Histone Methyltransferases (GLP, G9a, SETDB1) and H3K9me2; Regulation in Psychiatric Disorders

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THESIS

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LIST OF ABBREVIATIONS

EDTA	Ethylenediaminetetraacetic Acid
HBSS	Hanks Balanced Salt Solution
RPM	Revolutions per Minute
DMSO	Dimethyl Sulfoxide
VPA	Valproic Acid
TSA	Trichostatin A
EtOH	Ethanol Alcohol
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qRT-PCR	Real-Time Polymerase Chain Reaction/Quantitative Real Time Polymerase Chain Reaction
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
EHMT1	Euchromatic Histone-lysine N-methyltransferase 1
GLP	G9a-Like Protein
G9a	Euchromatic histone-lysine N-methyltransferase 2
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
SETDB1	SET Domain, Bifurcated 1
PMSF	Phenylmethanesulfonylfluoride
PBS	Phosphate Buffered Saline

LIST OF ABBREVIATIONS CONTINUED

H_2SO_4	Sulfuric Acid
Kb	Kilobases
RCF	Relative Centrifugal Force
H3K9me2	Di-methylated Lysine 9 of Histone H3
BSA	Bovine Serum Albumin
ECL	Electrochemiluminescence
ChIP	Chromatin Immunoprecipitation
SCID	Structured Clinical Interview for DSM-IV
ddH_2O	Double Distilled Water
PDL	Poly-D-lysine
DNMT	Deoxyribonucleic Acid Methyltransferase
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders
ICD-10	International Statistical Classification of Diseases and Related Health Problems
GAD	Glutamic Acid Decarboxylase
GAT1	GABA Membrane Transporter 1
PPI	Prepulse Inhibition
H1	Histone 1
H3	Histone 3
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase Inhibitor
HMT	Histone Methyltransferase

LIST OF ABBREVIATIONS CONTINUED

PANSS	Positive and Negative Syndrome Scale
DA	Dopamine
NMDA	N-methyl-D-aspartate
PCP	Phencyclidine
GPCR	G protein coupled receptors
PBMC	Peripheral Blood Mononuclear Cell
SFNC	Stanley Foundation Neuropathology Consortium
PMI	Postmortem Interval
RIN	Ribonucleic Acid Integrity
H3K9me3	Tri-methylated Lysine 9 of Histone H3
MDB	Methylated-DNA Binding Proteins
MeCP2	methyl-CpG-binding Domain Protein 2
BDNF	Brain Derived Neurotrophic Factor
HAT	Histone Acetyltransferases
Pol II	RNA polymerase II
KDAC	Lysine Deacetylase
SIRT	Sirtuins
NAD+	Nicotinamide Adenine Dinucleotide
SAM	S-Adenosyl Methionine
MAT	Methionine Adensoyltransferase
SAH	S-Adenosylhomocysteine

LIST OF ABBREVIATIONS CONTINUED

HP1	Heterochromatin Protein 1
H3K36me3	Histone H3 lysine 36 tri-methylated
H3K4me3	Histone H3 lysine 4 tri-methylated
H3K79	Histone H3 lysine 79
H3K9	Histone H3 lysine 9
H3K27	Histone H3 lysine 27
H4K20	Histone H4 lysine 20
HDM	Histone Demethylase
PRMT	Arginine-Specific Methyltransferase Family
SET	Suppressor of variegation (Su(var)3-9), Enhancer of zeste [E(z)], Trithorax
DOT1	Non-SET-Domain Protein Family

SUMMARY

Schizophrenia is a chronic and debilitating brain disorder, which occurs in 1% of the world-wide population. Although twin studies demonstrate an inherited risk to schizophrenia, genetic studies have heralded few absolute findings, prompting our lab to examine the effects of a more global lesion, epigenetics. Epigenetic changes are stable and long-lasting chemical modifications that regulate gene activity without altering the underlying DNA code. Through epigenetics, the impact of environmental factors on changes in gene expression can be examined. DNA is packaged into chromatin, and through post-translational modifications of histone protein n-terminal tails, the epigenomic environment can be either permissive restrictive. The addition of two methyl groups of the 9th lysine of histone H3 (H3K9me2) by histone methyltransferases (HMT) leads to a restrictive chromatin state, and thus reduced levels of gene transcription. Previous literature and the work presented in this thesis demonstrate that patients with schizophrenia have a more restrictive epigenome. H3K9me2, and the HMTs G9a and Setdb1 are increased in both lymphocytes and post-mortem brain tissue from patients with schizophrenia. Also demonstrated in this thesis, three known epigenetic modifier drugs, Valproic Acid, Trichostatin A (histone deacetylase inhibitors) and nicotine (a drug of abuse disproportionately used in patients with schizophrenia) decrease HMT mRNA and total and promoter-specific H3K9me2 levels. Decreases in H3K9me2 also result in up-regulation of Bdnf mRNA.

The central hypothesis is that chromatin is restrictive in schizophrenia. As a result these various modifications can be a therapeutic target for treatment. If, through pharmacological interventions, a reduction in the restrictive state of the chromatin can be relaxed, a process deemed ‘genome softening,’ thereby allowing for increased treatment outcomes.

I. INTRODUCTION

I.A. Introduction to Schizophrenia

Schizophrenia is a chronic and debilitating mental disorder that affects 0.4 to 1% of the total world population (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV, 2000; Saha, Chant, Welham, & McGrath, 2005). Schizophrenia-like illnesses have been reported and studied as early as the 19th century, with Benedict Morel first labeling the deterioration of mental functioning seen in various late teens and early adults as 'Dementia Praecox' (Palha & Esteves, 1997). In this definition, patients were thought to be experiencing a 'premature dementia.' Morel's dementia praecox, defined in the 1850's, would prove to be the first biological model of mental illness that identified a hereditary component to the illness, while still acknowledging the effects of the social environment (Palha & Esteves, 1997). The German psychiatrist Emil Kraepelin would broaden Morel's definition by incorporating other functional psychotic disorders through a detailed textbook description of the illness, thus standardizing the illness based on a specific set of psychiatric symptoms or characteristics (Kraepelin, 1913). The 'Kraepelinian dichotomy' was the first to identify a separation between dementia praecox, which would later be known as schizophrenia, and 'manic-depressive insanity,' which would be later known as bipolar disorder (Kraepelin, 1971; 1919). Prior to this partition, all forms of psychosis were considered variations of a single underlying disease, and thus a result of the same cause and subject to a single treatment (Decker, 2007). Through this delineation, Kraepelin was able to demonstrate a clear pattern and course outcome of the separate illnesses. Dementia praecox showed a persistent decline in mental functioning leading to poor outcome, while manic-depressive insanity was an episodic illness

with symptom-free periods. Perhaps most imperative to Kraepelin's thesis was his work concerning the underlying inheritable patterns of these diseases. Through studying familial patterns of psychiatric illness, Kraepelin determined dementia praecox was a disease of the brain due to biological and genetic malfunction, specifically a form of dementia similar to Alzheimer's disease (Zec, 1995).

From the criteria established by Kraepelin, the term 'schizophrenia,' translated as 'splitting of the mind,' was created by Paul Eugen Bleuler, to specifically emphasize that this illness was not simply dementia manifested early, but a unique and individual syndrome (Bleuler, 1950; Stotz-Ingenlath, 2000). In opposition to Kraepelin's observations, Bleuler realized that schizophrenia was not invariably incurable and did not always progress to a full dementia (Andreasen & Carpenter, 1993). Bleuler's concept expanded the psychological characteristics of schizophrenia as defined by Kraepelin to include the 'four A's', believed to be the fundamental primary symptoms of schizophrenia (Black & Boffeli, 1989). The four A's are defined as **A**utistic Withdrawal, which is a focus on internal stimuli, social withdrawal and loss of contact with reality; Inappropriate **A**ffect, which is an incongruence in emotional expression and thought content or flattened affective expression; **A**mbivalence is defined as simultaneous contradictory thinking and feelings about self and others; and **A**ssociational Disturbance, now known as 'thought disorder,' which is characterized by an unpredictable train of thought, incoherence, or illogical thought processes (Moskowitz & Heim, 2011). Some manifestation of these criteria is still used today in the diagnosis of schizophrenia.

In modern times, the diagnosis of Schizophrenia is based on criteria defined in the American Psychiatric Assosciation's *Diagnostic and Statistical Manual of Mental Disorders*

(DSM-IV-TR) (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV, 2000) or the International Statistical Classification of Diseases and Related Health Problems (ICD-10) (World Health Organization, 2009) through self-report and observation by a trained mental health clinician. The ICD-10 is typically used in European countries while the DSM is used in the United States. The two systems demonstrate a high reliability in the clinical diagnosis of schizophrenia (Jakobsen *et al*., 2005). The main purpose of these two systems is to provide a categorical classification system which can be used in clinical practice, research, and administration across health professions. Through this standardization, the DSM and ICD facilitate communication within the field of mental health by providing a nomenclature that supports the standardized identification of psychiatric symptoms for diagnosis, prognosis, treatment, research, reimbursement of services provided, and record keeping. It does not address causes of mental illness, but rather provides a framework for consistent description of various illnesses (Rosen *et al*., 2009). According to the DSM, to warrant a diagnosis of schizophrenia, the following three diagnostic criteria must be met (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV, 2000). The patient must have two or more of the following symptoms for at least a one-month period: Bizarre delusions, hallucinations, disorganized behavior and speech or negative symptoms. Additionally, the patient must have significant social or occupational dysfunction, which markedly abates interpersonal relationships, hygiene and ability to work. Finally, the duration of the illness needs to have persisted for at least six months (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV, 2000). To fully and reliably assess criteria for diagnosing a mental illness, the Structured Clinical Interview for DSM (SCID) is

used by a clinician or trained mental health professional (First, 1997). The SCID has high reliability and validity, (Fogelson *et al*., 1991; Spitzer *et al*., 1992; Williams *et al*., 1992) and has the highest inter-rater concordance observed for diagnosing schizophrenia (Skre *et al*., 1991).

Several reviews of the prevalence of schizophrenia have been published, but the general consensus among them is that the lifetime risk is just below 1% of the general population (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV, 2000; Jablensky, 2000; Saha *et al*., 2005). The rate of schizophrenia can vary wildly depending on definition (van Os & Kapur, 2009). The average life expectancy of patients with schizophrenia is 12 to 15 years shorter than those not affected, due to increased occurrence of physical health problems and a higher suicide rate (Caldwell & Gottesman, 1992).

There are specific gender differences seen in schizophrenia. Disease onset occurs between 3 to 5 years earlier in men than women. Men experience a more serious form of the illness, marked with more negative symptoms and cognitive deficits, all leading to a worse overall prognosis. Females generally present with more positive symptoms, which are more responsive to psychotropic medication, leading to a more favorable disease course (Leung & Chue, 2000; Riecher-Rossler & Hafner, 2000). Some studies report a higher incidence of schizophrenia in men, although this statistic is still controversial (McGrath *et al*., 2004; Saha *et al*., 2005).

Schizophrenia is diagnosed by patient self-report of abnormalities in behavior and thorough observations by a trained clinician. The generally recognized manifested symptoms are positive, negative, and cognitive, which all encompass higher brain functions. This plethora of symptoms challenges any attempt to reconcile pathophysiology to one or a few specific

neurotransmitter systems. This is demonstrated by current antipsychotic therapeutic strategies targeting neurotransmitter systems with less than satisfactory results, (Lieberman *et al*., 2005) furthering a model of genome-wide lesion or dysregulation. Positive symptoms are present in schizophrenia, but are not normally experienced by normal individuals. Positive symptoms include bizarre or paranoid delusions, disordered thoughts and speech (conceptual disorganization), auditory, visual and more rarely tactile, olfactory and gustatory hallucinations (Andreasen & Olsen, 1982; Lenzenweger, 1999). These symptoms are accompanied by significant social or occupational dysfunction.

Negative symptoms are defined as are deficits in normal emotional and cognitive responses seen in patients with schizophrenia when compared to the general population. Negative symptoms include: social withdrawal, blunted affect and emotion, the inability to experience pleasure (anhedonia), the inability to form close interpersonal relationships with others (asociality), poverty of speech (alogia) and a lack of motivation or drive (avolition). Cognitive impairment is recognized as a core characteristic of schizophrenia, with the vast majority of patients showing some type of deficit (Mohamed *et al*., 1999). Cognitive disturbance in schizophrenia spans multiple functions, including cognitive flexibility, learning, attention, memory, language, speed of processing, social cognition, and executive functioning (Mesholam-Gately *et al*., 2009). These deficits are not a result of neuroleptics, as impaired functioning can be seen in treatment naive subjects (Saykin *et al*., 1994). Neuropsychological studies have failed to support any model of localization, but rather support a more generalized impairment (Heinrichs & Zakzanis, 1998).

There have been many theories to the causes of schizophrenia. Although twin and

adoption studies seem to demonstrate a clear inherited risk to schizophrenia, previous genetic studies have heralded few absolute findings, resulting in a great deal of debate on the number of genes involved, the location and functions of any involved genes, and their interaction with environmental factors (McGue & Gottesman, 1991).

I.A.1 Environmental Factors

Attention has turned to a variety of environmental factors in an attempt to examine non-genetic causes of schizophrenia. These environmental factors include: maternal illness and viral infection, (Brown *et al*., 2000; Clarke *et al*., 2006; Yolken & Torrey, 1995) season of birth, (Boyd *et al*., 1986) urban birth/upbringing (Kelly *et al*., 2010; Krabbendam & van Os, 2005) and cannabis smoking (Bossong & Niesink, 2010). Maternal illness and viral infection are thought to increase the risk for schizophrenia in offspring (Yolken & Torrey, 1995). In pregnant mice given a respiratory infection, offspring displayed deficits in prepulse inhibition (PPI) and exploratory behavior in open-field and novel-object tests (Shi *et al*., 2003). In humans, influenza epidemics positively correlate with incidence of schizophrenia in offspring who were in utero during the maternal influenza illness (Kendell & Kemp, 1989). Urbanicity is also believed to be a significant environmental factor in the development of schizophrenia (Kelly *et al*., 2010; Krabbendam & van Os, 2005). Individuals living in an urban environment are at higher risk for developing schizophrenia than those living in rural areas. Exposure to pollution is an environmental factor rampant in urban life. Research has linked an increase in schizophrenia diagnosis and total number of psychiatric emergency room visits during days of increased air pollution has been demonstrated, further contributing to the hypothesis of environmental involvement in later disease development (Briere *et al*., 1983). Furthermore, heavy cannabis use

in adolescence increases the risk for later onset of schizophrenia. The association between cannabis consumption and development of schizophrenia was first reliably shown in a Swedish longitudinal cohort, and has been reliably replicated (Andreasson *et al*., 1987; Zammit *et al*., 2002). Additionally, the earlier beginning use of cannabis confers greater risk for development of psychotic symptoms (Fergusson & Horwood, 1997). Although the majority of adolescents that try cannabis do not develop schizophrenia, these findings suggest there may be an aspect of a vulnerable population in which cannabis use may be the precipitating factor (Arseneault *et al*., 2002).

These environmental factors have substantial lines of supporting evidence, but not one environmental effect can completely explain schizophrenia. The exact cause of schizophrenia remains a mystery, but various neurotransmitter dysregulation and dysfunction has been highly characterized in schizophrenia. These neurotransmitter based theories of schizophrenia attempt to explain the underlying molecular mechanisms for the etiology of the disease.

