is not sufficient for clozapine-induced histone methylation. Therefore, chromatin
remodeling mechanisms at GABAergic gene promoters, including MLL1-mediated histone methylation, operate throughout an extended period of normal human PFC development and play a role in the neurobiology of schizophrenia.

**Introduction**

Prolonged maturation of the prefrontal cortex (PFC), extending into or even beyond the second decade, plays a key role in normal human development and the neurobiology of major psychiatric disease, including schizophrenia (Weinberger, 1987). In the adolescent non-human primate PFC, dynamic changes in the levels of GABAergic marker proteins suggest a role for inhibitory circuitry (Lewis et al., 2004), but little is known about the underlying molecular mechanisms. Traditionally, expression studies on brain (including human) were confined to quantification of mRNA and protein, but this approach does not discriminate between various cellular mechanisms such as gene transcription as opposed to translational or other post-transcriptional regulatory pathways. There is evidence, however, that psychosis and other psychiatric diseases are accompanied by molecular alterations related to epigenetic control of gene expression
(Abdolmaleky et al., 2004; Petronis, 2004; Grayson et al., 2005; Tsankova et al., 2007). Here, we show that maturation of human PFC and rodent cerebral cortex is accompanied by progressive increases in GABAergic mRNA levels, including \textit{GAD1} which encodes a key enzyme for GABA synthesis that is regulated by neuronal activity (Benson et al., 1994) and which is frequently affected in schizophrenia and related disease (Akbarian et al., 1995; Heckers et al., 2002; Veldic et al., 2005; Lewis and Gonzalez-Burgos, 2006). These developmentally regulated changes in mRNA levels were associated with dynamic chromatin remodeling at \textit{GAD1}/\textit{Gad1} and other GABAergic gene loci, as reflected by increased methylation of histone H3-lysine 4. This included the trimethylated form, H3K4me3, a type of histone modification linked to transcriptional mechanisms and RNA polymerase II activity (Hampsey and Reinberg, 2003; Sims et al., 2003). We report that H3K4 methylation, including H3K4me3, was down-regulated at \textit{Gad1} and other GABAergic genes in mice heterozygous for a targeted allele of \textit{Mll1} (\textit{mixed leukemia-lineage 1}), a histone methyltransferase that is expressed at robust levels in cortical interneurons. Interestingly, treatment with the atypical antipsychotic, clozapine, resulted in increased Mll1-occupancy and H3K4me3
levels at the Gad1/GAD1 promoter. Finally, we demonstrate that schizophrenia subjects having biallelic for GAD1 haplotypes previously associated with schizophrenia and mood and anxiety disorders (Addington et al., 2005; Lundorf et al., 2005; Hettema et al., 2006; Straub et al., 2007a) had a deficit in prefrontal GAD1 mRNA, in conjunction with a shift from open (H3K4me3) towards repressive (H3K27me3) chromatin-associated histone methylation. These novel findings present the first evidence that MLL1-mediated histone lysine methylation is an important regulator of GABAergic chromatin structures that are dynamically regulated throughout an extended period of human PFC development and involved in the pathophysiology of chronic psychotic illness, including schizophrenia.

Results

Dynamic changes in GAD1 expression during human PFC development

To determine the regulation of GAD1 expression during maturation of human PFC, we monitored GAD1 transcript levels from the second trimester of pregnancy to old age, using qRT-PCR (GAD1 and 18S rRNA for normalization) in conjunction with
multiple primer pairs in 55 postmortem samples. There was a progressive increase in
GAD1 mRNA levels throughout pre- and postnatal development and childhood until
the peripubertal stage, which was followed by a plateau or mild decline during
subsequent aging (Fig. 2-1A). Developmental changes for GAD2, a glutamic acid
decarboxylase isoform encoded by a different gene, were similar to GAD1 but less
pronounced. Levels were lowest in the prenatal specimens, peaked before puberty
followed by intermediate levels in the adult (fetal (mean ± S.E.M.), 4.5 ± 0.9 vs. child
(<12 years old) 11 ± 1.4, p = 0.003, ANOVA Tukey HSD; adult 7.0 ± 1.0) (data not
shown). Gad25 and Gad44, two spliced forms of Gad1, express predominantly in a
short period of time in mouse embryonic brains (Gad25: E10.5-E12.5; Gad44:
E12.5-E15.5). Gad44, but not Gad25, has GABA synthesis activity (Chessler and
Lernmark, 2000; Varju et al., 2002). Maturation of GABAergic neurons occurs
postnatally instead of prenatally (Del Rio et al., 1992). Therefore, we didn’t address
whether an imbalance of Gad25 and Gad44 expression could contribute to the
etiology of schizophrenic.

Progressive methylation of H3-lysine 4 at GABAergic gene promoters
**during PFC development**

To determine whether a transcriptional mechanism, including chromatin remodeling, contributes to the extended regulation of GAD1 mRNA expression, we measured histone lysine methylation levels at the GAD1 locus in PFC nucleosomal preparations, using NChIP (O’Neill and Turner, 2003; Huang et al., 2006) in conjunction with modification-specific anti-histone antibodies, and rabbit serum as negative control (*Fig. 2-1B-E*). In adult PFC, open (H3K4) and repressive (H3K27) chromatin-associated methylation marks showed peak levels in nucleosomes positioned within the first 2kb upstream (H3K27) or downstream (H3K4) of the GAD1 transcription start site (TSS) (*Fig. 2-1C*). Very similar findings were obtained for Gad1 chromatin from mouse cerebral cortex (*Fig. 2-2A*). Therefore, regulation of histone methylation in human and rodent cerebral cortex bears similarities to various other tissues and cell lines for which clustering of histone methylation signals around TSS in chromosome-wide scans have been reported (Schneider et al., 2004; Bernstein et al., 2005). Next, we monitored levels of H3K4me3, and of H3-mono-methyl-lysine 4 (H3K4me1) – a related modification that partially overlaps with H3K4me3 in genome-wide screens
(Heintzman et al., 2007) – around the GAD1 TSS during the course of PFC development. Similar to the progressive up-regulation in GAD1 mRNA levels, H3K4me3 levels at the GAD1 promoter showed a several-fold increase from fetal to childhood and from childhood to postpubertal/adult stages (Kruskal-Wallis, p < 0.001 & Dunn’s test, p < 0.01) (Fig. 2-1D). Similar changes were observed for the monomethylated form, H3K4me1 (Kruskal-Wallis, p < 0.01 & Dunns’ test, p < 0.05) (Fig. 2-1E). This developmentally regulated increase in H3K4 tri- and mono-methylation at the GAD1 locus is not explained by a generalized increase in histone methylation levels, because nucleosomes positioned at the locus control region of the erythropoetic β-globin locus, and around a telomere-associated DNA repeat remained at very low levels throughout all stages of PFC development (Fig. 2-1D). To examine if GABAergic genes other than GAD1 undergo chromatin remodeling during the course of PFC development, we measured H3K4 methylation at the 65 kDa glutamate decarboxylase (GAD2), somatostatin (SST) and neuropeptide Y (NPY) gene loci. All GABAergic gene loci showed a developmental increase in methylated H3K4 (Fig.2-1D, E). For the tri-methylated form, H3K4me3, levels were again highest in the postpubertal/adult age group
(GAD2, SST, Dunn’s test, p <0.01) (Fig. 2-1D) while monomethylated H3K4 at these loci peaked during childhood (GAD2, SST, NPY, Dunn’s test, p <0.01) (Fig. 2-1E). We conclude that developmentally regulated chromatin remodeling in human prefrontal cortex is not limited to GAD1 but affects additional GABAergic gene loci.