I.A.2 Biochemical theories:

I.A.2.a Dopamine

The dopamine hypothesis of schizophrenia is the most influential and longest enduring explanatory model for psychosis. The dopamine (DA) hypothesis arose from the initial finding that antipsychotics exhibit dopamine-receptor D2 receptor blockade effects (Seeman *et al*., 1975; Stone *et al*., 2007). The theory suggests that over-activity in the mesencephalic DA projections to the limbic system may contribute to positive symptoms, while negative and cognitive symptoms are believed to be a result of hypo-functioning D1 receptors in the mesocortical DA pathway (Abi-Dargham & Moore, 2003).

The discovery of the revolutionary drug chlorpromazine, known in the United States as Thorazine, and its mechanism of action spurned the creation of the DA theory (Delay *et al*., 1952). Thorazine was the first drug in the phenothiazine class of drugs, which would serve as a platform for the creation of other antipsychotics. This group of drugs was shown to antagonize dopamine D2 receptors, which are predominately located in the striatum and nucleus accumbens, thus reducing the positive symptoms of psychosis (Lee *et al*., 1978; Seeman *et al*., 1975; Toda & Abi-Dargham, 2007). D2 receptor occupancy with antipsychotic drugs has been demonstrated by a large body of literature; however there is no clear relationship between receptor occupancy and clinical response (Talbot & Laruelle, 2002). Additionally, dopamine enhancing drugs such as amphetamine, cocaine, L-dopa and phencyclidine all have the ability to induce symptoms of psychosis. Chronic amphetamine abuse can produce positive symptoms exhibited in schizophrenia, such as paranoid psychosis, in normal control participants (Ellinwood *et al*., 1973). Upon administration of a subpsychotogenic psychostimulant challenge, a significant amount of patients with schizophrenia exhibited increased psychotic symptoms. Interestingly, non-amphetamine like psychostimulant drugs, such as methylphenidate had a greater psychotogenic effect than amphetamine-like drugs, even resulting in a reemergence of dormant positive symptoms (Lieberman *et al*., 1987; Sharma *et al*., 1990; Sharma *et al*., 1991). Furthermore, patients with schizophrenia release greater amounts of striatal dopamine upon amphetamine ingestion when compared to normal control participants, which corresponds directly with severity of positive symptoms exhibited (Laruelle *et al*., 1996). Increased density of striatal D2 receptors in patients with schizophrenia is the most constant post-mortem finding (Soares & Innis, 1999). But as chronic antipsychotic treatment can upregulate D2 receptor

density, it is very likely these findings are an artifact of years of neuroleptic treatment rather than inherent to the disease (Heinz, 2000).

Unfortunately, negative symptoms and cognitive impairments are resistant to D2 receptor antagonism. Other lines of evidence have demonstrated the importance of DA transmission at D1 receptors in the prefrontal cortex, lending to the idea that a deficit is responsible for said cognitive impairments (Weinberger, 1987). The level of prefrontal D1 receptor availability is a source of contention, with studies reporting both decreased D1 availability, as well as and no change in patients with schizophrenia compared to normal controls (Toda & Abi-Dargham, 2007). Atypical antipsychotics antagonize the D2 receptor with lower affinity and are rapidly displaced by dopamine, thus allowing for more normal dopamine transmission (Seeman, 2002). They also demonstrate more "limbic-specific" binding with D2 receptors, as well as a high ratio of serotonin 5-HT2 receptor binding, thus implicating other neurotransmitters in the disease course (Stone *et al*., 2000). Even with more complex receptor action on multiple receptors, there is no clear evidence that atypical antipsychotics are more effective or have higher tolerability than typical antipsychotics (Geddes *et al*., 2000; Lieberman *et al*., 2005). Furthermore, newer atypical antipsychotics are not significantly more effective at addressing cognitive deficits seen in patients with schizophrenia (Carpenter & Buchanan, 2008).

The DA theory has numerous shortcomings. First, D2 antagonists are only effective in treating the positive symptoms of schizophrenia. Additionally, there are a significant number of unpleasant side effects from these drugs, including sedation, tardive dyskinesia, and weight gain. More importantly, only around 30-40% of patients respond to D2 antagonists at any given time during the disease course, and this response is not always guaranteed in the long term, thus

suggesting alternative hypotheses need to be further explored (Lindsley *et al*., 2006). Due to these lines of evidence, the dopamine theory of schizophrenia is clearly insufficient to explain the complexity of schizophrenia.

I.A.2.b Glutamate

Alterations in glutamate transmission, the primary excitatory neurotransmitter, are also thought to be associated with schizophrenia. This theory is not meant to negate the dopamine theory, but rather supplement it and attempt to explain treatment-resistant negative and cognitive symptoms, as well as puberty-tied onset. Similar to dopamine, the N-methyl-D-aspartate (NMDA) glutamate receptor hypo-function hypothesis was developed in response to mechanisms of action in drugs of abuse.

Glutamate antagonists such as phencyclidine (PCP) and ketamine have been shown to reliably induce positive and negative symptoms as well as cognitive deficits in normal controls with striking similarities to schizophrenia (Javitt & Zukin, 1991). Chronic PCP abuse in humans can induce enduring cognitive deficits similar to those seen in schizophrenia, such as increased perservation, abnormalities in memory, attention and social interaction. Indeed, in both mouse and monkey models, chronic PCP administration is able to induce behavioral deficits that are similar to those observed in patients with schizophrenia (Jentsch *et al*., 1997; Jentsch & Taylor, 2001). Repeated low-dose PCP treatment led to a reduction in parvalbumin RNA expression in the prefrontal cortex of rats, (Cochran *et al*., 2003) which matches parvalbumin reductions seen in patients with schizophrenia (Hashimoto *et al*., 2003). Most importantly, these changes were maintained even after a reduction in PCP exposure, suggesting that cumulative chronic effects may lead to long-lasting changes in the mechanisms underlying gene expression. Furthermore,

the action of these drugs specifically targets NMDA receptors, thus distinguishing them from other psychoactive drugs (Vollenweider & Geyer, 2001).

Similar to amphetamine, glutamate antagonists can exacerbate psychotic symptoms in patients with schizophrenia (Olney & Farber, 1995). In post-mortem studies, reduction of mRNA levels of the NR1 NMDA receptor subunit has been reported in the hippocampus of patients with schizophrenia, and NMDA receptor Nr1 knockdown mice exhibit stereotypic locomotor behavior related to schizophrenia. These behaviors observed in the knockdown mice can be ameliorated by administration of the antipsychotics haloperidol and more effectively Clozapine (Mohn *et al*., 1999). Furthermore, administration of clozapine or raclopride can reverse ketamine induced prepulse inhibition (PPI) deficits, but not mGluR5 knockout PPI (Brody *et al*., 2004). Due to these findings, antipsychotic treatment focusing on this system seems to be rather promising. However, direct NMDA antagonists tend to be neurotoxic (Marino & Conn, 2002a; Marino & Conn, 2002b). Indirect action through activation of G protein coupled receptors (GPCRs) that regulate NMDA function are thus a more viable target for drug targeting. Indeed, initial drug trials using a selective agonist for metabotropic glutamate 2/3 receptors demonstrate significant improvement in both positive and negative symptoms (Patil *et al*., 2007). Although targeting this area for treatment does seem well founded, it seems unlikely that glutamate transmission abnormalities can fully explain the entire symptomology of schizophrenia. Glutamatergic dysfunction may stimulate some acute symptomatology, but not all, and there is little evidence that glutamate disturbances underlie lifelong debilitation and decline in function seen in patients with schizophrenia.

I.A.2.c GABA

GABA is the major inhibitory neurotransmitter found in the brain. Deficits in GABAergic transmission in patients with schizophrenia have been implicated in the etiology of the disease. Again, examination of the role of GABA in schizophrenia is not intended to negate the dopamine theory, but rather to augment it.

GABA is synthesized by the action of glutamic acid decarboxylase (GAD), which catalytically removes the carboxyl group of a glutamate molecule. The most reliably replicated finding has been that GAD67 mRNA expression is significantly decreased in post-mortem brain samples from patients with schizophrenia, but in the absence of overall cell loss (Akbarian *et al*., 1995; Guidotti *et al*., 2000; Impagnatiello *et al*., 1998; Veldic *et al*., 2007). The deficit in GAD67 mRNA is correlated with a significant decrease in GAD67 protein, but this has been less extensively studied (Guidotti *et al*., 2000). By contrast, mRNA and protein levels of a slightly smaller GABA synthesizing enzyme, GAD65, are not altered in patients with schizophrenia (Benes *et al*., 2000). GABA interneurons also preferentially express reelin, which is an extracellular matrix protein implicated in neuronal migration and synaptic plasticity. Decreased reelin mRNA and protein has also been demonstrated in prefrontal and temporal cortices, hippocampus, caudate nucleus and the cerebellum in post-mortem brain samples from patients with schizophrenia (Guidotti *et al*., 2000; Impagnatiello *et al*., 1998). Additionally, GABA membrane transporter 1 (GAT1) is responsible for terminating GABA signaling through clearance of GABA from the synaptic cleft (Borden, 1996). In the prefrontal cortex of patients with schizophrenia, there is a significant decrease in GAT1 mRNA in cortical layers 1-5, but not 6 (Volk *et al*., 2001). Taken together, these studies suggest the existence of profound deficits in

the regulation of GABA signaling in patients with schizophrenia.

$GABA_A$ receptors are pentameric ligand-gated ion channel that can be comprised of several different α and β subunits. $GABA_A$ receptors in the cortex generally contain one type of α subunit. Although presence of an α2 subunit is only present in about 15% of all cortical $GABA_A$ receptors, this subunit is present at over 95% of all inhibitory synapses (Fritschy & Mohler, 1995). $GABA_A$ receptors containing the α2 subunit have a higher affinity for GABA, resulting in faster activation of the receptor, hence allowing for more rapid inhibitory influence (Lavoie *et al*., 1997). This α2 subunit containing $GABA_A$ receptor is increased in patients with schizophrenia, (Benes *et al*., 1996) specifically layer II (Beneyto *et al*., 2011).

From these studies it can be understood that presynaptic markers of GABA synthesis (GAD67) and reuptake (GAT1) are decreased, while post-synaptic markers ($GABA_A$ receptors) are increased in the frontal cortex. Of particular interest for this thesis, it is now evident that the GABA interneuron is rich in epigenetic mechanisms to control its 'output', as discussed in greater detail later in this thesis (Costa *et al*., 2003; Tremolizzo *et al*., 2005; Veldic *et al*., 2007).

Given all the lines of evidence here, early development, neurobiological, social and psychological processes all play a critical role in the development of schizophrenia. Upon further examination, most of these lines of evidence were presented over fifty years ago, with minute advancement in the field since then. No single isolated organic or environmental cause has ever been identified, nor does all medication have similar efficacy rates in patients. Although positive symptoms are the most striking symptoms of schizophrenia, negative symptoms and cognitive disturbances are the core features of the illness, many of which are present in the prodromal phase, and are treatment resistant. Additionally, almost all of the neurotransmitters present in the

brain have been implicated in schizophrenia, thus lending to a more global, underlying dysregulation in gene expression.

I.B. Introduction to Epigenetics

Epigenetics is the study of environmentally induced changes in gene regulation that can be observed in the post-mitotic neuron, and can endure for the lifespan of the organism. These changes arise from post-transcriptional modifications to DNA packaging proteins (histones) or by covalent additions to the DNA sequence. Epigenetic modifications, termed the 'epigenetic code,' are dynamic, susceptible to environmental influence and determine the phenotype of the cell or organism through regulation of gene expression (Wolffe & Hayes, 1999). Some critical epigenetic processes include imprinting (Wood & Oakey, 2006), X chromosome inactivation (Chow *et al*., 2005), and cancer (Baylin & Jones, 2011). Epigenetic modifications result in protein assemblies that are commonly described as 'open' or 'closed.' Open assemblies or 'euchromatin' (wide spacing of nucleosomes, acetylation of histones) leave exposed the naked DNA sequences, permitting them to interact with DNA-binding proteins as a prelude to gene regulation. Closed assemblies (methylation of histones, DNA methylation) or 'heterochromatin' effectively seal the gene promoter from regulation by transcription factors and the transcriptional machinery. Chromatin assembly exists on a continuum, consisting of the two antithetical transcriptional states as noted, and an intermediate category titled 'facultative heterochromatin'. Facultative heterochromatin is localized to promoter regions, and can become decondensed upon signaling cues, thus leading to increased gene transcription (Oberdoerffer & Sinclair, 2007). It is often associated with the post-translational methylation of histone H3, lysine 9, which will be discussed in great detail later in this section (Rosenfeld *et al*., 2009). This intermediate state of

chromatin is the cornerstone of this thesis. "Facultative heterochromatin' may be crucial to disease pathology through continued chromatin dysregulation due to previous insult or overextension of a restrictive message.

I.B.1 DNA Methylation

DNA methylation is a heritable and reversible epigenetic modification that involves the enzymatic addition of a covalent methyl group to the pyrimidine ring of a DNA cytosine within a CpG dinucleotide. This modification is relatively stable and can be inherited through rounds of cell division (Stein *et al*., 1982). However, this modification also is reversible, thereby playing a role in life-long genomic adaption (Ramchandani *et al*., 1999; Robertson, 2002). As a result, DNA contains two layers of information, the ancestral genetic DNA sequence, and the additive methyl modifications of epigenetic information. Methylated and unmethylated cytosines within a CpG dinucleotide are tissue and function specific. DNA methylation is believed to be a repressive epigenetic modification, which influences many processes, including transcriptional modulation, genomic stability, chromatin structure, and is required for normal development. Repetitive and parasitic DNA sequences are generally hypermethylated, thus protecting the cell from their damaging effects (Noyer-Weidner & Trautner, 1993). X chromosome inactivation is also regulated via DNA methylation (Chow *et al*., 2005). CpG-rich regions, known as CpG islands, are defined as an area with GC content of greater than 60%, ranging in size from 0.5 to 5kb; they are generally hypomethylated, and are found in the promoter regions of many genes (Gardiner-Garden & Frommer, 1987; Saxonov *et al*., 2006; Takai & Jones, 2002). Methylation of CpG islands in a gene promoter region is inversely proportional to its transcriptional activity (Szyf, 1996). Alterations of DNA methylation have been recognized as an important component

of cancer development. Hypermethylation of normally unmethylated gene promoter CpG islands is the most extensively demonstrated epigenetic alteration in cancer (Baylin & Jones, 2011).

DNA methylation works to decrease gene transcription in a multitude of ways. The addition of a methyl group at the transcription regulatory sequence alters protein/DNA interactions, physically impeding the binding of various transcription factors to the underlying DNA sequence (Comb & Goodman, 1990; Iguchi-Ariga & Schaffner, 1989). DNA methylation also recruits various methylated-DNA binding proteins (MBD), such as methyl-CpG-binding domain protein 2 (MeCP2) and MBD1. These enzymes work cooperatively with other repressive enzymes, such as histone deacetylases (HDACs) and histone methyltransferases (HMTs) to create a co-repressor complex at gene promoter regions (Nan *et al*., 1997). HDACs and HMTs are chromatin modifying enzymes, thus, DNA methylation is able to spread its restrictive message to histone modifications, thus further restricting gene transcription.

The addition and maintenance of a covalently attached methyl group is catalyzed by DNA Methyltransferase (DNMT) enzymes. There exist three distinct enzymatically active DNMTs in mammals, consisting of DNMT1, DNMT3a and DNMT3b, all of which have a specific, unique and cooperative role in DNA methylation. DNMT1 was the first mammalian DNA methyltransferase cloned, (Bestor *et al*., 1988) and is believed to be the maintenance methyltransferase in mammals, demonstrating a 5 to 30 fold preference for hemi-methylated over unmethylated DNA *in vitro* (Yoder *et al*., 1997). DNMT1 also has de novo methyltransferase activity, and is responsible for the majority of DNA methylation exhibited in the mammalian genome (Chen *et al*., 2007). DNMT3a and DNMT3b are known as de novo methyltransferases, and methylate hemi-methylated and unmethylated cytosines at the same rate

(Okano *et al*., 1998). Mice lacking any of the DNMT enzymes display different developmental defects, but all die during development (Dodge *et al*., 2005; Meissner, 2010; Okano & Li, 2002). Active DNA demethylation is a controversial issue (Gavin *et al*., 2012; Ramchandani *et al*., 1999).

DNA methylation can be dynamically regulated through environmental influence. For example, the epigenetic effect of diet on phenotype was demonstrated in a landmark study of the mouse Agouti gene. Offspring promoter methylation patterns of the A_{VY} allele vary in response to maternal diet, resulting in changes in coat color, obesity and tumorigenesis (Cooney *et al*., 2002; Wolff *et al*., 1998). These results are specifically applicable to this thesis, as the effects of food deprivation during pregnancy increase the risk for schizophrenia in human offspring (Brown *et al*., 1996; Susser & Lin, 1992).

Additionally, DNA methylation plays a critical role in learning and memory. Fear conditioning increases DNMT gene expression, while memory formation can be blocked by DNMT inhibitors (Miller & Sweatt, 2007). DNA methylation also plays a critical regulatory role in depolarization-induced Brain Derived Neurotrophic Factor (BDNF) transcription. An up-regulation in Bdnf synthesis in neurons after depolarization is correlated with reduced levels of promoter CpG methylation (Martinowich *et al*., 2003). These findings are paramount in demonstrating that DNA methylation is dynamically regulated in the brain upon environmental stimulation.

I.B.2 Histone modifications

A nucleosome is the fundamental packaging structure of chromatin, and consists of >160 base pairs of DNA wrapped in a left-handed superhelical coil around an octamer of four core

histone proteins (two of each H3, H4, H2A and H2B) (Kornberg, 1974). As examined by X-ray crystallography, the H2A and H2B proteins lay near the ends of the connected DNA sequence, while the H3 and H4 proteins are connected to the internal DNA sequence (Klug *et al*., 1980). The short DNA sequence between the nucleosome is called a 'linker' sequence, and is where the H1 protein binds the DNA as it enters and leaves the nucleosome, effectively stabilizing the nucleosome. The 'linker' sequence can be of various lengths, can vary between cell types or even individual nucleosomes, and is determined by bound DNA binding proteins as well as the available energy of the bending DNA. As a result, histones generally attach at precise positions, such as promoter regions and critical regulatory elements in the underlying DNA sequence (Thoma, 1992). Nucleosomes are organized either in a condensed, transcriptionally silent state called 'heterochromatin' or a more relaxed, transcriptionally active state called 'euchromatin'. The nucleosome is the focal point of transcriptional control with each globular histone protein having a protruding amino tail which is subject to post-translational modification. Modifications include lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation and ubiquitination (Kouzarides, 2007). This 'barcode' of different post-translational modifications on the histone tail is called the "histone code" and regulates interactions between the histone protein octamer and the wound DNA strand, as well as provides an additional exposed surface for interactions and recruitment of other regulatory proteins (Strahl & Allis, 2000). These histone modifications are dynamic and integral in modifying levels of gene transcription based on the combinatorial or sequential modification on one or multiple residues.

I.B.3 Histone Acetylation

Post-translational acetylation of specific, highly conserved lysine residues reversibly

neutralizes the electrostatic interaction between the negatively charged DNA backbone and positively charged histone tails (Hong *et al*., 1993). This modification results in a relaxed chromatin structure (euchromatin), thereby increasing the accessibility of transcription factors and regulatory proteins to the DNA (Wolffe & Hayes, 1999). This effect can be examined *in vitro*, using hypo- and hyper-acetylated histone templates to demonstrate that the transcription factors USF and GAL4-AH preferentially interact with acetylated histones (Vettese-Dadey *et al*., 1996). Furthermore, in yeast, the global repressor enzyme Ssn6/Tup1 directly interacts with the tails of histones H3 and H4, and can be disassociated by increases in histone acetylation, further increasing gene transcription (Edmondson *et al*., 1996) (For a full review of associated transcription factors, refer to (Luo & Dean, 1999)). As a result, acetylation of histone tails is correlated with increased transcriptional activity (Grunstein, 1997; Kouzarides, 2007).

Histone acetylation is catalyzed by the actions of various histone acetyltransferases (HATs), which work to transfer an acetyl moiety from acetyl coenzyme A to a histone lysine residue. HATs can be grouped into two families; type A, which are localized in the nucleus and thus are closely tied to transcriptional regulation, and type B, which are found in the cytosol, and are responsible for acetylating newly synthesized histones prior to chromatin assembly (Brownell & Allis, 1996; Kuo & Allis, 1998). Some of the HATs have also been identified as key components and associates of the RNA polymerase II (Pol II) transcription machinery, (Kim, Lane, & Reinberg, 2002) providing a straightforward mechanism to account for increases in transcriptional activity (Wittschieben *et al*., 1999). Furthermore, some transcription factors also possess HAT activity (Mizzen *et al*., 1996).

Removal of histone acetylation is catalyzed by enzymes called histone deacetylases

(HDACs; or lysine deacetylases - KDAC), which facilitate re-establishment of the electrostatic connection between lysine and DNA, creating a more transcriptionally restrictive histone environment (Pazin & Kadonaga, 1997). Classical HDACs can be grouped into families; class I HDACs (HDAC 1, 2, 3 and 8) are found almost exclusively in multi-protein complexes in the nucleus, while class II HDACs (HDAC 4, 5, 6, 7, 9 and 10) are found in the cytoplasm, target both histone and non-histone proteins, and are able to shuttle into the nucleus in response to stimulation (Yang & Seto, 2008). Class III HDACs are known as sirtuins (SIRT 1-7) and deacetylate a broad range of substrates. These enzymes are dependent on the oxidizing cofactor nicotinamide adenine dinucleotide (NAD+) cofactor, and thus are linked to metabolic fluctuation and energy consumption (Rajendran *et al*., 2011). Class IV HDAC consists only of HDAC11. Little is known about HDAC11 besides the fact that it is highly conserved evolutionally (Gao *et al*., 2002).

I.B.4 Histone Deacetylase Inhibitors

The enzymatic activity of HDACs can be blocked by small molecule pharmacology. There are a multitude of pharmacological inhibitors known, including sodium n-butyrate, (Riggs *et al*., 1977) Valproic acid (VPA), (Gottlicher *et al*., 2001) and the most potent Histone Deacetylase Inhibitor (HDACi) known as Trichostatin A (TSA). TSA is the most potent reversible HDACi, and broadly inhibits class I, II and IV HDAC enzymes, and through this inhibition, histone acetylation, or ‘open’ chromatin is effectively increased (Finnin *et al*., 1999). TSA was originally reported as an antifungal antibiotic, (Tsuji *et al*., 1976) but was later demonstrated to result in increased *in vivo* histone acetylation and decreased *in vitro* HDAC activity at nanomolar concentrations (Yoshida *et al*., 1990).

TSA is commonly utilized as a reference substrate in new HDACi creation, and is useful in cell culture models of epigenetic regulation as doses up to 1μM are effectively utilized (Kozlowska & Jagodzinski, 2006; Mishra *et al*., 2001). TSA is also useful in animal models, as intraperitoneal injections of TSA are not toxic to adult pregnant mice nor result in embryo or developmental malformations to their fetus (Nervi *et al*., 2001). Treatment with HDAC inhibitors such as TSA result in highly specific and targeted changes in gene expression, increasing mRNA expression of only around 2% of genes expressed in mammalian cells, without affecting generalized function (Mishra *et al*., 2001; Nambiar *et al*., 2002; Van Lint, Emiliani, & Verdin, 1996). TSA treatment does not alter expression levels of GAPDH mRNA, (Van Lint *et al*., 1996) β-actin mRNA, (Camargo *et al*., 2011) or H1 linking protein, (Girardot *et al*., 1994) all of which are methods commonly used for as normalization. Furthermore, upon removal of TSA, gene expression can return to baseline levels (Hou *et al*., 2002). Perhaps more interestingly, the effects of TSA are amplified upon lymphocyte activation, (Dangond & Gullans, 1998) similarly to the 'genome-softener' concept that has been previously proposed (Sharma *et al*., 2005). Most pertinent to this thesis, previous studies have demonstrated that TSA inhibits H3K9 methylation, a restrictive chromatin mark (Zhang *et al*., 2007). This thesis utilizes this finding to further elucidate the effect of HDACis on histone methylation.

A second group of reversible HDACis are short-chain fatty acids, including butyrate and Valproic acid (VPA) (Monti *et al*., 2009). VPA is well tolerated in patients and has been used in epilepsy treatment (Loscher, 1999) and in treatment of mood disorders, particularly bipolar disorder (Emrich *et al*., 1980; Fountoulakis *et al*., 2005). Among its other functions in the body, VPA causes hyperacetylation of histones H3 and H4 both *in vitro* and *in vivo*. Additionally, it

inhibits Class I HDAC activity more efficiently than Class II HDAC enzymes, most likely through binding to the catalytic center of the enzyme (Gottlicher *et al*., 2001). Additionally, VPA uniquely induces proteasomal degradation of HDAC2 (Kramer *et al*., 2003). VPA also acts on the GABAergic system, even increasing GABA levels in human plasma, (Loscher & Schmidt, 1980) thus enhancing the inhibitory tone of the brain and increasing its complexity in epigenetic research (Macdonald & Bergey, 1979). VPA is less efficient than TSA, exerting HDACi effects at millimolar range rather than nanomolar range, and specifically inhibiting Class I HDACs with more efficiency than Class II HDACs, whereas TSA affects all HDAC classes equally (de Ruijter *et al*., 2003).

HDAC inhibitors, such as VPA, have recently shown usefulness in other clinical applications due to their ability to inhibit growth of several types of cancers. VPA can suppress tumor growth and metastasis, (Kuendgen & Gattermann, 2007) and can induce tumor differentiation and apoptosis (Blaheta *et al*., 2005). VPA has also been shown to markedly increase radiation-induced cell death and apoptosis in cancerous cells, supporting its clinical relevancy (Karagiannis *et al*., 2006).

I.B.5 Histone Methylation

Histone methylation is a more thermodynamically stable histone modification compared to the transient histone acetylation, and thus more attention has recently been focused on this methylation modification as an important regulator of gene transcription (Sharma & Chase, 2012). Histone methylation is a covalent post-translational modification commonly occurring on arginine and lysine residues of histone tails. Through utilization of the enzymatic donor s-adenosyl methionine (SAM), arginine sites can be either mono- or di-methylated, while lysine

residues can be mono-, di-, or tri-methylated (Zhang & Reinberg, 2001). Lysine methylation is the most notable of these residues for its diversity in its post-translational modifications, and thus binary-esque influence on gene expression. Methylation of unstructured n-terminal tails of histones was established close to 50 years ago, (Murray, 1964) although due to limitations in biological research tools, significantly less research has been conducted concerning its significance (Rice & Allis, 2001). Histone methylation happens to be a particularly durable and complex modification compared to acetylation and phosphorylation, with decreased turnover of methyl groups and slower rates of formation (Zee *et al*., 2010). Although relatively stable, histone methylation is a dynamic process that plays a significant role in chromatin remodeling.

Nucleosomes that are organized in a condensed transcriptionally silent state durably block the assembly of transcription factors to local gene promoter regions, thereby suppressing gene transcription or hermetically sealing the promoter from external stimuli and thus reprogramming (Kim *et al*., 2009). Heterochromatin can inactivate entire chromosomes, as in such cases as the inactive X chromosome in the human female (Barr body) (Heard *et al*., 2001) or entire single alleles (Goldmit *et al*., 2005). The transition from euchromatin to heterochromatin is a multi-step process. First, through the decline of environmental signals, transcriptional activators and stimulatory signals begin to decrease around the promoter region of a gene (Epsztejn-Litman *et al*., 2008). The di-methylation of euchromatic H3K9 (discussed in greater detail later) associated with active gene promoter regions, acts as an adaptor and recruits the adeptly named Heterochromatin Protein 1 (HP1) corepressor family. Identified in *Drosophila Melanogaster,* (James & Elgin, 1986) this highly conserved family binds directly to the methylated H3K9, thus repressing transcription in both heterochromatic and euchromatic

regions (Kwon & Workman, 2008). The HP1 family consists of HP1α and HP1β, which are localized to repressed chromatin, and HP1γ, which is associated with transcriptionally active chromatin (Lomberk *et al*., 2006). HP1 function is further defined by its post-translational modifications, adding another layer of complexity to its role in histone regulation (Lomberk *et al*., 2006). Upon recruitment to a restrictive chromatin modification, HP1 can act as a platform and recruit other restrictive chromatin enzymes such as histone methyltransferases (discussed later in greater detail), deacetylases, and DNMTs. All known catalytically active DNMTs interact with histone deacetylases and methyltransferases to repress gene transcription. Through this multi-protein recruitment, the restrictive mark propagated through HP1 can spread to other neighboring promoters, effectively decreasing their transcription. This process is known as 'chromatin creep,' and may be a significant incubator for disease, as dysregulation of one gene can easily spread to healthy promoters expressing no dysregulation (Sharma *et al*., 2012).

Histone methylation at specific residues is generally associated with a restrictive chromatin state, although this post-translational modification can lead to gene activation. As a forewarning, such generalized roles of these modifications are a gross simplification of their overall functioning in the cell. Histone modifications are correlated with changes in gene expression, not all encompassing indicators of gene expression. Additional interplay between recruitment of various corepressor/transcription factors and DNA methylation result in a highly regulated and complex method of manipulating gene expression that is still being explored. Histone H3 lysine 36 (H3K36me3) and histone H3 lysine 4 (H3K4me3), are commonly associated with increased transcription, euchromatin, and transcriptional elongation (Eissenberg & Shilatifard, 2010; Wagner & Carpenter, 2012). Histone H3 lysine 79 (H3K79) is still poorly

understood, but has been linked to transcriptional activation, with implications in leukemia (Frederiks *et al*., 2011; Nguyen & Zhang, 2011; Nguyen *et al*., 2011). Further adding to the complexity, H3K79 mono-methylation has been linked to gene activation, while tri-methylation has been linked to repression (Barski *et al*., 2007). Histone H3 lysine 9 (H3K9), histone H3 lysine 27 (H3K27) and histone H4 lysine 20 (H4K20) are all associated with transcriptional repression (Zee *et al*., 2010). H3K27 has been implicated in repression of HOX genes and silencing of many developmental genes throughout both euchromatic and heterochromatic regions of the genome (Barski *et al*., 2007; Swigut & Wysocka, 2007). Very little is known about H4K20 methylation other than its role in DNA repair and formation of constitutive heterochromatin, and thus continues to be under active investigation (Balakrishnan & Milavetz, 2010).