**Chromatin remodeling at GABAergic gene loci during neuronal differentiation**

Our studies on human postmortem PFC suggest that histone methylation at GABAergic gene promoters is dynamically regulated during an extended period of development. Next, we wanted to determine whether similar mechanisms operate in developing mouse cerebral cortex. Indeed, H3K4me3 levels at the proximal Gad1, Sst and Npy promoters, and the corresponding gene transcripts, were progressively up-regulated during development of mouse cerebral cortex (Fig. 2-2B, D). Remarkably, these dynamic changes continued beyond postnatal day 18 (P18), or 10 days after the density of cortical GABAergic neurons had reached adult levels (One way ANOVA with Tukey HSD, p < 0.05 – 0.001) (Fig. 2-2B, C).
These developmental changes in GABAergic mRNA expression and histone methylation shifts at the corresponding gene promoters were further tested in neural precursor cells cultured from E14.5 rat brain which differentiate into GABAergic and other neurons upon withdrawal of fibroblast growth factor 2 (FGF2) from the medium (Fig. 2-3A, B) (Laeng et al., 2004). As expected, Gad1 and Npy mRNA levels were up-regulated in cultures following FGF2 withdrawal, while changes in Sst transcript were not significant (Fig. 2-3C). Interestingly, these effects on Gad1 and Npy expression were further enhanced in cultures exposed to valproate (0.5 mM) or phenylbutyrate (0.5 mM), two short chain fatty acids acting as histone deacetylase inhibitors (HDACi) (Fig. 2-3C). We conclude that expression of a subset of GABAergic genes in neuronal culture is affected by exposure to chromatin modifying drugs. Furthermore, the up-regulation in Gad1/Npy transcripts in these differentiated cultures was associated with significant, 24-fold (Gad1) and 4-fold (Npy) increases in the open chromatin mark, H3K4me3, at the corresponding promoter (Fig. 2-3D). In contrast, differentiation-related histone methylation changes at the Sst gene locus were not significant (Fig. 2-3D). Finally, the repressive chromatin mark, H3K27me3, was
down-regulated at all 3 gene promoters (Gad1/Npy/Sst) upon differentiation although these decreases didn’t reach the level of significance (Fig. 2-3E). Further study for the crosstalk between histone acetylation and methylation is needed.

The histone methyltransferase mixed-lineage leukemia 1 (Mll1) is expressed in cortical interneurons and regulates H3K4 methylation at GABAergic gene promoters

To explore the molecular mechanisms that regulate histone methylation at the GAD1 locus, custom-made oligonucleotide arrays were used to profile the developmental expression pattern of approximately 30 histone methyltransferases and associated co-factors, including 4 transcripts encoding enzymes with H3K4 methyltransferase activity (MLL1, MLL2, SMYD3, SET7) and several co-factors specifically associated with methylation of H3-lysine 4 (Fig. 2-4Ba). Among the methyl-transferase encoding transcripts, only MLL1 (mixed lineage leukemia factor 1) was expressed at robust levels in both the adult human prefrontal cortex (Fig. 2-4Ba) and mouse cerebral cortex (Fig.
Expression of Mll1-immunoreactivity was observed in numerous nuclei positioned across the full vertical thickness of the mouse cerebral cortex (Fig. 2-4Ba). These included a significant portion of GABAergic interneurons as evidenced in sections double-labeled for Mll1 protein and Gad1 protein (Fig. 2-4Ab) or Gad1 transcript (Fig. 2-4Ac).

These high levels of Mll1 immunoreactivity in cortical interneurons suggest a role for that enzyme in the regulation of GABAergic chromatin. Indeed, there was robust Mll1 occupancy at the proximal Gad1 promoter in mouse P8 and P105 cortical chromatin extracts prepared by formaldehyde crosslinking and sonication (Fig. 2-4C). It is thought that MLL1-mediated H3K4 trimethylation is under the control of the transcriptional process (Hampsey and Reinberg, 2003; Eissenberg and Shilatifard, 2006). In dividing cells, the genome-wide positioning of Mll1 overlaps to 90% with RNA polymerase II occupied regions (Guenther et al., 2005). Notably, mice homo- or heterozygous for a truncated Mll1 allele lacking the methyltransferase (SET) domain showed deficiencies in H3K4 tri- and di-methylation (H3K4me3 and H3K4me2) at selected Hox and other gene loci (Milne et al., 2005). Homozygosity for this Mll1 allele is lethal at E10.5 and mutant
embryos exhibit segmentation defects in cranial ganglia and branchial arches (Yu et al., 1995; Yu et al., 1998). Interestingly, recent analysis of newborn brains and adult hippocampus from viable Mll1+/- mice identified increased Mll1 protein levels in conjunction with decreased Mll1 mRNA expression, suggesting a post-translational mechanism of Mll1 upregulation (Kim et al., 2007). Therefore, we examined H3K4me3 and H3K4me2 at Gad1/Sst/Npy in cerebral cortex of adult Mll1+/- animals. In the mutants, H3K4me3 was decreased by 42-57% and H3K4me2 by 15-31%, in comparison to controls (Fig. 2-4D). These methylation changes at GABAergic promoters were, as a group, significant (Binomial Test [6/6], p = 0.03). Notably, decreased promoter histone methylation in Mll1+/- mice was not accompanied by consistent reductions in the corresponding gene transcripts (data not shown), consistent with the hypothesis that Mll1-mediated histone methylation operates downstream from the transcriptional process (Hampsey and Reinberg, 2003; Eissenberg and Shilatifard, 2006). Furthermore, bulk histone preparations revealed no statistically significant difference in H3K4me2 and H3K4me3 levels between wild-type and Mll1 heterozygous brain (Fig. 2-4E). In the context of normal bulk histone methylation, decreased H3K4
methylation at \textit{Gad1/Sst/Npy} suggests that \textit{Mll1} functions at a select number of target loci in adult brain.

\textbf{\textit{GAD1} SNPs are associated with chromatin alterations in prefrontal cortex of schizophrenia subjects}

We profiled \textit{GAD1} and \textit{GAD2} histone methylation patterns (H3K4me3 and H3K27me3) and mRNA levels in a postmortem collection of 36 schizophrenia subjects (12 females, 24 males), using a case/control design. There were no significant alterations in \textit{GAD2} mRNA and histone methylation levels (\textit{Supplemental Figure 2-1, panel A}). In contrast, significant deficits were observed for \textit{GAD1} mRNA and H3K4me3 levels in female, but not male schizophrenia subjects (\textit{Fig. 2-5A}). Notably, schizophrenia in females is associated with a later age-of-onset as compared to males (Lieberman et al., 2001). Therefore, the deficits in \textit{GAD1} mRNA and H3K4me3 in the schizophrenia cohort of the present study showed a highly robust, inverse correlation with age-of-onset (\textit{Fig. 2-5B}). The molecular mechanisms mediating this gender- and age-of-onset related effect remain to be explored. Interestingly, prefrontal \textit{MLL1} expression was significantly
higher in females as compared to males; levels were similar in cases and controls (Supplemental Figure 2-1, panel B). Furthermore, the steep and progressive increase in GAD1 H3K4me3, which was observed during pre-pubertal development (Fig. 2-1D), continued on a moderate level also throughout subsequent maturation and aging of normal prefrontal cortex (Fig. 2-5C). Therefore, histone methylation at the GAD1 locus – in addition to its regulation during early brain development – is also a molecular mechanism potentially operating throughout early and late stages of psychotic illness.

Several GAD1 SNPs confer genetic risk for increased rate of frontal lobe gray matter loss, in conjunction with childhood-onset and other types of schizophrenia (Addington et al., 2005; Straub et al., 2007a), and bipolar illness (Lundorf et al., 2005). Given the proximity of these SNPs to the transcription start site (TSS) of GAD1 (Fig. 2-5D), we hypothesized that changes in GAD1 chromatin structure could contribute to cortical pathology in genetically susceptible individuals. We genotyped GAD1 SNPs and found that allele frequencies were not significantly different between disease and control brains (data not shown). A subset of 3 SNPs were selected for further analyses because >10% of cases were
homozygous for the rare allele, indicating sufficient statistical power. We then
determined the frequencies of cases that, in comparison to the matched controls,
showed decreased prefrontal GAD1 mRNA levels, in conjunction with a decrease
in the open chromatin mark, H3K4me3 and elevated levels of the repressive mark,
H3K27me3. Given these criteria, 35% of schizophrenia subjects (male and female,
see legend of Fig. 2-5D) biallelic for the GAD1 SNPs previously identified as risk
factors for schizophrenia (Addington et al., 2005; Straub et al., 2007a) or bipolar
disorder (Lundorf et al., 2005) (genotype 1/1 in Fig. 2-5D) were affected. Strikingly,
neither of the other two genotypes (genotypes 1/2 and 2/2, Fig. 2-5D) was
detected in the patients meeting these criteria. These differences were significant
(Fisher-Freeman-Halton, p< 0.05). For the matched controls, there were no
significant differences in distribution of allelic loads (data not shown). We
conclude that genetic polymorphisms around the proximal GAD1 promoter play
an important role for chromatin alterations and transcriptional dysregulation in
schizophrenia subjects.