This thesis focuses specifically on the ninth lysine of histone H3 (H3K9). This residue has been shown to receive the diametrically opposed functional groups of acetylation and methylation post-translational modifications, thus serving as a molecular 'switch' for gene activation. Methylation of H3K9 is a highly conserved post-translational modification of both transcriptionally facultative and 'closed' chromatin, known as heterochromatin (Barski *et al*., 2007). H3K9 modifications are found specifically in gene-rich areas of the genome, and are inversely associated with gene expression (Wen *et al*., 2009). Levels of methylated H3K9 expression vary by cell type, with much more extensive H3K9 methylation in the liver compared to the embryonic stem cell. Interestingly, the adult brain only represses ~10% of its total genome through H3K9 methylation, (as opposed to ~46% in the liver) supporting the idea that the brain utilizes a high number of genes, thus demonstrating more genomic plasticity and dynamic use of

gene regulation. Alternatively, the brain may be more susceptible to increasing H3K9 modifications across previously 'healthy' genomic territory (Wen *et al*., 2009).

Histone methylation is a dynamic process that is susceptible to environmental influences, as highlighted in the previous paragraph. Contextual fear-conditioning is able to induce H3K9me2 histone modifications, an effect that is reversible with HDACi treatment (Gupta *et al*., 2010). This finding will be corroborated later in this thesis, as the HDACis TSA and VPA will be demonstrated to have an effect on H3K9me2 and participating enzymes. These findings further support the importance and dynamic regulation of histone modifications in learning and memory.

In a paper paramount to this thesis, Maze *et al* critically examined histone methylating and demethylating enzymes following chronic cocaine exposure (Maze *et al*., 2010). Of all enzymes studied in the rat nucleus accumbens, only the HMTs G9a and GLP demonstrated consistent down-regulation following chronic cocaine administration. As a result, global H3K9me2 levels were significantly decreased (Renthal *et al*., 2009). Transcription of numerous genes are increased upon chronic cocaine treatment, a finding perhaps explained in part by this mechanism. Genes targeted by the histone methyltransferase (HMT) enzyme G9a become more transcriptionally active following chronic cocaine use as G9a is more repressed; a point further elucidated in nucleus accumbens G9a knockdown animals. These animals show enhanced drug-associated behavior, while G9a overexpression inhibits this response (Maze *et al*., 2010). Through examining drugs of abuse, the importance of epigenetic mechanisms on long-term changes in gene expression and subsequent effects on behavior can be demonstrated. From this study, two critical modulators of the restrictive chromatin mark H3K9me2 were identified as

being manipulated through environmental factors, and thus chosen for further study in this thesis.

I.B.6 Histone Methyltransferases

Methylation status of histone tail residues are subject to dynamic modifications through the activities of histone methyltransferases (HMTs) and histone demethylases (HDMs). These enzymes are highly conserved and exhibit specificity for mono, di, or tri-methylation. These enzymes belong to three distinct families: the arginine-specific methyltransferase family (PRMT family), the set domain-containing protein family, and the non-set-domain proteins (DOT1). The arginine-specific methyltransferase family (PRMT) consists of nine members, that either mono-methylate, asymmetric di-methylate or symmetric di-methylate an arginine residue on a histone tail, and are grouped in families based on the type of methylation pattern they create (Di Lorenzo & Bedford, 2011). The DOT1 methyltransferase family specifically methylates the lysine 79 on histone H3 (H3K79), and is associated with increased gene transcription (Gao & Liu, 2007; van Leeuwen *et al*., 2002).

Histone lysine methylation is almost exclusively catalyzed by enzymes in the SET domain-containing family of methyltransferases. This highly conserved, 130 amino acids long sequence was first identified in three separate *Drosophila* proteins, Suppressor of variegation (Su(var)3-9), (Tschiersch *et al*., 1994) the polycomb-group protein Enhancer of zeste [E(z)], (Jones & Gelbart, 1993) and trithorax, (Stassen *et al*., 1995) which is where the acronym 'S.E.T.' originates from. Seven subfamilies of SET domain-containing proteins exist: SUV4-20, (Sakaguchi *et al*., 2008; Schotta *et al*., 2004; Yang *et al*., 2008) SMYD, (Brown *et al*., 2006; Hamamoto *et al*., 2004) EZ, (Cao & Zhang, 2004) SET2, (Krogan *et al*., 2003) SET1 (Martin & Zhang, 2005) and SUV39 (Dillon *et al*., 2005). These enzymes all utilize the transfer of a

methyl group from s-adenosylmethionine to a lysine residue of a histone tail, but differ in their substrate and product specificity.

The SUV39 family has been most extensively researched, as they were the first SET-domain methyltransferases identified. Enzymes in this family include G9a, G9a-like protein (GLP) and SETDB1, although this list is not all inclusive (Kim *et al*., 2003; Krishnan *et al*., 2011). These proteins specifically methylate lysine 9 of histone H3 (H3K9), a signature and dynamic modification of restrictive chromatin.

SETDB1 (ESET) is a SET domain-containing methyltransferase that is the only euchromatic HMT to specifically di- and tri-methylate lysine 9 of histone H3, effectively repressing gene transcription. The SET domain is interrupted by an insertion, 347 amino acids long, to create a 'bifurcated' domain. This unique insertion is not demonstrated in any other histone methyltransferase, and is highly conserved between species (Harte *et al*., 1999). SETDB1 acts in a progressive manner, binding to a previously di-methylated residue and remaining bound until subsequent methylation occurs (Wang *et al*., 2003; Zee *et al*., 2010). SETDB1 consists of 22 exons, and is 36kb long. There exists one splice variant which consists of only the first 12 exons, thus lacking the catalytic SET domain (Blackburn *et al*., 2003). The full length SETDB1 mRNA is ubiquitously expressed, and has been demonstrated to be measurable in resting B lymphocytes (Baxter *et al*., 2004). Tri-methylation of H3K9, acts as a platform for recruitment of HP1 proteins, previously discussed, and proteins associated with DNA methylation, such as MBD1 and DNMTs (Li *et al*., 2006; Schultz *et al*., 2002).

SETDB1 null mutants are embryonic lethal between 3.5 to 5.5 days post coitum, demonstrating this methyltransferase's role in early development (Dodge *et al*., 2004).

Spontaneous mutations due to endogenous retroviruses widely scattered throughout euchromatic regions of the genome are silenced by H3K9me3 mediated repression through SETDB1 action (Karimi *et al*., 2011; Matsui *et al*., 2010). Perhaps similarly, SETDB1 has been implicated in cancer. Its status as an oncogene was shown through SETDB1-mediated increases in melanoma formation on *zebrafish* (Ceol *et al*., 2011). Additionally, SETDB1 has been shown to directly interact with tumor suppressor genes p53 and RASSF1A, both of which are down-regulated through increased H3K9me3 levels in cancer (Li *et al*., 2006). SETDB1 has been implicated in other illnesses besides cancer; for example. SETDB1 mRNA expression and resulting H3K9me3 protein levels are significantly increased in both patients with Huntington Disease and transgenic Huntington mice. Down-regulation of SETDB1 through mithramycin, an antibiotic, and cystamine, (Fox *et al*., 2004) in transgenic Huntington mice, reduces increased levels of H3K9me3, improves body weight and motor performance and extends survival. As such, SETDB1 has been identified as a promising target for treatment in Huntington disease (Ryu *et al*., 2006).

G9a (eu-HMTase-2 or ehmt2) is the predominant euchromatic methyltransferase of H3K9 (Tachibana *et al*., 2002). G9a has a closely related enzyme titled G9a-like-protein (GLP or ehmt1), with which it forms a functional dimer. The human G9a gene has 28 exons, and is 17.3 kilobases (kb) long, while GLP is 25 exons and 120 kb. GLP has 24% sequence homology to G9a, with GLPs first three and last 20 exons being identical in both enzymes (Dillon *et al*., 2005; Volkel & Angrand, 2007). G9a and GLP can form homomeric or heteromeric complexes, demonstrating a preference for a heteromeric interdependent grouping (Tachibana *et al*., 2005). This G9a/GLP dimer is responsible for the bulk of H3K9me2 modifications across the genome,

although they do not substitute for each other, and their function is not redundant (Shinkai & Tachibana, 2011). In mammals, loss of either GLP or G9a protein can result in embryonic lethality and a severe reduction of H3K9 mono- and di-methylation (Tachibana *et al*., 2005). Additionally, G9a is a crucial regulator of end point embryonic stem cell determination by silencing the undifferentiated-specific transcription factor Oct3/4 through G9a mediated H3K9 heterochromatinization (Feldman *et al*., 2006). Re-activation of the pluripotency factor Oct3/4 can be catalyzed through multiple epigenetic mechanisms, including G9a knockdown or overexpression of the histone demethylase Jhdm2a. G9a inactivation through catalytic domain point mutation can also result in Oct3/4 re-activation (Epsztejn-Litman *et al*., 2008; Ma *et al*., 2008). G9a has been implicated in cancer, and is highly expressed in aggressive lung cancer cells, and this increase in expression is correlated with a poorer prognosis (Chen *et al*., 2010). Specifically, increased H3K9 methylation and resulting DNA methylation via G9a has been shown to exert aberrant repression of various tumor repressor genes, thereby promoting cell proliferation (Dong *et al*., 2012; Wozniak *et al*., 2007). G9a and GLP can also methylate non-histone proteins, as is the case with lysine 373 of the tumor suppressor gene p53. Di-methylated p53 is an inactivating post-translational modification, thereby implicating GLP and G9a as oncogenes (Huang *et al*., 2010).

H3K9me2 has not been extensively studied in the brain, and until recently the regulation and role of G9a or GLP was not known. Additionally, in mice, GLP and G9a conditional knockouts produce a significant decrease in global H3K9me2 levels, and inappropriate gene expression, which result in deficits in learning, reduction in exploratory behaviors, motivation and environmental adaption such as contextual fear conditioning (Schaefer *et al*., 2009; Shinkai

& Tachibana, 2011; Tachibana *et al*., 2005; Tzeng *et al*., 2007). These effects appear to be neuron specific. Mouse GLP/G9a postnatal, neuron-specific conditional knockdown results in decreases of H3K9me2, but not H3K9me3 levels, leading to increases in both non-neuronal and neuronal progenitor genes. In *Drosophila,* GLP/G9a knockouts impair courtship behavior as well as short and long term memory (Kramer *et al*., 2011). In humans, deletions or loss-of-function mutations of G9a results in Kleefstra Syndrome, a genetic disorder which results in learning disability and developmental delay (Nillesen *et al*., 2011). Haploinsufficiency as a result of microdeletions in the human GLP gene result in a clinically recognizable illness titled 9q subtelomeric deletion syndrome (Kleefstra *et al*., 2005). Symptoms of this syndrome include severe mental retardation, hypotonia (reduced muscle strength), brachycephaly (flat head syndrome), epileptic seizures, flat face, hypertelorism (abnormally increased distance between two organs or parts), synophrys (unibrow), anteverted nares (tipping forward of the nose), carp mouth with macroglossia, and heart defects. Mouse GLP/G9a postnatal, neuron-specific conditional knockdown results in decreases of H3K9me2, but not H3K9me3 levels, leading to increases in both non-neuronal and neuronal progenitor genes. These increases in transcription are correlated with behavioral phenotypes, such as deficits in learning, reduction on exploratory behaviors, motivation and environmental adaption, such as contextual fear conditioning, similarly to the human 9q34 mental retardation syndrome (Schaefer *et al*., 2009).

As previously mentioned, the H3K9 can be methylated or acetylated, thus acting as a 'switch' between open and closed chromatin modifications. Upon G9a knockout, and resulting decreases in H3K9me2 levels, there are coordinate increases of acetylated H3K9. This, perhaps, is indicative of a competitive interaction between the acetylation and methylation modification

(Tachibana *et al*., 2002). Further suggesting the dynamic interplay between histone modifications, increases in methylation of H3K4, the open chromatin modification, inhibits H3K9me2 formation (Wang *et al*., 2001).

I.B.7 Histone Methyltransferase Inhibitors

Although histone methyltransferases act in a similar repressive mechanism as HDACs and DNMTs, there have been very few compounds that selectively inhibit HMTs. BIX-01294 is a small molecule, recently discovered, which antagonizes both G9a and GLP, resulting in a decrease in levels of the H3K9me2 protein. BIX-01294 has an IC50 of 1.7 mM and is noncompetitive with the cofactor s-adenosylmethionine (Chang *et al*., 2009). One other HMT inhibitor has been recently identified, titled UNC0224, which is a more potent inhibitor of G9a, demonstrating an IC50 of 15nM (Liu *et al*., 2009). Very little research has been conducted using these drugs; although, based on the research presented here, they may have great clinical application. Interestingly, through mechanisms unknown, the HDAC inhibitor TSA inhibits specifically H3K9 di-methylation *in vitro,* (Dai & Rasmussen, 2007; Gavin *et al*., 2009) an effect that will also be demonstrated and discussed later in this thesis.

Together, these studies indicate that environmental effects have profound impact on the epigenetic modifications of histone acetylation, methylation and DNA methylation. Additionally, these data support the role for epigenetic regulation in the manipulation of synaptic plasticity and behavior, implicating dysregulation in human disease. If dysregulation of epigenetic mechanisms is indeed the underlying cause of various diseases, then perhaps in reprogramming of these pathways through pharmacological approaches lies the cure.

I.C. Epigenetics and Schizophrenia

Identifying causative factors in the development of schizophrenia have been shortcoming, even with years of targeted research. The most powerful predictor of schizophrenia is an identical twin or first degree relative with the illness, suggesting a component of heritability (Gottesman & Bertelsen, 1989). Although concordance of monozygotic twins is significantly increased compared to dizygotic twins, it is not 100%, (Davis *et al*., 1995) implicating non-familial environmental factors in disease occurrence (Franzek & Beckmann, 1998). Furthermore, twin and family genome wide studies have heralded very little in the way of pinpointing specific candidate gene sequence variation related to schizophrenia (Crow, 2008; McGue & Gottesman, 1991). Furthermore, there are a plethora of features of schizophrenia that are difficult to explain using a purely genetic approach. These include: first and foremost, disease non-concordance in monozygotic twins, (Davis *et al*., 1995; Franzek & Beckmann, 1998) gender-specific disease course, (Leung & Chue, 2000; Riecher-Rossler & Hafner, 2000) and hormone-dependent illness onset (Galdos *et al*., 1993). Given all these factors, epigenetics is the ideal scaffold to examine underlying non-mendelian etiology of schizophrenia, as it is the interface between underlying genetic code and environmental impact. As discussed previously, environmental influences, such as drugs of abuse, maternal interactions and stress, can result in significant, lasting and heritable changes in gene expression.

A hallmark abnormality in schizophrenia is aberrant gene regulation, including glutamic acid decarboxylase (GAD67) and reelin, which can result directly from abnormalities in epigenetic processes (Torrey *et al*., 2005). Additionally, studies have demonstrated significant decreases in Brain Derived Neurotrophic Factor (Bdnf) in patients with schizophrenia (Buckley

et al., 2007; Favalli *et al*., 2012; Jindal *et al*., 2010; Thompson Ray *et al*., 2011; Torrey *et al*., 2005). In fact, most studies in schizophrenia report a down-regulation of gene transcription which is most suggestive of a restrictive epigenome. Glutamic acid decarboxylases (GAD) exist in two isoforms, GAD65 and GAD67, and catalyze the decarboxylation of glutamate to GABA, the inhibitory neurotransmitter. GAD65 is found in axon terminals, while GAD67 is readily detectable in cell bodies and dendrites (Erlander *et al*., 1991). Reelin is a secreted extracellular matrix protein that regulates neuronal migration in the developing brain, as well as modulates synaptic plasticity, and dendritic spine development in the mature brain. Currently little is known regarding the regulation of reelin in mature neurons (Forster *et al*., 2010; Hattori, 2011). Down-regulation of GAD67 and reelin mRNA and protein is one of the most replicable findings in schizophrenia (Akbarian *et al*., 1995; Akbarian & Huang, 2006; Benes *et al*., 2007; Eastwood & Harrison, 2003; Fatemi *et al*., 2005; Guidotti *et al*., 2000; Hashimoto *et al*., 2003; Hashimoto *et al*., 2008; Impagnatiello *et al*., 1998; Volk *et al*., 2000). Critically, patients with schizophrenia have normal levels of cortical GABAergic neurons, and demonstrate no differences in prefrontal gray and white matter volume. Differences lie in the reduced number of dendritic spines in patients with schizophrenia, possibly occurring as a result of this Reelin hypo-expression (Akbarian *et al*., 1995; Bennett, 2011).

GAD67, Reelin and Bdnf are all epigenetically regulated. All three genes have promoter regions that fall within CpG islands, and thus changes in gene expression are subject to DNA methylation patterns. In a landmark study, through using a neuronally committed human teratocarcinoma cell line (NT2) reelin promoter methylation and mRNA expression can be examined both pre and post differentiation. Upon differentiation, the reelin promoter region

becomes hypo-methylated, which is associated with increases in reelin mRNA expression (Chen *et al*., 2002). Methionine is an amino acid that upon ingestion is converted to s-adenosylmethionine, which serves as a methyl-donor in enzymatic reactions, including histone methylation. Treatment with methionine induces a down-regulation of reelin and GAD67 mRNA, which is correlated with increases in promoter methylation (Noh *et al*., 2005). Additionally, decreases in DNA methylation at the gene promoter play a crucial role in increases in depolarization-induced increases in Bdnf mRNA transcription (Martinowich *et al*., 2003). Furthermore, methylation patterns of the Bdnf gene established in childhood can result in altered gene expression in the mature brain (Roth *et al*., 2009).

The possible role that epigenetics could play in disease course and treatment was first demonstrated over 50 years ago through experiments with methionine. It was initially believed that increases in methionine in the diet of patients with schizophrenia would be therapeutically beneficial through increasing the amount of methylated dopamine, thereby inactivating the perceived surplus of neurotransmitter in the brain (Antun *et al*., 1971; Berlet *et al*., 1965). Unfortunately, treatment with methionine worsens symptoms in patients with schizophrenia (Nestoros *et al*., 1977). Furthermore, methionine treatment in mice elicits various schizophrenia endophenotypes, such as decreases in social interaction, and impairments in prepulse inhibition and attention; all of which could be rectified through epigenetic manipulation by administration of Valproic acid (Tremolizzo *et al*., 2002; Tremolizzo *et al*., 2005). In both post-mortem brain tissue and erythrocytes from patients with schizophrenia, methionine adenosyltransferase (MAT), the enzyme which catalyzes the conversion of methionine to s-adenosylmethionine (SAM), demonstrates an increased affinity towards methionine (Gomes-Trolin *et al*., 1998).

Furthermore, increased levels of SAM have been demonstrated in Broadmanns area 9 of the prefrontal cortex in patients with schizophrenia (Guidotti *et al*., 2007). Finally, levels of s-adenosyl-L-homocysteine (SAH), created after SAM donates its methyl group in a catalytic reaction, and are higher in leukocytes in patients with schizophrenia compared to normal controls (Bromberg *et al*., 2008). SAM serves as the methyl-donor for the histone methyltransferases, thus these studies concerning this enzymatic reaction, when taken together, demonstrate an increased propensity for the existence of a hyper-methylated state, which could be leading to epigenetic changes in the psychotic brain, specifically histone methylation (Costa *et al*., 2002).

Indeed, these increases in methyl-catalyzing agents have been demonstrated to have epigenetic consequences in patients with schizophrenia. In post-mortem brain tissue from patients with schizophrenia, the reelin promoter has increased methylation patterns, which are correlated with decreases in reelin mRNA expression (Abdolmaleky *et al*., 2005; Grayson *et al*., 2005). Further support for an overall restrictive epigenome in the psychotic brain, DNMT1 (Veldic *et al*., 2005) and DNMT3a (Zhubi *et al*., 2009) mRNA expression is increased in GABAergic neurons from schizophrenic post-mortem tissue. These increases in DNMT expression are also correlated with decreases in GAD67 and reelin mRNA (Veldic *et al*., 2004).

As previously discussed, histone deacetylases (HDAC) are enzymes which remove an acetyl group from a histone tail. Upon removal of this post-translational modification, the chromatin is more condensed, leading to a decrease in gene transcription (Pazin & Kadonaga, 1997). Patients with schizophrenia show high levels of restrictive chromatin through low levels of acetylated histones. In post-mortem brains of patients with schizophrenia, HDAC1 mRNA is up-regulated (Benes *et al*., 2007; Sharma *et al*., 2008). Interestingly, female patients had almost

twice the amount of HDAC1 mRNA in their prefrontal cortex than their male counterparts (Sharma *et al*., 2008). Furthermore, GAD67 mRNA was negatively correlated with mRNA expression levels of HDAC1, 3 and 4 (Sharma *et al*., 2008). Early studies in peripheral lymphocytes extracted from drug free patients with schizophrenia have demonstrated increases in condensed heterochromatin compared to normal controls (Issidorides *et al*., 1975; Stefanis & Issidorides, 1976). Lymphocyte treatment with 5-azacytidine, led to a lesser degree of heterochromatin decondensation in patients with schizophrenia compared to control participants (Kosower *et al*., 1995).

In a pioneering study, the Sharma lab sought to examine the characteristics of chromatin remodeling in psychiatric patients treated with the histone deacetylase inhibitor (HDACi) Valproic Acid (VPA) *in vivo* (Sharma *et al*., 2006). As stated previously, patients with schizophrenia exhibit increases in HDAC1 mRNA, contributing to an overall more restrictive chromatin state. Treatment with an HDACi would then negate these intrinsic increases, thus leading to a more plastic and open epigenome. Patients with schizophrenia and bipolar disorder were both administered a clinically relevant dose of VPA for a 4 week treatment period. Upon study termination, both diagnostic groups demonstrated increases of the 'open' chromatin mark acetylated histone H3 in extracted lymphocytes. VPA treatment was less efficacious in increasing acetylated H3 levels in patients with schizophrenia compared to patients with bipolar disorder, which is consistent with other published work (Kosower *et al*., 1995). Additionally, the percent change in acetylated H3 levels was positively correlated with percent change in General Psychopathology scores on the Positive and Negative Symptom Scale (PANSS), and remained highly significant even after controlling for blood levels of VPA. Levels of acetylated H4 were

also increased after 4 weeks of VPA treatment. Similarly to acetylated H3 levels, patients with schizophrenia showed a muted increase of acetylated H4 levels compared to patients with bipolar disorder (Sharma *et al*., 2006). The data suggests that patients with schizophrenia have a global restrictive chromatin state that is less receptive to remodeling in response to external stimuli, which could be contributing directly to the disease phenotype.

To further expand upon the results described above, the Sharma lab utilized an *in vitro* cultured lymphocyte technique from patients with schizophrenia and normal controls (Gavin *et al*., 2009). Through culturing lymphocytes, chromatin assemblies and histone modifications can be examined and manipulated without confounds of interacting medications and absorption rates. Additionally, epigenetic changes as a result of experimental drugs at doses that may not be acceptable in a living participant can be utilized. Using this technique, lymphocytes from healthy controls were treated in culture with the HDACi Valproic acid (VPA) or Trichostatin A (TSA), eliciting significant dose dependent increases in the 'open' chromatin mark histone H3, acetylated at lysine 9 and lysine 14 (H3K9,K14ac). Furthermore, VPA or TSA administration resulted in a significant increase of H3K9,K14ac bound to the GAD67 promoter, resulting in dose dependent increase of GAD67 mRNA. Through this study, gene expression was shown to occur directly through chromatin remodeling. In a complementary *in-vivo* study, GAD67 mRNA expression and H3K9,K14ac levels were measured in patients treated with VPA for 4 weeks (Gavin *et al*., 2009). In these sets of studies, patients with schizophrenia had significantly lower H3K9,K14ac levels compared to patients with bipolar disorder. Clinically relevant VPA serum levels significantly increased GAD67 mRNA levels among patients with schizophrenia. By utilizing two separate approaches, epigenetic mechanisms, such as the increase of 'open'

chromatin (H3K9,K14ac) through HDAC inhibition and resulting up-regulation of the schizophrenia candidate gene GAD67, chromatin remodeling is demonstrated as a potential pathophysiological mechanism. Additionally, *in-vitro* lymphocyte culture is an effective and legitimate method for parsing apart the properties of chromatin remodeling and epigenetic regulation of schizophrenia candidate genes (Gavin *et al*., 2009). The approach is powerful and introduces molecular pharmacological techniques to studying the physiology of cells from living patients.

Furthering the results that patients with schizophrenia have a more restrictive chromatin that was more resistant to manipulation, (Sharma *et al*., 2006) a potent HDAC inhibitor Trichostatin A (TSA) was administrated in culture to lymphocytes extracted from patients with schizophrenia and normal controls (Gavin *et al*., 2008). From these culture studies, it was established that patients with schizophrenia had lower baseline levels of the 'open' chromatin mark histone H3, acetylated at lysine 9 and lysine 14 (H3K9,K14ac) compared to normal controls. Additionally, after 24 hour incubation with TSA, percent changes in H3K9,K14ac differed between patients and control participants, showing that in patients, 'open' chromatin was less responsive to pharmacological intervention. In lymphocytes of normal control participants, TSA induced changes in H3K9,K14ac were correlated with increases in GAD1 mRNA expression. These results were not the effect of age, gender, duration of illness, age of illness onset or type of ongoing antipsychotic treatment. These GAD1 mRNA increases were not seen in lymphocyte cultures in patients with schizophrenia. This study further demonstrated that patients with schizophrenia not only have a more restrictive chromatin, but it is less resistant to dynamic modification (Gavin *et al*., 2008).

While the previous papers examined histone acetylation, a mark of 'open' chromatin, histone methylation or 'closed' chromatin has also been examined (Gavin *et al*., 2009). Di-methylation of the ninth lysine of histone H3 (H3K9me2) is a repressive mark associated with decreased promoter activity specifically in euchromatic regions of the genome. As the H3K9 can be either methylated or acetylated, this histone residue acts as a 'switch' between repressive and expressive chromatin. Using the lymphocyte cell culture technique utilized in previous papers, (Gavin *et al*., 2009) H3K9me2 protein levels were established to be higher in patients with schizophrenia compared to normal controls (Gavin *et al*., 2009). Additionally, in lymphocyte culture, upon administration with Trichostatin A (TSA) for 24 hours, patients with schizophrenia exhibit blunted decreases in H3K9me2 protein levels. This study again provides further evidence of the preexisting restrictive chromatin state in patients with schizophrenia that is less modifiable due to increases in this more thermodynamically stable histone modification. This thesis builds off the findings of this paper, initially examining levels of di-methylated H3K9 in post-mortem brain tissue to complement the lymphocyte findings explained here. This thesis also aims to elucidate possible mechanisms of this increase, through examining histone methyltransferases, the enzymes that specifically add this restrictive chromatin mark.

The central hypothesis is that chromatin is restrictive in schizophrenia. As a result these various modifications can be a therapeutic target for treatment. If, through pharmacological interventions, a reduction in the restrictive state of the chromatin can be relaxed, a process deemed "genome softening," then neuronal gene expression can be enhanced, thus allowing for increased treatment outcomes.

II. METHODS

II.A. Aim I: H3K9me2 Protein Levels in Patient Post-mortem Brain Samples

II.A.1. Post-mortem Brain Database

The neuropathology consortium of the Stanley Foundation Neuropathology Consortium (SFNC) (Bethesda, USA) generously allowed use of fresh-frozen parietal cortex post-mortem tissue for this study. Patient demographic and clinical characteristics, methods of tissue harvest, preparation, and storage, have been previously described in detail elsewhere (Torrey *et al*., 2000), and selected data are presented in Table I. We found no significant diagnostic differences in post-mortem interval (PMI), sample pH, age or RIN score.

Table I: Demographic Characteristics of Post-Mortem Brain Samples

SFNC	N	Sex	Age	PMI	pH	RIN
Non-Psychiatric Controls	15	6F/ 9M	48±10.6	23.7±9.9	6.3±.24	4.9±0.97
Psychotic Subjects - Patients with Schizophrenia and Bipolar Disorder	26	11F/ 15M	44±12.4	30.4±14.0	6.2±.23	5.5±0.90

II.A.2. Protein Extraction

Protein from human post-mortem samples was extracted using the TRIzol method. First, the brains were suspended and homogenized in 1mL of TRIzol. 200µL of chloroform was added, and centrifuged for 15 minutes at 12,000g (at 4°C). The top clear aqueous phase containing RNA was discarded, and the bottom pink phenol phase was retained. 300µL of 100% cold EtOH was added to the lower pink phenol phase to precipitate DNA. After centrifuging for five minutes at 2,000g at 4°C, the supernatant was transferred to a new tube and the pellet was discarded. 1.5mL of 100% isopropanol of was added to the supernatant. After incubating the sample at room temperature for 15 minutes, the samples were centrifuged at 12,000g for ten minutes at 4°C. Supernatant containing protein was discarded, and three volumes of cold acetone were added to the pellet. Samples were centrifuged for ten minutes at 12,000g at 4°C, and supernatant was discarded. 2mL of 300mM guanidine HCl/95% ethanol solution was added, and a homogenizer was used to disrupt the pellet. The mixture was incubated at room temperature for 20 minutes then centrifuged at 12,000g for five minutes at 4°C. The supernatant was then discarded and the pellet was washed twice more with 500uL of the previously mentioned solution. The protein pellet was then washed with ice cold 100% acetone, centrifuged at 12,000g for five minutes at 4°C, then allowed to air dry for 15 minutes. 100uL of 10% SDS was added, and protein concentration was measured (Kirkland *et al*., 2006).

II.A.3. Western Blot Analysis

An equal concentration of protein was boiled in Laemmli buffer (Laemmli, 1970) for ten minutes and loaded onto 10-20% Tris-glycine gel (Invitrogen EC61355BOX) in 1x running buffer (0.125 M TRIS base, 0.95 M glycine, 0.5% SDS) for 90 minutes at 125V and 35 mA.

Proteins were transferred to a Nitrocellulose membrane 0.45µm pore size (Invitrogen LC2001) in transfer buffer (20% methanol, 0.05% SDS, glycine, TRIS base) at 25V and 100mA overnight at 4°C. The membrane was treated with H3K9me2 mouse monoclonal antibody (Abcam Ab1220) at a dilution of 1.5:2,000 for 72 hours in 1X PBS. The membrane was then washed with 1X PBS and treated with anti-mouse secondary antibody (Amersham Biosciences NA934V) at a dilution of 1:2,000 for five hours at room temperature. All membranes were developed with chemiluminescence methodology using ECL Plus (Amersham Biosciences W319851) after incubation with the secondary antibody. After the H3K9me2 assay, membranes are further processed for Beta-actin protein (Sigma A5316) for internal control. Membranes were treated with an antibody/10% blotto ratio of 1:4000 for one hour at 4°C. The incubation with anti-mouse secondary antibody (Amersham Biosciences NA931V) was for one hour at room temperature. Gel loading and membrane transfer is monitored using Coomassie blue staining and unsatisfactory transfers were discarded.