Mll1 occupancy and H3K4 trimethylation at the Gad1 promoter in cerebral
cortex is selectively increased after treatment with the atypical antipsychotic clozapine

Previous studies reported increased \textit{Gad1} mRNA expression in rodent cerebral cortex after chronic antipsychotic drug treatment (Lipska et al., 2003; Zink et al., 2004; Chertkow et al., 2006). In order to test if chromatin remodeling is involved, \textit{Gad1} H3K4me3 levels were monitored after daily injections of clozapine (5mg/kg) for 21 days. Clozapine is an atypical antipsychotic drug with relative efficiency in treatment-resistant schizophrenia and for some of the cognitive deficits of that disorder (Meltzer, 2004). Indeed, after chronic clozapine treatment, \textit{Gad1}-associated H3K4me3 was increased 3-fold in cerebral cortex, in comparison to vehicle-treated controls (One way ANOVA with Tukey HSD, $p < 0.05$) (\textit{Fig. 2-6A}). Because clozapine's pharmacological profile extends beyond antagonism of dopamine D$_2$-like receptors, we also monitored \textit{Gad1} H3K4me3 after chronic treatment with the conventional antipsychotic and D$_2$-like antagonist, haloperidol (0.5 mg/kg/day x 21 days). No significant changes were observed (\textit{Fig. 2-6A}). Furthermore, no changes in H3K4 methylation were observed in cortical \textit{Gad1} chromatin from compound mutant mice homozygous for null alleles of the
Drd2 and Drd3 dopamine receptor genes (Glickstein et al., 2002) (Fig. 2-6A). Therefore, blockade of D2-like receptors is not sufficient to explain the clozapine-induced increase in histone methylation at the Gad1 promoter.

Notably, Mll1 mRNA expression in mouse cerebral cortex was increased after chronic clozapine treatment (Fig. 2-6B). In addition, Mll1-occupancy at the Gad1 promoter showed a significant 2-fold increase after a single dose of 5mg/kg clozapine (Fig. 2-6B). This clozapine-regulated Mll1 occupancy at Gad1 chromatin was evident both at 30 min (236% ± 72% relative to controls, N=12) and 120 min after the injection (291% ± 110% relative to controls, N=12). In contrast, Gad1/Mll1-occupancy 30 min after a lower dose of clozapine (1mg/kg) remained unchanged relative to controls (97% ± 16%, N=6). Furthermore, the clozapine-mediated Mll1 recruitment at Gad1 chromatin was specific for that locus, because Mll1 occupancy at the Sst promoter was indistinguishable between drug-treated animals and controls (data not shown). We conclude that clozapine-induced histone methylation at the Gad1 locus involves two steps, which are increased Mll1 expression and recruitment to the GABAergic promoter.

In order to investigate whether clozapine’s effects on Gad1 methylation, as
described above for mouse cerebral cortex, also apply to the human prefrontal cortex, we obtained postmortem specimens from another 9 schizophrenia subjects that were treated with clozapine prior to death. In comparison to schizophrenia subjects treated with typical antipsychotics but otherwise closely matched for age, gender, RNA quality and allelic load for GAD1 risk alleles (Supplemental Table 2-1), the clozapine-treated subjects showed on average a 2-fold higher level of H3K4me3 at the GAD1 locus. This difference between the two groups was significant (Fig. 2-6C). In contrast, there were no consistent differences in H3K27me3 levels between the two treatment groups. Furthermore, the clozapine-treated subjects showed a modest, albeit non-significant increase in mRNA levels for GAD1 and MLL1 (Supplemental Figure 2-1, panel C, D). There is no change of Gad1 mRNA levels after either acute or chronic clozapine treatment (data not shown). Taken together, the animal studies and the human data support the notion that clozapine positively regulates MLL1-mediated histone methylation at the GAD1 locus.
Discussion

We report that maturation of human and rodent cerebral cortex is reflected by a progressive increase in histone H3-lysine 4 methylation at GABAergic gene promoters. Regulation of chromatin structures at a subset of GABAergic gene loci, including \textit{GAD1}/\textit{Gad1}, was dependent on normal gene dosage for \textit{Mll1}, a histone methyl-transferase expressed in GABAergic interneurons. These chromatin remodeling mechanisms are likely to play an important role for schizophrenia-related pathophysiology, because \textit{Gad1}/\textit{GAD1}-associated H3K4 methylation, and \textit{Mll1} occupancy, were upregulated after treatment with the atypical antipsychotic clozapine, a drug that improves working memory and other frontal lobe-associated cognitive functions (Meltzer, 2004). Notably, polymorphisms around the \textit{GAD1} promoter previously associated with schizophrenia and accelerated loss of frontal lobe gray matter (Addington et al., 2005; Straub et al., 2007a) also correlate with, according to the present study, deficits in gene expression and histone methylation alterations in schizophrenia brain. Therefore, \textit{Mll1}/\textit{MLL1}-mediated histone lysine methylation at GABAergic gene loci emerges as a molecular link that interconnects three major factors in the
neurobiology of psychosis: developmental mechanisms, interneuron dysfunction and antipsychotic pharmacotherapy.

Importantly, the present study faces two limitations. First, it remains unclear why the observed deficits in GAD1 mRNA levels and H3K4 methylation occur predominantly in female subjects with schizophrenia (Fig. 2-4A). Notably, the genetic association of GAD1 haplotypes with schizophrenia is reportedly more robust in females than in males (Addington et al., 2005; Straub et al., 2007a). It remains possible that the gender-specific effects in these genetic studies and in the present postmortem study are, at least in part, due to higher levels of MLL1 expression in PFC of females as compared to males (Supplemental Fig. 2-1, panel B). Second, it remains unclear if and how the observed upregulation of H3K4 methylation at the Gad1/GAD1 promoter of clozapine-treated mice (Fig. 2-5A) and human subjects (Fig. 2-5B) reflects functional changes in the cortical GABAergic system, given that the corresponding changes in steady state GAD1 mRNA levels were more subtle and without statistical significance (Supplemental Figure 2-1, panel D). Notably, evidence from animal models suggest that clozapine, in contrast to conventional antipsychotic drugs blocking D2-receptors, selectively
improves GABAergic circuitry functions in cerebral cortex (Pillai-Nair et al., 2005) possibly by enhancing inhibitory inputs onto pyramidal neurons through a network-based mechanism (Gao, 2007). In agreement with this hypothesis, clozapine-regulated methylation of GABAergic chromatin – as reported here for the animal and human brain – requires functional brain circuitry, because Gad1-H3K4 methylation remains unaffected in neuronal cultures treated with clozapine (Huang and Akbarian, 2007). Therefore, further studies will be necessary to explore the link between GABAergic chromatin remodeling and the electrophysiological changes (Gao, 2007) after clozapine treatment.

Beyond its biochemical function as a histone methyltransferase with specificity for H3 lysine 4 (Milne et al., 2002), recent evidence points to a distinct developmental role of Mll1 in the central nervous system. This was evident from segmentation defects in cranial ganglia in Mll1-/- embryos (Yu et al., 1998), as well as abnormal synaptic plasticity in the hippocampus of Mll1+/- adult mice (Kim et al., 2007). In support of multiplicity of function, Mll1 undergoes highly regulated proteolytic cleavage (Yokoyama et al., 2002) and was recently detected in a complex with the Polycomb protein Eed and repressive chromatin remodeling in
the adult brain (Kim et al., 2007). This suggests that Mll1 regulates CNS circuitry through several mechanisms operating at different stages of development, as well as in adult brain. Furthermore, the dynamics and molecular machineries regulating histone methylation and de-methylation (Etchegaray et al., 2006; Tahiliani et al., 2007) in CNS await further investigations.

It will also be of interest to find out whether or not GAD1 H3K4 histone hypomethylation in schizophrenia, as reported here, is accompanied by epigenetic modifications of the genomic DNA, including methylation of CpG dinucleotides. However, according to a preliminary study on a small subset of CpG’s positioned in the 5’ portion of GAD1, DNA cytosine methylation frequencies at the GAD1 promoter are very low in normal human PFC (<5%) and potentially even further decreased in repressive (H3K27me3) but not open (H3K4me3) chromatin of schizophrenic subjects (Huang and Akbarian, 2007). Therefore, comprehensive mapping of multiple histone modifications, and DNA CpG methylation tags, at GAD1 and other GABAergic gene loci will be necessary in order to better understand epigenetic regulation of GABAergic gene expression in healthy and diseased human PFC.
It is remarkable that, according to the present study, chromatin structures in prefrontal cortex are subjected to progressive changes from prenatal to peripubertal stages. In addition, this process continues at some gene loci, including \textit{GAD1}, throughout adulthood into old age. This implies that chromatin remodeling and transcriptional mechanisms function as part of a molecular “clock” that regulates, over the course of many years, the protracted maturation, and perhaps also subsequent aging, of prefrontal GABAergic circuitry. To gain further insight into this clock bears great promise for a better understanding of normal human development, including possible alterations in schizophrenia and related disease.