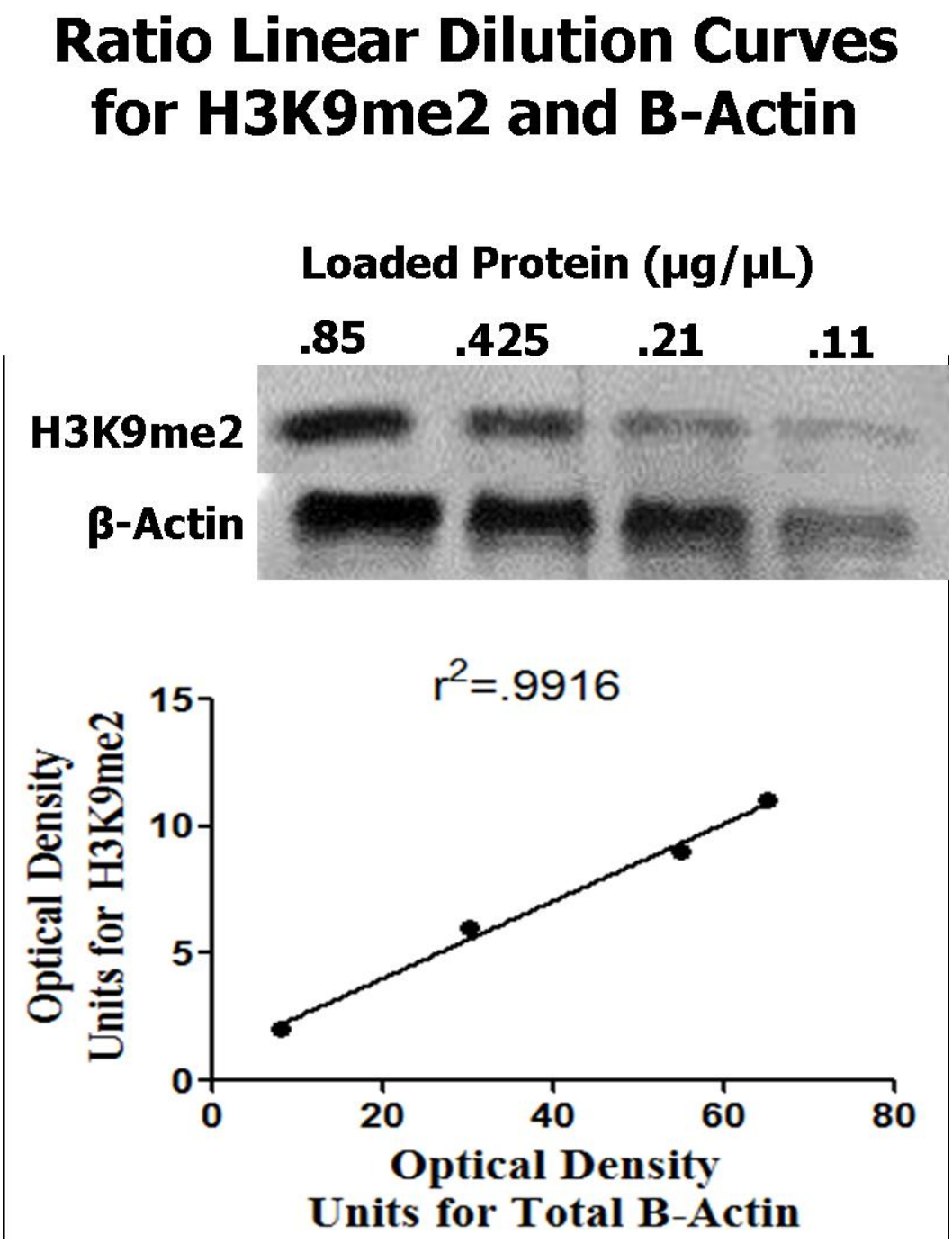

Figure 1 - Linear Dilution Ratio for H3K9me2 and B-actin Western Blot - demonstrating linear range for the ratio with B-actin under optimized Western blot conditions. The Western blot demonstrates both bands at each of four serial dilutions. The graph depicts the linear range of the ratio across the concentrations

II.B. Aim II: mRNA Levels of HMT Gene Expression in Lymphocytes and Post-Mortem Brain Samples

II.B.1 Patient Selection for Lymphocyte Harvest

Subjects were recruited from the inpatient and outpatient programs of the University of Illinois at Chicago medical center, located in the Illinois Medical District (IMD) of Chicago, Illinois, after receiving approval from the UIC Institutional Review Board. All subjects provided written informed consent before participating in any study procedures. Healthy individuals with no Axis I disorder (as assessed by a SCID interview), and no known first-degree familial history of psychosis were recruited via advertisements in local papers and bulletin boards, and were matched to patient groups on age, sex, socioeconomic status and education. General inclusion criteria for all subjects was: good physical health, no history of neurological disease or head trauma, no lifetime history of substance/alcohol dependence or recent (2 months) substance abuse, age range of 18-65 years, and not pregnant. For clinical subjects (patients with schizophrenia and bipolar disorder), patients could not have been treated with Valproic acid, carbamazepine, or clozapine in the previous 30 days. Also, patients with prior non-response to Valproic acid product or carbamazepine were excluded. Patients were assessed via SCID interview, DSM-IVR criteria and any and all known information by the two diagnosticians. Clinicians established a consensus before final assignment to diagnostic group.

II.B.2 Clinical Measures for Harvested Lymphocytes

Clinical measures included: age of illness onset, lifetime cumulative dose of antipsychotic treatment, patterns of weight change, as well as standard physical and laboratory screens such as blood chemistry and metabolic indices (cholesterol, HDLs and LDLs), liver and

renal function tests, pregnancy tests, Body-Mass Index (BMI) the Structured Clinical Interview for DSM Disorders (SCID), and The Positive and Negative Symptom Scale (PANSS)

II.B.2.a. SCID

The Diagnostic Statistical Manual of Mental Disorders (DSM) is a comprehensive reference defining the standard criteria for the classification of mental disorders (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV, 2000). By creating standard criteria of mental disorders, diagnosis, research, treatment and potential prognosis can also be identified. In order to best diagnose a patient, a Structured Clinical Interview for DSM-IV (SCID) is used (First, 1997). Using a "decision tree approach", the administering clinician can record current or lifetime occurrence of Axis I disorders of interest. The SCID has high reliability and validity, (Fogelson *et al*., 1991; Spitzer *et al*., 1992; Williams *et al*., 1992) and has the highest interrater concordance observed for diagnosing schizophrenia (Skre *et al*., 1991).

II.B.2.b. PANSS

The Positive and Negative Syndrome Scale (PANSS) was administered to all clinical participants. The PANSS is a clinician administered, 30-item, 7-point rating questionnaire assessing symptom severity in patients with schizophrenia. Through using a 7 point likert scale, with ratings ranging from 1 (symptom is absent) to 7 (symptom is extreme). The PANSS is separated into three smaller scales a positive scale, a negative scale and a general psychopathology scale. Symptom severity is assessed through summation of likert scores for the smaller scales. The PANSS can be administered in approximately 40 minutes. The interview begins with an open-ended discussion with the patient concerning their mental health history,

hospitalizations, and symptoms, from which the clinician can assess the patients thought content and process, judgment, insight, communication, affect and body movement. Symptom severity is measured through frequency of occurrence and impact on functioning. Abstract reasoning abilities are also examined through proverb interpretation (what does "one in the hand is worth more than two in the bush" mean?). Non-verbal clues are noted and recorded throughout the entire interview, and include posture, blunted affect, attention, hostility and uncooperativeness, conceptual disorganization, spontaneity and flow of conversation, delusions, depression and anxiety and difficulty in abstract thinking (Kay, Fiszbein, & Opler, 1987).

II.B.3. Lymphocyte Cell Culture

Eighty mL of blood was collected in two 50mL falcon tubes (rinsed with 0.5M EDTA, pH 8.0) via sterile venipuncture. Blood was then diluted 1:1 with Hanks Balanced Salt Solution (HBSS; GIBCO 14170-112) (without calcium). 25ml of diluted blood (blood + HBSS) was carefully layered over 25ml of Ficoll-Paque® (Amersham; a sterile endotoxin tested (<0.12 EU/ml) solution of Ficoll™ 400 and sodium diatrizoate with a density of 1.077 ± 0.001g/ml). Samples were then centrifuged at 1,800RPM for twenty minutes at room temperature. After centrifugation, the resulting cream-colored opaque middle interface containing the lymphocytes cells, was diluted 1:3 with HBSS and pelleted at 2,000RPM for ten minutes at 10°C. Cells were counted with trypan blue using 1:1 dilution. Isolated lymphocytes were suspended at a concentration of $1x10^6$ cells/mL in complete media consisting of RPMI 1640 (Gibco 21870) supplemented with 100 U/mL of penicillin, 100µg/mL of streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 0.1mM nonessential amino acids, and 10% FBS. Cells were plated in 6 well plates, (Becton Dickenson 353046) and incubated at 37°C and 5% CO_2. Over time, the

mononuclear cells will settle to plate bottom. The T-Lymphocytes (which constitute up to about 65% of the population of cells) will remain and the B-Lymphocytes cells (subtype of lymphocytes) will not survive, and neither will platelets or red blood cells. This technique provides a homogeneous suspension of T-lymphocytes, as only suspended and surviving cells are harvested. Lymphocytes were incubated with optimal concentrations of Valproic Acid, Trichostatin A, Nicotine or vehicle (DMSO for Trichostatin A and water for nicotine and Valproic acid) in media at various time points (Gavin *et al*., 2009; Jayaraman *et al*., 1999).

II.B.4. <u>mRNA Extraction in Human Lymphocytes</u>

Total RNA was isolated using TRIzol reagent techniques (Life Technologies). 500µL of TRIzol reagent was added to each sample. The sample was then thoroughly vortexed, and 100µL of Chloroform was added. Samples were left at room temperature for two minutes, and then centrifuged at 12,000g for 15 minutes at 4°C. The top aqueous phase (clear layer) was extracted, kept and added to 250µL of 2-Propronol at room temperature. After incubation at room temperature for ten minutes, the samples were centrifuged at 12,000g for ten minutes at 4°C. The pellet was then resuspended in 70% EtOH, and centrifuged at 7,500g for five minutes at 4°C. The pellet was resuspended in 30µL DNase RNase Deionized water, and treated with DNase (Amersham 27-0514-03). Only total RNA extracts with an OD_{260}/OD_{280} ratio above 1.96 indicating relatively pure RNA were processed for real-time RT-PCR, the remainder undergoing re-extraction (Mannhalter *et al*., 2000).

II.B.5. <u>Real Time RT-PCR Quantification in Human Lymphocytes</u>

Total RNA was converted to cDNA using the Applied Biosystems High Capacity Archive Kit (4368813). For detection and measurement of expression, Fermentas Maxima

SYBR Green/ROX qPCR Master Mix (K0222) was used. SYBR Green is only fluorescent when bound to double-stranded DNA (dsDNA), thus the amount of fluorescence is directly proportional to amount of dsDNA. The real-time qRT-PCR was set up in a reaction volume of 20µl. Gene expression was measured with a non-significant variation between triplicates. PCR mixtures were run on a Stratagene Mx3005P™ QPCR System. The cycling conditions are as follows: 10 min 95°C, 40 cycles at 95°C for 30 seconds, 60°C for 1 min, 72°C for 1 min. Cycle threshold (C_T) value was used for relative quantification of target gene expression, and all values were normalized to three housekeeping genes, GAPDH, TFRC and B-Actin, using a geometric mean (Vandesompele *et al*., 2002). Primers were designed specifically to cross over one intron, yielding an amplicon of between 70-150 base pairs. Primers were designed this way as to only amplify cDNA. Primers were also designed to have a Tm between 58°C and 62°C, with the GC content between 45-55%. Dissociation curves with control samples were conducted to establish the presence of a single amplicon at the predicted melting temperature, and a lack of primer-dimer formation. Primer sequences are listed in Table II.

Table II: Primer Sequences Used

mRNA Expression Primers	
Primer Name/Location	***Sequence***
hGLP 5' +1801	5' TCCTGGCTGTGGCTACTTCT 3'
hGLP 3' +1875	5' AAACGGTGAGAGATGCTGCT 3'
hG9a 5' +610	5' TTCCGCATGAGTGATGATGT 3'
hG9a 3' +745	5' TCGTCAGGGTCACTTCTCCT 3'
hSetdb1 5' +463	5' AAGACCAGAAGCTCCGTGAA 3'
hSetdb1 3' +561	5' CCTGGGAACTGCTCTTCTTG 3'
hBDNFIXabcd 5'	5' AACCTTGACCCTGCAGAATG 3'
hBDNFIXabcd 3'	5' TGGTCATCACTCTTCTCACCTG 3'
hGAPDH 3' +340	5' CGAGATCCCTCCAAAATCAA 3'
hGAPDH 5' +509	5' TTCACACCCATGACGAACAT 3'
hB-Actin 5' +787	5' TCCCTGGAGAAGAGCTACGA 3'
hB-Actin 3' +922	5' TGAAGGTAGTTTCGTGGATGC 3'
hTFRC 5' +1534	5' AAAATCCGGTGTAGGCACAG 3'
hTFRC 3' +1670	5' CACCAACCGATCCAAAGTCT 3'
mmGlp 5' + 3569	5' ATTGACGCTCGGTTCTATGG 3'
mmGlp 3' +3799	5' ACACTTGGAAGACCCACACC 3'
mmG9a 5' +3112	5' TGCCTATGTGGTCAGCTCAG 3'
mmG9a 3' +3247	5' GGTTCTTGCAGCTTCTCCAG 3'
mmSetdb1 5' +794	5' GATTCTGGGCAAGAAGAGGA 3'
mmSetdb1 3' +989	5' GTACTTGGCCACCACTCGAC 3'
mmBdnfI 5'	5' GTCTTCTGTAGTCGCCAAGGTGG 3'
mmBdnfI 3'	5' GCACACCTGGGTAGGCCAAG 3'
mmBdnfIV 5'	5' GTCTTCTGTAGTCGCCAAGGTGG 3'
mmBdnfIV 3'	5' TCGCTGAAGGCGTGCGAGTA 3'
mmBdnfIXa 5'	5' GCAGCTGGAGTGGATCAGTAA 3'
mmBdnfIXa 3'	5' TGGTCATCACTCTTCTCACCTG 3'
mmGapdh 3' + 13	5' ACGGCCGCATCTTCTTGTGCAGTG 3'
mmGapdh 5' + 238	5' GGCCTTGACTGTGCCGTTGAATTT 3'
Chromatin Immunoprecipitation Primers	
Primer Name/Location	***Sequence***
hBDNFIX 5'	5' CCTCTGGCAAACAGGAAGAG 3'
hBDNFIX 3'	5' CGCGCTCTGAGTTTATCCTA 3'
mmBdnfI 5'	5' CAAAATAGGGCAGCGACTCT 3'
mmBdnfI 3'	5' CTGAGCGAAAAGGTGTAGGC 3'
mmBdnfIV 5'	5' CCCTGGAACGGAATTCTTCT 3'
mmBdnfIV 3'	5' AGTCCTCTCCTCGGTGAATG 3'
mmBdnfIXa 5'	5' CATGAGACCGGGCAAGTC 3'
mmBdnfIXa 3'	5' CCTTGGGAGGAATGTGTGAT 3'

II.B.6. Post-Mortem Brain Database

We obtained fresh-frozen inferior parietal lobule tissue from the neuropathology consortium of the Stanley Foundation Neuropathology Consortium (SFNC) (Bethesda, USA), as described in II.A.1.