\textbf{Materials and methods}

\textbf{Human subjects}

Human postmortem samples included in this study were obtained from the dorso-rostral pole of the frontal lobe, corresponding to Brodmann’s Area 10 of adult cortex. All procedures were approved by the Institutional Review Boards of the participating institutes. All brains were fresh-frozen and stored at -80\textdegree{}C. The
fetal, neonatal and child samples were obtained through the Brain and Tissue Banks for Development Disorders, University of Maryland (NICHD contract no. NO1-HD-8-3284). In addition, 50 subjects diagnosed with schizophrenia and matched controls were obtained from a brain bank located at the University of California at Davis (UCD) and a brain bank at Maryland Psychiatric Research Center, Baltimore, Maryland (Akbarian et al., 2005). Matching was done for gender and also for age and autolysis time (± 25%). All matching procedures were completed prior to any experiment, including genotyping and determination of RNA integrity numbers (RIN). When an RIN value of 4.0 was chosen as the cut-off for acceptable RNA quality (Lipska et al., 2006), 14/50 matched pairs were removed from this study. Demographics, medication status and postmortem confounds, including tissue pH and RIN for the remaining 36 matched pairs are provided in Supplemental Table 2-1. Because none of these 36 cases had received clozapine, an additional set of 9 cases (all treated with clozapine prior to death) was obtained from the Maryland Psychiatric Research Center and also the Bronx VA Medical Center, Mount Sinai School of Medicine. The serum levels of clozapine in the toxicology screens after death ranged from 0.7 – 5.6 mg/L. The
36 matched pairs of this study include 10 matched pairs (4 female, 6 male) from a previous study focused on GAD1 DNA cytosine methylation in PFC of schizophrenia subjects (Huang and Akbarian, 2007).

**Animal studies**

For antipsychotic drug studies, adult male C57BL/6J mice (JAX), 10-15 weeks of age, were treated acutely or for 21 days with once daily i.p. injections of saline or haloperidol (0.5 mg/kg) or clozapine (5 mg/kg) (Sigma), then sacrificed 0, 30 and 120 min (acute) or 60 min (chronic) after the last treatment. Mutant mice included in this study (Mll1+/−, Drd2−/−Drd3−/−) were 10-15 weeks old and gender-matched to animals from an outbred colony (Mll1) (Kim et al., 2007) or inbred strain (Drd2−/−Drd3−/−, C57Bl6) (Glickstein et al., 2002). Cerebral cortex, without hippocampus, was isolated, for RNA extraction (left hemisphere) and chromatin immunoprecipitation (right hemisphere).

**Cell cultures**

Neural precursor cells were prepared from forebrain of E14.5 SASCO SD rat
embryos (Charles River). Live cells were plated out at 1.2-1.4x10^6 cells per 100-mm poly-l-lysine coated dishes pre-coated with 15 μg/ml poly-l-ornithine (Sigma) and 1μg/ml fibronectin (R&D Systems), and treated daily with 10 μg basic fibroblast growth factor 2 (FGF2 - R&D Systems). At DIV5, cells were passaged and plated out at 0.8-1.0 x10^6 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 x10^6. After 1-2 days FGF2 was removed, cells washed once with medium and then resuspended in medium without FGF2 ± VA (or PB) (0.5 mM each), and harvested after 4 days.

**Chromatin immunoprecipitation, mRNA studies and histone preparation**

Cortical gray matter (70-100mg, human; 30-50 mg, mouse) was stripped of adjacent white matter from the frozen blocks, then homogenized and digested with micrococcal nuclease for subsequent native chromatin immunoprecipitation (NChIP) followed by qPCR, exactly as described (Huang et al., 2006). For NChIP, anti-H3-trimethyl-lysine 4 (anti-H3K4me3) and anti-H3-trimethyl-lysine 27 (anti-H3K27me3) antibodies (Upstate) were used, with rabbit serum as negative
control. Specificity of each antibody was monitored using dot blot assays with synthetic peptide (*Supplemental Figure 2-2*). Primers pairs chosen for quantitative analyses had to pass 2 filters (i) reliable amplification of specific (sequence-verified) product, (ii) primer slope with less than 25% variation from the expected value, 1.4427. Primer and genomic sequences are listed in *Supplemental Table 2-2*. To study Mll1 and RNA pol II occupancy, chromatin from extracted nuclei was prepared by crosslinking in 1% buffered formaldehyde for 10 min, followed by sonication and immunoprecipitation with anti-Mll1 (Bethyl Laboratories) and anti-RNA pol II (Santa Cruz) antibodies.

Total RNA was isolated and purified from brains by using RNeasy Lipid Tissue Mini Kit (Qiagen). Samples were treated with DNase I to avoid DNA contamination, then processed with TaqMan One-Step RT-PCR (Applied Biosystems) with suitable primers (*Supplemental Table 2-2*). From additional samples, biotinylated cRNA was hybridized to custom-made Oligo GEArray® Microarrays (Superarray BioScience) according to the manufacturer’s instructions and analyzed using the GEArray Expression Analysis Suite.

Bulk histone preparations from cerebral cortex, Western blot analysis and
quantification of H3K4me2 and H3K4me3 levels were performed as described before (Kim et al., 2007).

**In situ hybridization and cell counting**

Brains were fixed in 4% phosphate-buffered paraformaldehyde (4 hours), and 14 µm sections were hybridized to DIG-11-UTP sense and antisense probes (15 ng/25ml hybridization buffer/section) at 60°C overnight, then washed and digested with RNAse A and developed with the DIG Nucleic Acid detection kit (Roche). The proportion of Gad1 mRNA expressing cells, relative to the β-actin-expressing cells cut through the level of the nucleus, was determined in 0.75x 1.25 mm counting frames positioned across the full thickness of the somatosensory cortex, using Bioquant software.

**Immunocytochemistry**

For immunocytochemistry, cultured cells were fixed with phosphate-buffered 4% paraformaldehyde /0.2% glutaraldehyde, then permeabilized with 0.2% Triton X-100 and blocked with 10% goat serum, then incubated with primary antibody
(anti-Nestin, /-NeuN, -GABA (Chemicon and Sigma)) for 1 hr, then washed and incubated with Alexa 488- or 594-conjugated secondary antibodies (Molecular probes). For immunohistochemistry, brain sections were prepared as described above and then processed free-floating for Mll1- and Gad1 immunoreactivity in conjunction with Texas-Red and FITC-labeled secondary antibodies (VectorLabs) using standard protocols.

**Genotyping**

*GAD1* SNP genotyping was performed using direct sequencing and also matrix-assisted laser desorption/ionization mass spectrometry (Sequenom), in conjunction with SpecroDesign software for PCR and MassEXTEND primers (*Supplemental Table 2-2*).

**Statistical analysis**

Methylation and transcript profiles across genomic loci, or age groups, or treatments in animals were evaluated by one-way analysis of variance (ANOVA) followed by post hoc *Tukeys HSD* or, in the case of non-normal distribution, by
*Kruskal-Wallis statistic* followed by *Dunn’s test*. Frequencies of cases that, in comparison to matched control, showed mRNA deficits in conjunction with histone methylation alterations were assessed with $2 \times 3$ contingency tables and *Fisher-Freeman-Halton Exact test*. Case/control (clinical samples) or mutant/wildtype (mice) comparisons with non-normal distribution were done by Wilcoxon signed-rank test, otherwise Student’s Paired t-test was applied. Postmortem confounds, including tissue pH, were assessed with *Pearson’s correlation coefficient* and did not show a significant effect on any of the chromatin markers of this study. Generalized effects of *Mll1* gene dosage on H3K4 methylation at GABAergic gene promoters were assessed in *Mll1*+/+ and +/- mice by the *Binomial test*. Levels of H3K4me2 and H3K4me3 between wild-type and Mll1+/- samples were analyzed statistically as described (Kim et al., 2007).
Figure 2-1. Chromatin remodeling at GABAergic gene loci during development and maturation of human prefrontal cortex (PFC). (A) GAD1 mRNA levels in 55 subjects ranging in age from second trimester of pregnancy to old age (x-axis, log scale, B = birth, or “0.77 years”, P = puberty arbitrarily set at 12.77 years), expressed for each sample as mean ± S.E.M from 3 independent qPCR amplicons/primer pairs after normalization to 18S rRNA (y-axis). Values of GAD1 mRNA from 55 subjects were weighted in each GAD1 mRNA primer and then averaged from 3 different GAD1 mRNA primers. Three GAD1 mRNA primers were used to make sure that the developmental trend of GAD1 mRNA is not due to the artificial effect of primer itself. Notice progressive increase during prenatal and postnatal development until puberty. (B) Representative qPCR data from ChIP with anti-methyl-histone antibodies. Top, amplification curves of input (black), immunoprecipitates with anti-H3K27me3 (blue) and -H3K4me3 (red) antibodies and no antibody (as negative control, “no Ab”, green) as indicated. Bottom, SYBR Green-based melting curve and ethidium bromide-stained 3% agarose gel, showing a single peak in melting curve corresponding to the 122-bp product (GAD1, bp -440 to -319). Arrowheads = 100 and 200 bp DNA ladder. (C), ChIP-to-input ratios for 10 primer pairs positioned across 20 kb of GAD1 sequence (x-axis) surrounding the transcription start site (TSS). CpG islands and exons I-III are indicated. N=8-12 samples/primer pair. Notice increased histone methylation in proximity of the TSS (Kruskal-Wallis, H3K4me3, p < 0.001 and H3K27me3 p < 0.05). (D, E) GAD1 mRNA and histone lysine 4 methylation, (D)
H3K4me3, and (E) H3K4me1, for GAD1, GAD2, SST and NPY promoters, the β-Globin locus control region and Telomere-associated repeat 1, TAR1. Data are expressed as mean ± S.E.M for 3 age groups (fetal, 0-12 years old and > 12 years of age). Notice progressive and significant increase in GAD1 mRNA (black) and GAD1-H3K4me3 and H3K4me1 levels (red) from prenatal to postnatal/child and from postnatal/child to adolescent/adult stages, and similar profiles for GAD2, SST and NPY. * p < 0.01 compared to prenatal group, Dunn’s test. Micrococcal nuclease (MNase)-digested PFC chromatins were used as inputs.
Figure 2-2. Histone H3 methylation at GABAergic gene promoters in developing mouse cerebral cortex. (A), Histone methylation profile of adult mouse cerebral cortex at Gad1 locus (x-axis) Exons I-III and the three alternative promoters P1, P2 and P3 as indicated. N=5 samples/primer pair. Notice sharp rise in histone methylation in proximity of TSS (Kruskal-Wallis, H3K4me3, p = 0.001; H3K27me3, p < 0.05). (B), H3K4me3 at 5’ end of Gad1 (bp + 1048 to + 1145) (red) and Gad1 mRNA (black) levels at different stages of mouse cortical development (x-axis). Notice marked increase for both markers after P8 (One way ANOVA with Tukey HSD, * p < 0.05 – 0.005). (C), Representative images through the full thickness of somatosensory cortex from P8 (left) and P105 (right) sections hybridized with digoxigenin-labeled β-actin or Gad1 antisense probes. The ratio of Gad1+: β-actin+ cells across the vertical thickness of cortex was similar for both age groups (mean ± S.E.M. P8, 0.107 ± 0.003; P105, 0.127 ± 0.033. N = 3/group). Bar = 50 μm. (D) Co-regulation of mRNA levels and histone H3K4-trimethylation during maturation from P8 to P105 cortex for Npy (top) and Sst (bottom) (two-sample t test, * p < 0.05).
Figure 2-3: Neuronal differentiation is accompanied by differential changes in H3K4 and H3K27 methylation at GABAergic gene loci. (A), Flowchart for expansion, passage (P) and treatment of cultured neural precursors from E14.5 rat forebrain. (B) (a-c) digitized images showing Nestin and H3K4me3 immunofluorescence and DAPI counterstain of undifferentiated cells, (d,e) GABAergic neurons (arrows) and (f,g) H3K4me3 and NeuN immunoreactivities in differentiated cultures. (C) Levels of Gad1, Npy and Sst1 mRNA, normalized to 18S rRNA (y-axis) from 4 different culture conditions as indicated. Notice robust increase in Gad1 and Npy mRNA levels in differentiated cultures (-FGF2 ± PB or VA), in comparison to undifferentiated (+FGF2) cells. (D, E) Levels of (D) H3K4me3 and (E) H3K27me3 in nucleosomes positioned within 500 bp of rat Gad1, Npy and Sst transcription start sites (see Supplemental Table 2). Data expressed as mean ± S.E.M., N = 3 – 8 / treatment / assay. Notice robust increase in open (H3K4me3) but not repressive (H3K27me3) chromatin methylation at Gad1 and Npy promoters. * (C) = One way ANOVA with Tukey HSD (four groups were compared with each other), p < 0.05 – 0.005; (D, E) = two-tailed Student’s t-test, p < 0.05 – 0.005.
Figure 2-4. Histone methylation in GABAergic chromatin is regulated by Mll1. (A, a-c) Sections from P105 somatosensory cortex illustrating (a) distribution of Mll1 immunoreactive nuclei across cortical layers, and (b,c) expression in GABAergic neurons, (b) anti-Mll1 (red) /anti-Gad1 (green) immunoreactivity, (c) anti-Mll1/ digoxigenin-labeled Gad1 cRNA probe. Scale bars in (a), 50 µm; (b) & (c), 25 µm (B, a-b) Relative expression pattern (gray scale) of 30 histone methyltransferase enzymes and co-factors in (a) human fetal, child and adult PFC and in (b) mouse developing (E15.5) and adult (P105) cerebral cortex, as determined by custom-made oligonucleotide array. Notice high levels of MLL1/ Mll1 expression in comparison to other transcripts regulating H3K4 trimethylation. (C) Bar graphs summarizing data from chromatin immunoprecipitation assays, confirming Mll1 and RNA pol II occupancy at Gad1 locus in P8 and P105 cortex (N=5/assay). (D) Levels of (left) H3K4me3 and (right) H3K4me2 at Gad1/Npy/Sst promoters in cerebral cortex of adult Mll1 +/- and wildtype (wt) controls, after normalization to the B2M housekeeping gene. N = 11/genotype (H3K4me3), 5 (H3K4me2). Notice decreased methylation in Mll1+/- animals (black columns) in 6/6 comparisons (Binomial test, p = 0.03); for Gad1- and Npy-H3K4me3, p < 0.05, Wilcoxson signed-rank test (data are not normal distribution); Npy-H3K4me2, p < 0.05, Student’s Paired t-test (data are normal distribution). (E) Levels of H3K4me2 and H3K4me3 in bulk histone preparations form wild-type (light column) and Mll1+/- (dark column) brain. For each histone modification, the ratio of methylated histone versus total histone H3 was normalized against the respective wild-type
ratio expressed as 100% (N= 8 mice/genotype, data expressed as mean ± S.E.M.).
Figure 2-5. *GAD1* histone methylation and gene expression changes in a subset of subjects with schizophrenia. (A) Levels of *GAD1* mRNA, and open (H3K4me3) and repressive (H3K27me3) histone methylation in the 12 female (top) and 24 male (bottom) case-control pairs, after normalization to the *B2M* housekeeping gene. Notice that 9/12 female schizophrenia subjects have a decrease in *GAD1* mRNA and 11/12 by a reduction in H3K4me3, in comparison to control. Notice no consistent changes in males. P-values shown for Wilcoxon signed-rank test. (B) *GAD1* mRNA and H3K4me3 levels (y-axis, relative to matched control) show an inverse correlation with age-of-onset (x-axis; data were available from 27/36 schizophrenia subjects; male (female) = open black circle (open red circle) symbols). (C) Direct comparison of *GAD1* H3K4me3 in 18 control subjects between 15 and 80 years of age reveals progressive, age-related increases. Data in (B) and (C) shown after removal of extreme outlier (one/graph) (Stem-and-Leaf plot was done to determine the extreme outliers). (D) [Top] Map for 5' portion of *GAD1*, including transcription start site (TSS), CpG islands, exons I-III and location of 5 SNPs associated with increased risk for psychiatric disease (Addington et al., 2005) (Lundorf et al., 2005; Straub et al., 2007a). [Bottom]
Effects of allelic load for SNP 2, 4 and 5 (x-axis) on frequency (y-axis) of schizophrenia cases that show decreased $GAD1$ mRNA levels together with decreased $GAD1$ H3K4me3 and increased $GAD1$ H3K27me3, in comparison to matched control. Notice that 35.5% (6 [4 male, 2 female] /18 [14 male, 4 female]) of schizophrenia subjects homozygous for the common allele (1/1) are affected by above event, but none with other genotypes (total N of schizophrenia subjects per genotype: 1/1 = 17 [SNP 2,4] or 18 [SNP 5]; 1 /2 = 12 [SNP 2,5] or 13 [SNP 4]; 2/2 = 7 [SNP 2] or 6 [SNP 4,5]).
Figure 2-6. Histone methylation at Gad1/GAD1 locus is positively regulated by the antipsychotic, clozapine. (A) Bar graphs for mouse cerebral cortex, showing H3K4me3 levels at Gad1 promoter. Left graph, wildtype mice after 3 weeks of daily injections of 5mg/kg clozapine (Clz, black bar), 0.5 mg/kg haloperidol (Hal, shaded bar), or vehicle (white bar); right graph from Drd2−/−, Drd3−/− double null mutant and control mice. (B) Graphs (left) show cortical Mll1 mRNA expression and (right) Mll1 occupancy at GAD1 promoter in cortex of mice after a single dose of clozapine (5 mg/kg) or vehicle. Data are expressed as mean ± S.E.M. after normalization to housekeeping gene, β2-microglobulin (B2m); N=6-24/treatment or genotype. (C) Bar graphs showing levels of (left) H3K4me3 and (right) H3K27me3 at GAD1 locus in subjects treated with clozapine prior to death (black bars), relative to B2M housekeeping gene and compared to subjects treated with a typical antipsychotic (TA) (white bars). Notice significant, >2-fold increase in H3K4me3 in clozapine treated cohort (N=9), * = p < 0.05 (One way ANOVA with Tukey HSD (A, left), Two-sample t test (B, right) and Wilcoxon signed- rank test (B, left; C)).
Supplemental Figure 2-1. Profiles of histone methylation and gene expression in schizophrenics and their matched controls