II.B.7. mRNA Extraction in Post-Mortem Brain Samples

mRNA was extracted using methods listed in section II.B.4. In addition, RNA was further purified using Qiagen RNeasy Minikit (Qiagen Inc., Valencia, CA). RNA integrity (RIN) was assessed with an Aglient 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

II.B.8. Real Time RT-PCR Quantification in Post-Mortem Brain Samples

Real time RT-PCR quantification on post-mortem brain samples was performed using methods described in section II.B.5.

II.C. Aim III: Pharmacological Manipulations of HMT Gene Expression and Comparative Alterations in H3K9me2

II.C.1. Primary Cortical Neuronal Cell Culture

Primary cortical neurons were dissected from E14 Harlan ICR strain fetuses. Microsurgery was performed on mouse fetus, on ice, extracting the cortex and the striatum. Tissue was dissociated to single neuron suspensions using sequentially smaller bore sized glass pipette tips, polished by firing on the day of surgery. Neurons were grown on 1% poly D-lysine (PDL) coated 6-well plates in 5% CO_2 at 37°C. The growth media consisted of 200µM l-glutamine, 50mg/ml of Gentamycin Sulfate Reagent solution (Cellgro 61-098-RF), 2% B-27 Supplement (Gibco 17504-044), dissolved in Neurobasal medium (Gibco 21103) without

Glutamine. Cells were plated at an average of one fetus per 6-well plate, and spiked with 500µM Cytosine beta-D-Arabinofuranoside (Sigma C-1768) at 2 days in vitro (DIV). Three days after dissection, 50% of media was freshly replaced. Cells were incubated with optimal concentrations of Valproic Acid, Trichostatin A, Nicotine or vehicle (control) in media at various time lengths 9-10 days after dissection (Noh & Gwag, 1997; Noh *et al*., 2005; Sharma *et al*., 2008).

II.C.2. Lymphocyte Cell Culture

Lymphocytes were cultured using methods listed in section II.B.3.

II.C.3. Culture Drug Treatment

(−)-Nicotine hydrogen tartrate salt (Sigma N-5260) was dissolved in double distilled water and was added to cells in a 1:1000 dilution in media. Nicotine was added in increasing concentrations, and cells were incubated six hours before harvest. All doses are expressed in terms of the salt, not the nicotine base. Nicotine base is roughly equivalent to one third of the nicotine tartrate salt weight, thus 3.0 mg is equivalent to 1.0 mg of nicotine base (Schreiber *et al*., 2002).

Valproic acid sodium salt (Sigma P4543) was dissolved in double distilled water and was added to cells in a 1:1000 dilution in media. VPA was added increasing concentrations, and cells were incubated for 24 hours before harvest (Kaiser *et al*., 2006). Trichostatin A (Sigma T8552) was dissolved in dimethyl sulfoxide (DMSO) and was also added to cell media in a 1:1000 dilution. TSA was added in increasing concentrations, and cells were incubated for 24 hours before harvest (Yoshida *et al*., 1990).

II.C.4. Mouse Treatment

Adult Swiss albino mice were treated with either Valproic acid (Sigma P4543) or Nicotine (Sigma N-5260), given via intraperitoneal injections (IP). There were three different conditions of Valproic treated mice: one injection of 150mg/kg four hours before harvest, one injection of 150mg/kg 24 hours before harvest, (Tremolizzo *et al*., 2002) or twice daily for 7 days of 150mg/kg (Yildirim *et al*., 2003). In a separate group of animals, 3mg/kg of nicotine was given IP two hours before sacrifice (Schreiber *et al*., 2002; Stolerman *et al*., 1973). Valproic acid and Nicotine were dissolved in 0.9% saline to a concentration of 0.01mL/10g. All control mice were injected with 0.01mL/10g of 0.9% saline IP at identical time points.

II.C.5. mRNA Extraction

mRNA was extracted using methods listed in section II.B.5.

II.C.6. Real Time RT-PCR Quantification

Real time RT-PCR Quantification was performed using methods listed in section II.B.5. In addition, a comparative threshold cycle (C_T) validation experiment was done using a serial diluted sample to check whether the efficiencies of the target primers and reference amplifications were approximately equal (the slope of the log input amount versus $\Delta C_T < 0.1$) (Figure 2, Figure 3). A significant effort was made to establish efficiency above 95%. Cycle threshold (C_T) value was used for relative quantification of target gene expression, and normalized to GAPDH. Fold changes relative to control condition are calculated as ΔC_T (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Primer sequences are listed in Table II, with human primers (h) used on the lymphocyte cultures and mouse primers (mm) used with primary neuronal culture and mouse cortex extracts.

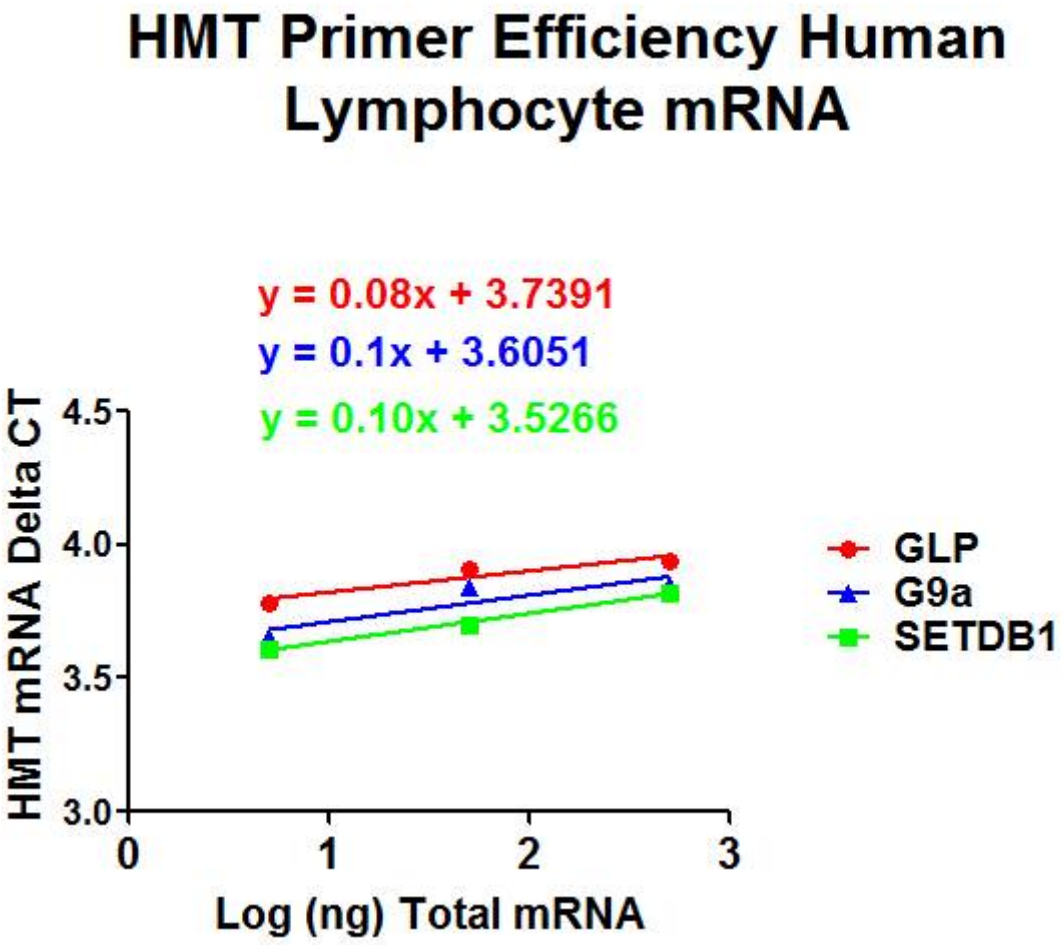

Figure 2 - Relative primer efficiency of real-time RT-PCR in HUMAN lymphocyte mRNA. Plot depicts delta C_T values for GLP, G9a, and SETDB1 to GAPDH used for normalization at different input amounts

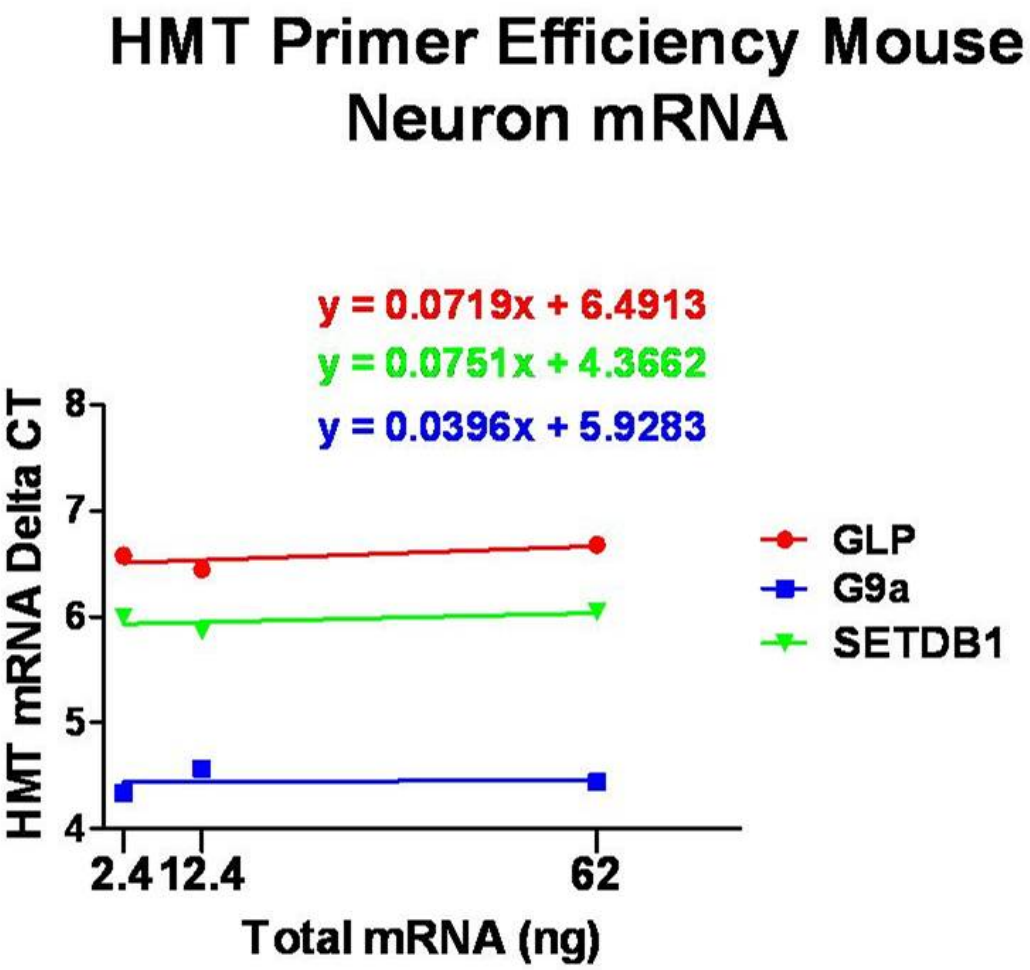

Figure 3: Relative primer efficiency of real-time RT-PCR in MOUSE Neuron mRNA. Plot depicts delta C_T values for GLP, G9a, and Setdb1 to GAPDH used for normalization at different input amounts

II.C.7. Acid Histone Extraction

Histone proteins were extracted by first homogenizing the sample in 500µL of 0.25M sucrose, 3.3mM calcium acetate, and 1mM PMSF solution and centrifuging at 1,000g for ten minutes. All centrifugations in this protocol were performed at 4°C. The pellet was washed twice with 500µl of 1X PBS and PMSF solution (495µL PBS + 5µL 100mM PMSF) and centrifuged at 800g for ten minutes. The pellet was then vortexed in 200µL of cold 0.4N H_2SO_4 and placed on an orbital shaker for 60 minutes at 4°C. After centrifuging at 10,000g for 15 minutes, the supernatant was collected and 100µL of 33% TCA is added. The sample was placed on ice for 30 minutes and then centrifuged at 12,000g for ten minutes. The pellet was washed first with 100µL of 100% acetone/0.05M HCl then with 100µL of 100% acetone and centrifuged at 10,000g for 2 minutes after each wash. The acetone was then allowed to evaporate by placing the micro-centrifuge tube on a hot plate at 40°C for 30 minutes. Pellets were re-suspended in 100µL of ddH_2O. The extract yields a relatively pure sample of basic histone proteins. Protein levels were measured using the Bradford Method with BSA (10mg/ml) as the standard (Sharma *et al*., 2006; Simonini *et al*., 2006).

II.C.8. Western Blot Analysis

Equal concentrations of protein from each sample were boiled in Laemmli buffer (Laemmli, 1970) for ten minutes and loaded onto 10-20% Tris-glycine gel (Invitrogen EC61355BOX) in 1x running buffer (0.125M TRIS base, 0.95M glycine, 0.5% SDS) for 90 minutes at 125V and 35 mA. Proteins were then transferred to a Nitrocellulose membrane 0.45µm pore size (Invitrogen LC2001) in transfer buffer (20% methanol, 0.05% SDS, glycine, TRIS base) at 25V and 100mA overnight at 4°C. The membrane was washed with 1xPBS 3

times for ten minutes and incubated in blotto buffer (3% nonfat dry milk, 0.1% Tween 20, 1xPBS) for one hour at room temperature. The membrane was then treated with anti-Dimethylated histone 3, Lysine 9 (H3K9me2) rabbit polyclonal antibody (Millipore 05-768) for human samples and anti-H3K9me2 mouse monoclonal antibody (Abcam 1220) for mouse samples at a dilution of 1:2,000 overnight. Twenty-four hours later, the membrane was washed with 1X PBS, and treated with species-specific secondary antibody (Amersham Biosciences NA934V) at a dilution of 1:2,000 for two hours at room temperature. Membrane was then washed with 1X PBS, and all membranes were developed with chemiluminescence methodology using ECL Plus (Amersham Biosciences W319851) after incubation with the species appropriate secondary antibody. After the H3K9me2 assay, membranes were further processed for H1 protein as an internal control for normalization across samples. Membranes were treated with anti-histone-H1 (Millipore 05-457) with an antibody/blotto ratio of 1:1000 for three nights at 4°C. The incubation with anti-mouse secondary antibody (Amersham Biosciences NA931V) was for two hours at room temperature. Gel loading and membrane transfer was monitored using Coomassie blue staining and unsatisfactory transfers were discarded. Figure 4 demonstrates a typical result for H3K9me2 using 4 two-fold dilutions that encompass a dilution range up to a 16 fold difference in magnitude. Interassay coefficients for the H3K9me2 ratio was performed by repeated measurements of the same protein samples in independent assays and computed at 5.4% for H3K9me2. Intraassay coefficient for these same ratios was performed by measurement of the same sample at the same concentration within the same gel; these were computed as 5.2% for H3K9me2 (Sharma *et al*., 2006).