(A) Bar graphs showing levels of (left) GAD2 mRNA, (middle) GAD2-H3K4me3 and (right) GAD2-H3K27me3 in 11(22) female (Male) schizophrenia subjects (black bars) and matched controls (white bars). Data normalized to the B2M housekeeping gene. (B, C) Levels of Mll1 mRNA (normalized to B2M) in (B) schizophrenia subjects vs. controls (same cohort shown in A) and (C) schizophrenia subjects treated with clozapine (Clz., black bars) or typical antipsychotic (TA, white bars). (D) Levels of GAD1 mRNA (normalized to B2M) in Clz. And TA-treated subjects. (C, D) N= 9/group. Data shown as mean +/- S.E.M., *p < 0.05 (Mann-Whitney Test)
Supplemental Figure 2-2. Specificity of the antibodies against mono-, di-, and tri-methyl histone H3 lysine 4 (H3K4) and tri-methyl histone H3 lysine 27 (H3K27)

Dot-blot assays were performed for testing the specificity of (A) mono-, di-, and tri-methyl H3K4 and (B) tri-methyl H3K27 antibodies when exposed to mono-, di-, and tri-methyl (A) H3K4 and (B) H3K27 peptides. Peptides and antibodies were used as the indicated concentrations and amounts. Spot peptides directly to the wetted PVDF membrane and allow it to dry. Membrane was wetted again with methanol and TBST (Tris-buffered saline containing 0.1% Twen-20), incubated with 5% dry milk in TBST, and then probed with primary and secondary antibodies.
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*: The amount of peptide is 2.5 μg

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### Supplemental Table 2.1. Postmortem tissue (prefrontal cortex, Brodmann Area 10)

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RIN: RNA integrity number
APD: antipsychotic drugs included the following typical antipsychotics (neuroleptics): fluphenazine, haloperidol, loxapine, mesoridazine, perphenazine, trifluperazine,thioridazine and the following atypicals: olanzapine, risperidone
EWG, estimated weeks of gestation
PMI, postmortemp interval

### Clozapine-treated schizophrenia cases vs. neuroleptic (typical antipsychotic)-treated cases

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**SNPs: 1/1 (homozygosity for risk allele) marked in red**
**all other genotypes (1/2 and 2/2) marked in black**

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### Genomic sequences for chromatin immunoprecipitation studies

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CHAPTER IV: GAD1 MRNA EXPRESSION AND DNA METHYLATION IN PREFRONTAL CORTEX OF SUBJECTS WITH SCHIZOPHRENIA

This work presented here in this chapter is reproduced from a study by Huang et al., published in PLoS ONE (Huang et al., 2007).

This work was conducted under the direction of Dr. Schahram Akbarian, and it is with gratitude to him and the other authors that I reproduce these data for the purposes of this dissertation. My contributions to this work consisted of rat cell culture, immunofluorescence staining, native chromatin immunoprecipitation, mRNA extraction, RT-PCR, DNA sodium bisulfite conversion, subcloning, DNA sequencing and data analysis. Silvia Materna contributed by assisting in Methprimer design and tests, DNA sodium bisulfite conversion, subcloning, and DNA sequencing. Anouch Matevossian contributed by assisting in PCR and native chromatin immunoprecipitation. Catheryne Whittle contributed by assisting in establishing and maintaining rat differentiated neuron cultures. The manuscript was primarily prepared by Dr. Akbarian, while I contributed to the materials and methods, figures and legends, and provided feedback on other sections.
Abstract

Expression of glutamic acid decarboxylase 1 (GAD1), a key enzyme for GABA synthesis, is decreased in schizophrenic PFC. However, the molecular mechanisms contributing to the deficit of GAD1 are unclear. Alterations of DNA methylation as an epigenetic factor of gene regulation could play a role. However, it is difficult to test this hypothesis because no techniques are available to selectively extract DNA from GABAergic neurons efficiently in human postmortem brain.

Here, we present an alternative approach that is based on immunoprecipitation of mononucleosomes with anti-methyl-histone (H3K4me3 and H3K27me3) antibodies differentiating between sites of potential gene expression and silent genes. Methylation of CpG dinucleotides at the GAD1 proximal promoter and intron 2 was measured from two separate chromatin fractions. In a case-control design, all 14 schizophrenia subjects had 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
GAD1 proximal promoter and 30% within intron 2. Strikingly, schizophrenia subjects showed a significant 8-fold decrease of DNA methylation at the proximal promoter in repressive chromatin. The results indicate that chromatin remodeling mechanisms are associated with dysregulated GAD1 expression in schizophrenia.

Introduction

Deficits in GAD1 mRNA in schizophrenic PFC have been reported in different studies by using distinct human brain collections (reviewed in Akbarian and Huang, 2006). Recently, studies on prefrontal cortex of schizophrenia subjects identified several genes including SOX10 (Iwamoto et al., 2005), REELIN (Abdolmaleky et al., 2005; Grayson et al., 2005) and membrane-bound catechol-O-methyltransferase (MB-COMT) (Abdolmaleky et al., 2006), which are affected by altered DNA methylation in conjunction with changes in mRNA levels. Furthermore, increased Gad1 mRNA is accompanied by decreased Dnmt1 expression and DNA methyltransferase activity after treatment with DNA methyltransferase inhibitors in NT-2 neuronal precursor cells (Kundakovic et al.,
Interestingly, levels of \textit{GAD1} mRNA are inversely correlated with levels of \textit{DNMT1} mRNA in the PFC of non-psychiatric subjects and psychiatric patients (Veldic et al., 2005). Moreover, \textit{DNMT1} mRNA is overexpressed in GABAergic neurons of schizophrenia subjects (Veldic et al., 2004).

According to those pieces of evidence, a deficit of \textit{GAD1} mRNA in GABAergic neurons of schizophrenia subjects could be associated with increased DNA methylation at \textit{GAD1} promoter. However, it is difficult to test this hypothesis because no reproducible technique can be used for extracting genomic DNA specifically from GABAergic neurons in postmortem brains. Here, we show an alternative approach that is based on the rationale that active \textit{GAD1} expression is associated with the tri-methylation of histone H3-lysine 4 (H3K4me3), a chromatin mark that defines open chromatin at sites of active transcription (Hampsey and Reinberg, 2003; Sims et al., 2003). Therefore, we separated open and repressive chromatin from human prefrontal cortex with specific anti-methyl-histone antibodies, followed by measuring DNA methylation frequencies for each separate chromatin fraction. Furthermore, we focus on the potential role of DNA methylation at the \textit{GAD1} proximal promoter in schizophrenia subjects affected by
a deficit of _GAD1_ mRNA.

**Results**

**Differential changes of H3K4me3 and H3K27me3 levels during neuronal differentiation**

To determine whether 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) (Hampsey and Reinberg 2003; Sims et al. 2003) chromatin-associated histone methylation at the _Gad1_ locus in a neural differentiation assay for precursor cells from rat embryonic forebrain (**Fig. 3-1A**, 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 (Laeng et al., 2004). In comparison to undifferentiated precursor cells (“+FGF2” in **Fig. 3-1A, B**), neurons (“-FGF2/+VA” in **Fig. 3-1A, B**) showed, on average, a 168-fold fold increase in _Gad1_ mRNA levels by qRT-PCR, and this was associated with a 30-fold increase in H3K4me3 levels at the proximal _Gad1_ promoter (**Fig. 3-1B**). 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. 3-1B**). Furthermore, cultured neurons treated with the
antipsychotic, clozapine (“-FGF2 +VA +Clz” in Fig. 3-1B) for four days, 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 (Straub et al., 2007a) – 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 in vitro for four days.

Selective changes of DNA methylation at the GAD1 promoter in schizophrenic PFC

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 (Stadler et al., 2005; Huang et al., 2006). 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 the GAD1 proximal promoter and 5’ end sequences were tested; 67 primer 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 chromatin preparation technique that produces mononucleosomes with less than 148 bp of genomic DNA. The remaining 3 primer pairs (Supplemental Table 3-1) covered altogether 12 CpG’s positioned between -1120 to +3400 bp from the GAD1 transcription start site (Fig. 3-2A). 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. 3-2A upper panel). Two CpG’s located within 200-250 bp upstream of the 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. 3-2A 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. 3-2A lower panel, and Fig. 3-2B) and a higher methylation frequency (approximately 15%) within intron 2 (Fig. 3-2A lower panel, and Fig. 3-2C). Unexpectedly, however, GAD1 DNA methylation in repressive chromatin (H3K27me3) of schizophrenia subjects was significantly different from control subjects: CpG methylation frequencies were on average 3.5% in the control cohort but only 0.4% in the disease cohort (Fig. 3-2A lower panel, and Fig. 3-2B). This DNA methylation deficit in repressive GAD1 chromatin of schizophrenia subjects affected 5/8 GpG nucleotides (Fig. 3-2A 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. 3-2A, lower panel, and Fig. 3-2C) 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. 3-2, 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 2 kb 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 (Addington et al., 2005). 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 cohorts showed no significant difference in the number of subjects bi-allelic for the common allele (1/1), which defines the at risk haplotype (Addington et al., 2005) (Pearson chi-square $\chi^2 = 0.47$, df =1, p = 0.5).
Furthermore, overall allele frequencies were not significantly different between the two cohorts (Fisher’s Exact Test).

**Discussion**

To the best of our knowledge, we are the first to assess DNA methylation in human brain for open and repressive chromatin separately. In the open chromatin fractions of the present study, which were defined by trimethylation of histone H3 lysine 4 (H3K4me3) (Hampsey and Reinberg, 2003; Sims et al., 2003), *GAD1* DNA methylation was overall much lower, in comparison to repressive chromatin that is defined by trimethylation of histone H3 lysine 27, (H3K27me3) (Sims et al., 2003). 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. Unexpectedly, DNA methylation at the *GAD1* proximal promoter in repressive chromatin showed significant decreases in schizophrenia subjects affected by the deficit of *GAD1* mRNA.

Our profiling data for DNA methylation across the proximal promoter and intron 2 of the *GAD1* gene in human brain are consistent with the idea that gene
promoters are more resistant to DNA methylation (Caiafa and Zampieri, 2005). Moreover, it has been shown that regulation of gene activity is coordinated at both the histone and DNA levels (Fuks et al., 2003; Wang et al., 2005; Zhang et al., 2005). Therefore, it would not be so surprising that higher frequencies of methylation at CpG dinucleotides occurred in repressive chromatin, which is defined by the H3K27me3 mark here.

Given that DNA methylation around the proximal promoter typically contributes to transcriptional repression, it was expected that schizophrenia subjects would show increased GAD1 DNA methylation. Instead, we observed a significant decrease in CpG methylation at the GAD1 proximal promoter in the repressive chromatin fraction of schizophrenia subjects. 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 controls (see Methods and Fig. 3-3). 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. 3-4).
Therefore, repressive chromatin-associated histone methylation at the *GAD1* locus in schizophrenia appears to be, at least in part, independent of 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 determine 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.

**Materials and methods**

**Human brains**

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 3-1. 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 3-1 and Fig. 3-3). In addition, for 3 of the matched pairs, the decrease in GAD1 mRNA levels in the schizophrenia subjects had been observed in a previous study that used in situ hybridization histochemistry (Akbarian et al., 1995). 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 (Akbarian et al., 1995).

**DNA methylation**

For DNA methylation studies, nucleosomes first were extracted from prefrontal cortex gray matter and then immunoprecipitated with two anti-methyl-histone specific 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 (Stadler et al., 2005; Huang et al., 2006). Salmon sperm as a blocking agent was omitted and instead all samples were first pre-cleared by 30 min agarose pre-incubation prior to the immunoprecipitation procedure. DNA purified from immunoprecipitates was subjected to sodium
bisulfite conversion followed by purification and elution, using the EZ DNA methylation kit (Zymo Research), according to the manufacturer’s instructions. Methprimer software (http://www.urogene.org/methprimer) was used to design primer pairs for GAD1 sequences. Three primer pairs (Supplemental Table 3-1) altogether covering 12 CpG’s positioned between -1120 to + 3400 bp from the GAD1 transcription start site, were choosen to profile DNA methylation patterns in control brains (Fig. 3-2A upper panel). PCR amplicons were gel-purified, subcloned into pCR4-TOPO vector, purified in 96 well plates (Qiagen Turbo Miniprep) and for each subject, 12 clones from each immunoprecipitate were analyzed by sequencing. For each subject, DNA methylation levels were expressed as % methylated CpG’s separately for each primer pair. Differences between schizophrenia and control subjects were evaluated by two sample t-test, or in the 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 (Gabriel and Ziaugra, 2004).

**Cell cultures**

Neural precursor cells were prepared from forebrain of E14.5 SASCO SD rat embryos (Charles River). Live cells were plated out at 1.2-1.4x10⁶ cells per
100-mm poly-l-lysine coated dishes pre-coated with 15 μg/ml poly-l-ornithine (Sigma) and 1 μg/ml fibronectin (R&D Systems), and treated daily with 10 μg basic fibroblast growth factor 2 (FGF2 - R&D Systems). At DIV5, cells were passaged and plated out at 0.8-1.0 x10^6 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 x10^6, and after 1-2 days FGF2 was removed, cells washed once with medium and then resuspended in DMEM/M2/F12 medium (Invitrogen) without FGF2 but with 0.5 mM sodium valproate and with or without 1 μM clozapine, and harvested after 4 days.
Figure 3-1: 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 20x10 (200X) 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 or *Gad1* gene expression in cultured neurons. VA = Valproic acid, Clz = Clozapine. * = P< 0.01 (One way ANOVA with Tukey HSD, -FGF2+VA (or –FGF2+VA+Clz)-treated cells were compared with +FGF2-treated cells). When comparisons were performed in *Gad1* H3K4me3 or H3K27me3 levels, none of them reached level of significance.
A

Nestin  DAPI  H3K4me3

+FGF2

a  b  c

d  e  f

-FGF2+VA

NeuN  DAPI

g  h

GABA  DAPI

i  j
**B**

![Bar chart](#)

**Gad1 mRNA**
- Bar chart showing expression levels of Gad1 mRNA with different groups labeled: +FGF2, -FGF2, -FGF2 +VA, and -FGF2 +VA +Clz.
  - Asterisks indicate statistical significance.

**Gad1 H3K4me3 (ChIP/input)**
- Bar chart showing expression levels of Gad1 H3K4me3 with different groups labeled: +FGF2, -FGF2, -FGF2 +VA, and -FGF2 +VA +Clz.

**Gad1 H3K27me3 (ChIP/input)**
- Bar chart showing expression levels of Gad1 H3K27me3 with different groups labeled: +FGF2, -FGF2, -FGF2 +VA, and -FGF2 +VA +Clz.
**Figure 3-2:** GAD1 DNA methylation in prefrontal cortex of subjects with schizophrenia and comparison subjects. **(A)** (upper panel) GAD1 DNA methylation profile in open (H4K4me3) and repressive (H3K27me3) chromatin. Percentage of methylated GAD1 CpG residues in DNA from immunoprecipitates (y-axis, ●, anti-H3K4me3; △, anti-H3K27me3) for 12 CpG’s (shown in red) positioned between bp -1150 to +3400 relative to the GAD1 transcription start site. Primer pairs marked by arrows (N = 3-15 control subjects/CpG dinucleotide); (lower panel) averaged levels of methylation for each of the 12 GAD1 CpG residues (see Fig. 3-2, upper panel) in schizophrenia subjects (S), expressed relative to control, C (S/C, y-axis). Notice decreased methylation of 5/8 CpG’s at the GAD1 promoter of schizophrenia subjects (B,C) Averaged frequency of DNA methylation across the 8 CpG’s at the GAD1 (B) proximal promoter (see Fig. 3-2A, upper panel) and (C) intron 2 in schizophrenia and matched control subjects, as indicated (N=12 clones/primer pair/case or control). Data shown for open (“K4” = H3Kme3) and repressive (“K27” = H3K27me3) chromatin separately. Notice significant deficit in repressive chromatin-associated DNA methylation at the GAD1 promoter in schizophrenia subjects (P = 0.018, Wilcoxon Signed Ranks Test).
Figure 3-3: GAD1 mRNA levels in schizophrenia subjects and matched controls. Levels of GAD1 mRNA in 14 controls (C) matched to 14 schizophrenia (S) subjects, as determined by qRT-PCR separately for 3 different primer pairs spanning different exons. Data shown after log-transformation and normalization with the housekeeping gene transcript, β2-microglobulin.
Figure 3-4: DNA methylation changes at the *GAD1* locus in schizophrenia.

(Top) schematic presentation of *GAD1* CpG dinucleotides (open labels, unmethylated and filled labels, methylated) in normal prefrontal cortex, showing higher levels of DNA methylation in repressive (“K27” = H3K27me3) as opposed to open (“K4” = H3K4me3) chromatin. Notice overall low DNA methylation at the proximal *GAD1* promoter. (Bottom) In schizophrenia, *GAD1* promoter DNA methylation in repressive chromatin (“K27”) is further decreased from control values. A hypothetical but plausible mechanism would be the conversion from open chromatin to repressive chromatin that becomes tagged with the histone mark, H3K27me3, but without additional DNA methylation.
Table 3-1. Postmortem collections

<table>
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<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>Gender F/M,</th>
<th>APD</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>14</td>
<td>58.7 ± 5.5</td>
<td>13.8 ± 2.4</td>
<td>6.44 ± 0.09</td>
<td>6.1 ± 0.4</td>
<td>0.023 ± 0.008</td>
<td>0.038 ± 0.015</td>
<td>0.085 ± 0.018</td>
<td>5/9</td>
<td>86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>14</td>
<td>60.5 ± 5.2</td>
<td>13.7 ± 2.5</td>
<td>6.40 ± 0.09</td>
<td>5.6 ± 0.6</td>
<td>0.036 ± 0.010</td>
<td>0.054 ± 0.018</td>
<td>0.143 ± 0.017</td>
<td>5/9</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RIN, RNA integrity number; APD, antipsychotic drug; PMI, postmortem interval
### Supplemental Table 3.1. Primer for bisulfite-converted GAD1 DNA (sense strand)

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Chromosomal location</th>
<th>Primer location</th>
<th>Length (bp)</th>
<th>Primer sequence</th>
<th>Source</th>
<th>Gene transcription start site</th>
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<tbody>
<tr>
<td>Rat</td>
<td>mRat</td>
<td>E3 (4-4)</td>
<td>1 (268-132)</td>
<td>136</td>
<td>TGTGCCTCTCTGTCATCCCA TGTGCCTCTCTGTCATCCCA</td>
<td>54567-51257</td>
<td>15-AUG-2001</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>Human</td>
<td>E3 (4-4)</td>
<td>1 (268-132)</td>
<td>136</td>
<td>TGTGCCTCTCTGTCATCCCA TGTGCCTCTCTGTCATCCCA</td>
<td>54567-51257</td>
<td>15-AUG-2001</td>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Rat</td>
<td>E5 (4-4)</td>
<td>1 (268-132)</td>
<td>136</td>
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<td>15-AUG-2001</td>
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CHAPTER V: GENERAL DISCUSSION

In this dissertation, I investigated the potential roles of histone modifications and DNA methylation in the etiology of schizophrenia. We show that methylation of histone H3 lysine 4, a chromatin mark associated with active transcription, increased progressively at GAD1 and other GABAergic gene promoters (GAD2, NPY and SST) in human prefrontal cortex (PFC) from prenatal to peripubertal ages and throughout adulthood. Alterations in schizophrenia including decreased GAD1 expression and H3K4-trimethylation, predominantly in females, and in conjunction with increased H3K27-trimethylation, are associated with a risk haplotype at the 5’ end of GAD1. Heterozygosity for a truncated lacZ knock-in allele of mixed-lineage leukemia 1 (Mll1), a histone methyltransferase expressed in GABAergic and other cortical neurons, resulted in decreased H3K4 methylation at GABAergic gene promoters. In contrast, Gad1 H3K4-trimethylation and Mll1 occupancy at Gad1 promoter were increased in the cerebral cortices of mice after treatment with the atypical antipsychotic, clozapine. These effects were not mimicked by haloperidol or genetic ablation of dopamine D2 and D3 receptors, suggesting that blockage of D2-like signaling is not sufficient for clozapine-induced
histone methylation. As to DNA methylation, schizophrenia subjects with a deficit in \textit{GAD1} mRNA also showed decreased methylation of CpG dinucleotides at the \textit{GAD1} promoter in repressive chromatin.

Here, I would like to point out fertile research areas for epigenetic mechanisms in CNS neurons.

After the first histone demethylase (HDM), lysine-specific demethylase 1 (LSD1), was discovered in 2004 (Shi et al., 2004), 7 other HDMs were discovered within 2 years (Shi and Whetstine, 2007). These findings on HDMs revolutionized the previous idea that histone methylation was irreversible. Indeed, histone demethylation by HDMs might play a role in the deficit of \textit{GAD1} H3K4me3 levels found in female schizophrenics. The deficit of \textit{GAD1} H3K4me3 could be due, at least in part, to an imbalance in expression or coordination of HDMs and histone methyltransferases (HMTs). This possibility needs to be further investigated. Moreover, the interactions among different histone modifications need to be addressed by additional studies. For example, it has been known that methylation at histone H3 arginine 2 (H3R2) can exclude the methylation at histone H3 lysine 4 (H3K4) as well as the recruitment of Mll1 (Guccione et al., 2007).
Histone modification marks and their corresponding gene activities have already been defined in different organisms (Peterson and Laniel, 2004), but the molecular mechanisms linking these marks and their corresponding gene activities are still unknown. Therefore, the molecules recognizing and interacting with specific histone marks need to be further determined. Another important issue to be addressed is the downstream mechanisms that are activated after modulators bind to their corresponding histone marks. I believe that the results of such studies could clarify why mRNA levels of GABAergic genes in $\textit{Mll1}$ mutant mice did not change even though H3K4me3 levels significantly decreased in a subset of GABAergic genes.

Another interesting question to be addressed is whether $\textit{Mll1}$ mutant mice show schizophrenia-like phenotypes. Behavioral experiments with $\textit{Mll1}$ mutant mice have been limited due to embryonic lethality in the homozygous mutant strain and inactivity and sickness in the heterozygous mutant strain (Yu et al., 1995). Fortunately, conditional $\textit{Mll1}$ knockout mice have recently been generated (Jude et al., 2007). These mice could be engineered to delete $\textit{Mll1}$ in specific brain regions by mating with transgenic mice expressing Cre in different brain
regions. These mice could be behaviorally tested for potential schizophrenia-like phenotypes. If these behavioral tests showed that the conditional Mll1 knockout mice had defects in working memory, which is one core symptom of schizophrenia, then an even more meaningful question to address would be whether the PFC of these mutant mice show dysfunction of synaptic plasticity. Taken together, the results of such experiments would definitely move us to another level of understanding of the roles epigenetic factors play in brain functions.

My results show that clozapine increases levels of Gad1 H3K4me3, which was not affected by blockade of D2-like signaling such as by haloperidol treatment or D2/D3 deletion. These results may be confirmed by treating D2/D3-knockout mice with clozapine. Clozapine can block a wide range of receptors including dopaminergic (D4), adrenergic, cholinergic, histaminergic and serotonergic receptors. The receptor(s) involved in changing Gad1 chromatin structure need to be explored. This exploration might shed light on why clozapine is more effective than other antipsychotic drugs in treating negative symptoms of schizophrenia.

My results suggest that female schizophrenics have severe deficits in the levels of GAD1 mRNA and GAD1 H3K4me3, whereas male schizophrenics do not.
Furthermore, in our cohort schizophrenic patients, females had later age of onset than males, in agreement with previous findings (Hafner et al., 1998; Lieberman et al., 2001). Here, I hypothesize that these differences in age of onset and deficit of GAD1 chromatin structure could be mediated by estrogen. For example, estradiol-17-beta, the female sex hormone, has neuroprotective properties and could be involved in ameliorating schizophrenia symptoms (Rao and Kolsch, 2003). Nearly 30% of individuals affected by mental illness perceive menopause to worsen symptoms of mental illness (Sajatovic et al., 2003). Interestingly, estradiol-17-beta can activate estrogen receptors and then this activated complex can recruit menin, one integral component of Mll1, to trimethylate at histone H3 lysine 4 (Dreijerink et al., 2006).

The results of my dissertation work may lead to a better understanding of how environmental inputs influence the pathogenesis of schizophrenia through epigenetic factors, e.g., histone modifications and DNA methylation. These results may provide information to improve the treatment of schizophrenia.
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