Optimized Linear Dilution curve for H3K9me2 Western Blot

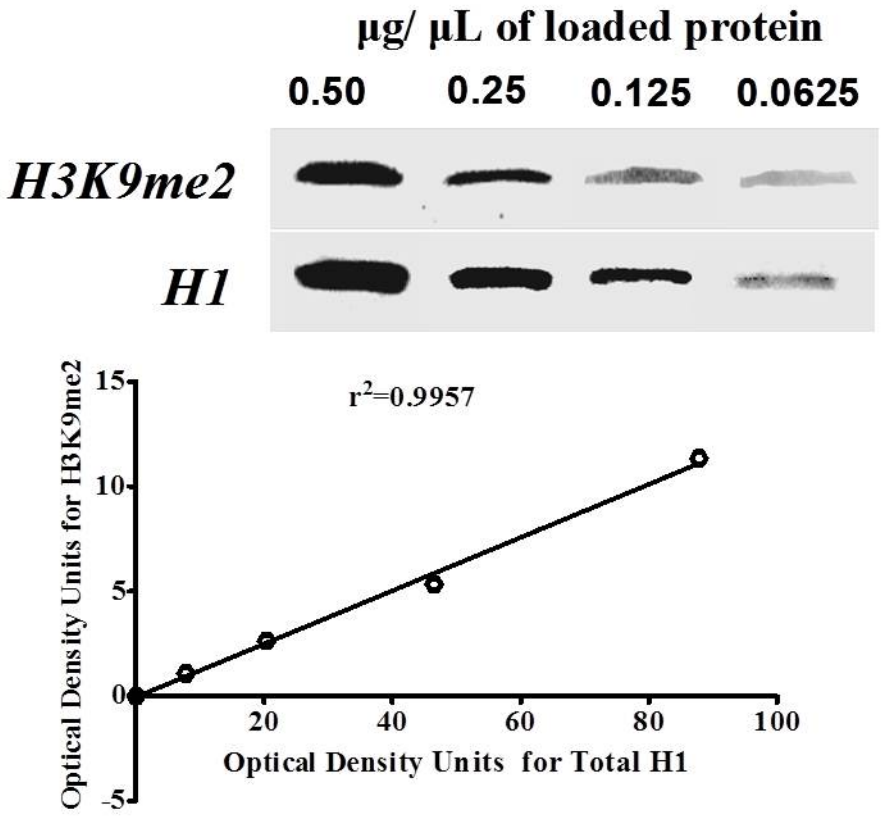

Figure 4 - Linear Dilution Ratio for H3K9me2 and H1 Western Blot - demonstrating linear range for the ratio with H1 under optimized Western blot conditions. The Western blot demonstrates both bands at each of four serial dilutions. The graph depicts the linear range of the ratio across the concentrations

II.C.9. Chromatin Immunoprecipitation

The protocol used in this thesis was a modified version of the fast Chromatin Immunoprecipitation (ChIP) protocol (Nelson, Denisenko, & Bomsztyk, 2006). Forty µL of 37% formaldehyde per 1mL of media (for a total concentration of 1.5% formaldehyde) was added to culture media and incubated at room temperature for 15 minutes. The formaldehyde was quenched with 125mM glycine for five minutes. Cells were centrifuged for 2,000g for five minutes at 4°C. Supernatant was discarded, and pellets were washed with 500µL of a 1X PBS and 5µL protease inhibitor mixture (Calbiochem Protease Inhibitor Cocktail Set III, EDTA-free 539134) and centrifuged at 800g for ten minutes at 4°C. From this point on, all procedures were performed on ice. Cells were lysed in 1mL IP buffer (150mM NaCl, 50mM Tris-HCl (pH 7.5), 5mM EDTA, IGEPAL 100% (0.5%vol/vol), and Triton X-100 (1.0% vol-/vol)) with added PMSF and protease inhibitors, and centrifuged at 12,200g for 2 minutes at 4°C. 400uL of IP buffer + PMSF + protease inhibitor were added to the pellet and sonicated for 15 minutes on the "High" setting (Diagenode Bioruptor). Samples were then centrifuged at 12,000g for ten minutes at 4°C. The supernatant was divided into three equal 133µL volume tubes, one for input, one for negative control, and one for anti-H3K9me2 mouse monoclonal antibody (Abcam ab1220). 5µL of the Anti-H3K9me2 antibody was added to precipitate H3K9me2 associated DNA. Input samples were frozen while negative control and H3K9me2 pull-down samples were placed overnight on an orbital shaker. The following day, negative control and H3K9me2 samples were placed in an ultrasonic bath for two hours at 4°C. Negative control and H3K9me2 samples were then centrifuged at 12,200g for ten minutes at 4°C, and supernatant was transferred to a separate 1.5mL tube. 30µL of agarose beads (Protein A/G PLUS-Agarose, Santa Cruz SC-

2003) (previously washed at 2,000g for 1 minute at room temperature with 1mL of IP buffer) was added to the supernatant and placed for two hours on an orbital shaker at 4°C. Negative control and H3K9me2 samples were centrifuged at 2,000g for 1 minute at 4°C and supernatant was aspirated, being careful not to disturb the agarose pulldown pellet. Agarose bead pellet was then washed with 1mL IP buffer (without protease inhibitors or PMSF) at 2,000g for 1 minute at 4°C, six times total. 100µL of 10% Chelex100 slurry was then added to the pelleted beads and boiled for ten minutes then allowed to cool. 5µL of 20ug/µL of Proteinase K was added to each sample, placed in a 55°C hot water bath for 30 minutes, followed by another ten minutes of boiling. Samples were then centrifuged at 12,200g for five minutes at 4°C, and supernatant was collected. **Previously frozen input samples were extracted separately as follows**. DNA was extracted first with 3 volumes of cold EtOH and centrifuged at 7,500g for five minutes at 4°C. Supernatant was aspirated and three volumes of cold 70% EtOH was added and centrifuged at 7,500g for five minutes at 4°C. Pellet was allowed to air dry, and then redissolved in 100µL of 10% Chelex100 slurry. Sample was boiled for ten minutes, and then allowed to cool. 5µL of 20ug/µL of Proteinase K was added to the input sample, and then placed in a 55°C water bath for 30 minutes. Samples were then boiled for another ten minutes and centrifuged 12,200g for five minutes at 4°C and supernatant was collected into a new 1.5mL tube. Real time PCR was used to amplify H3K9me2 bound DNA promoter sequences, as listed in Table II.

III. RESULTS

III.A. Aim I: H3K9me2 Protein Levels in Patient Post-mortem Brain Samples

SPSS statistical package (version 15.0 for Windows) was used for all statistical analyses.

Di-methylated lysine 9 of histone 3 (H3K9me2) is significantly increased in lymphocytes of patients with schizophrenia (Gavin *et al*., 2009). As will be shown in Specific Aim 2 (III.B), G9a and Setdb1 histone methyltransferase mRNA levels are significantly increased in both lymphocytes and post-mortem brain tissue of patients with schizophrenia compared to normal controls. This thesis aims to extend the increases in H3K9me2 results shown in lymphocytes to post-mortem brain samples.

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H3K9me2) in fresh-frozen parietal cortex tissue from the neuropathology consortium of the Stanley Foundation Neuropathology Consortium (SFNC). An experimental replicate for post-mortem brain was defined as a ~100mg of brain tissue from a single participant. We performed analysis on 7 patients with bipolar disorder with psychotic symptoms, 15 patients with schizophrenia, and 15 normal controls. The demographics associated with each patient population are presented in Table I. The demographic and clinical characteristics of the population, as well as methods of tissue harvest, preparation, and storage, have been described in detail in (Torrey *et al*., 2000). We found no significant diagnostic differences in post-mortem interval (PMI), pH, age or RIN score. Optical density levels of H3K9me2 protein levels in control participants, patients with schizophrenia and patients with bipolar disorder were analyzed using one-way analysis of variance (ANOVA). Group

differences were further elucidated by Tukey post hoc comparison. All data is presented as percent change from control ± standard error of the mean. In post-mortem brain tissue, the ANOVA revealed an effect of diagnosis on global H3K9me2 protein (ANOVA, $F_{2,44}$=5.74, p>0.01). Tukey post hoc comparisons indicated that patients with schizophrenia had significantly higher levels of global H3K9me2 protein levels (Figure 5). Figure 6 is a representative western blot of H3K9me2 in post-mortem brain tissue. Increased H3K9me2 protein levels and HMT mRNA indicates a global restrictive chromatin state in patients with schizophrenia. Additionally, elevated H3K9me2 levels can be found in both lymphocytes and post-mortem brain tissue, thus further validating the lymphocyte model as a viable biomarker model for examining epigenetic modifications in patients with schizophrenia.

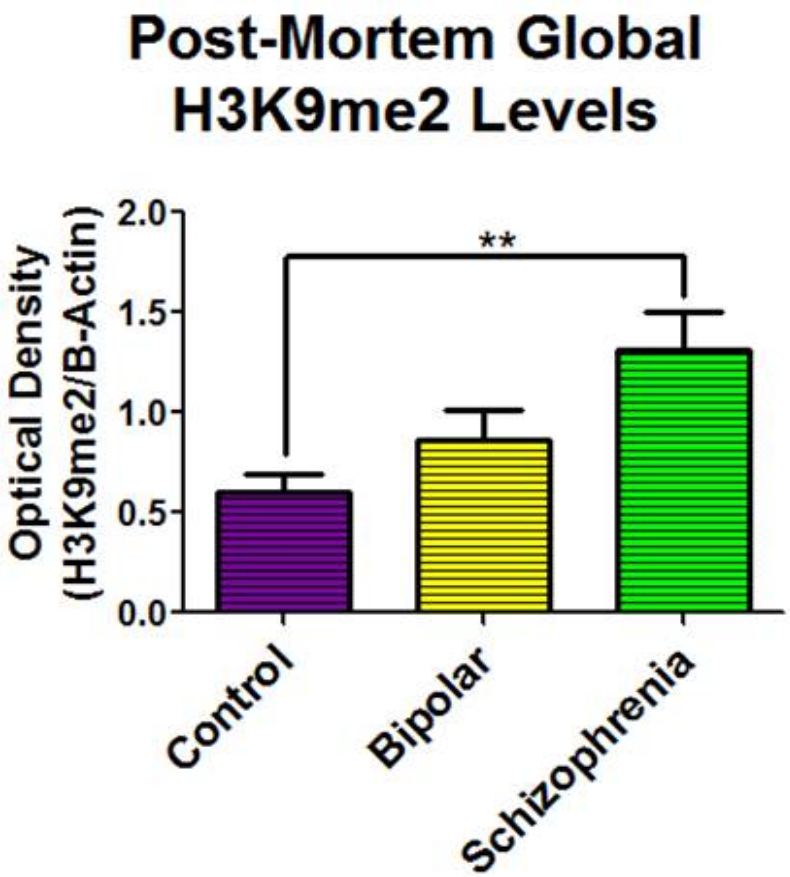

Figure 5 - Global H3K9me2 protein levels - are significantly increased in patients with schizophrenia when compared to normal controls. Data is shown as means for independent experiments ± standard error. *****p*<0.01, as determined by Tukey post-hoc.

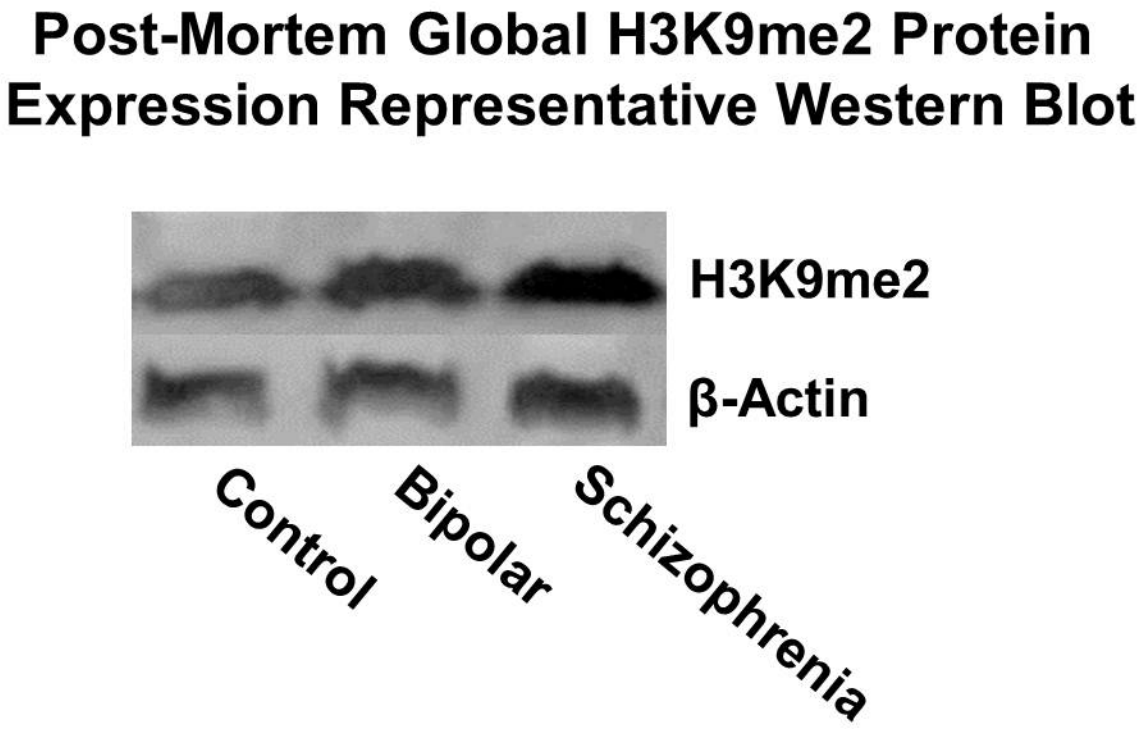

Figure 6 – Representative western blot for H3K9me2 protein levels in post-mortem brain tissue normalized to β-Actin.

III.B. Aim II: mRNA levels of HMT Gene Expression in Lymphocytes and Post-Mortem Brain Samples

III.B.1 mRNA Levels of HMT Gene Expression

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and SETDB1. We compared HMT mRNA levels between normal controls, patients with schizophrenia and patients with bipolar disorder. HMT mRNA was measured in both lymphocytes and post-mortem brain tissue extracts. Using lymphocytes to identify abnormalities and dysregulation in chromatin structure is both a viable and advantageous model for studying mental illness. Lymphocytes can be obtained from living patients, thus their use as a biomarker to characterize subsets of patients with schizophrenia could be established to lead to targeted pharmacological interventions (Gavin & Sharma, 2009). Experimental replicates for lymphocyte analysis were defined as a single blood draw from a participant. Patient data and sample size was described in Table III. An experimental replicate for post-mortem brain was defined as a ~100mg from a single participant. Patient data and sample size was described in Table I. Geometric mean (Vandesompele *et al*., 2002) of Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression in lymphocytes and post-mortem brain was analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance.

Based on participants diagnosis, the ANOVA revealed that GLP mRNA levels did not significantly vary in lymphocytes (ANOVA, $F_{2,44}=1.58$, $p=0.22$) or in post-mortem brain samples (ANOVA, $F_{2,44}=1.0$, $p=0.12$) (Figure 7).

Table III: Demographic Characteristics of Lymphocyte Samples

			Gender		Race			
Diagnosis	**N**	**Age (median)**	**Male**	**Female**	**White**	**Black**	**Hispanic**	**Other**
Control	18	29	61%	39%	84%	4%	12%	0%
Bipolar Disorder	5	26	57%	43%	27%	36%	10%	27%
Schizophrenia	23	29	74%	26%	35%	50%	12%	3%

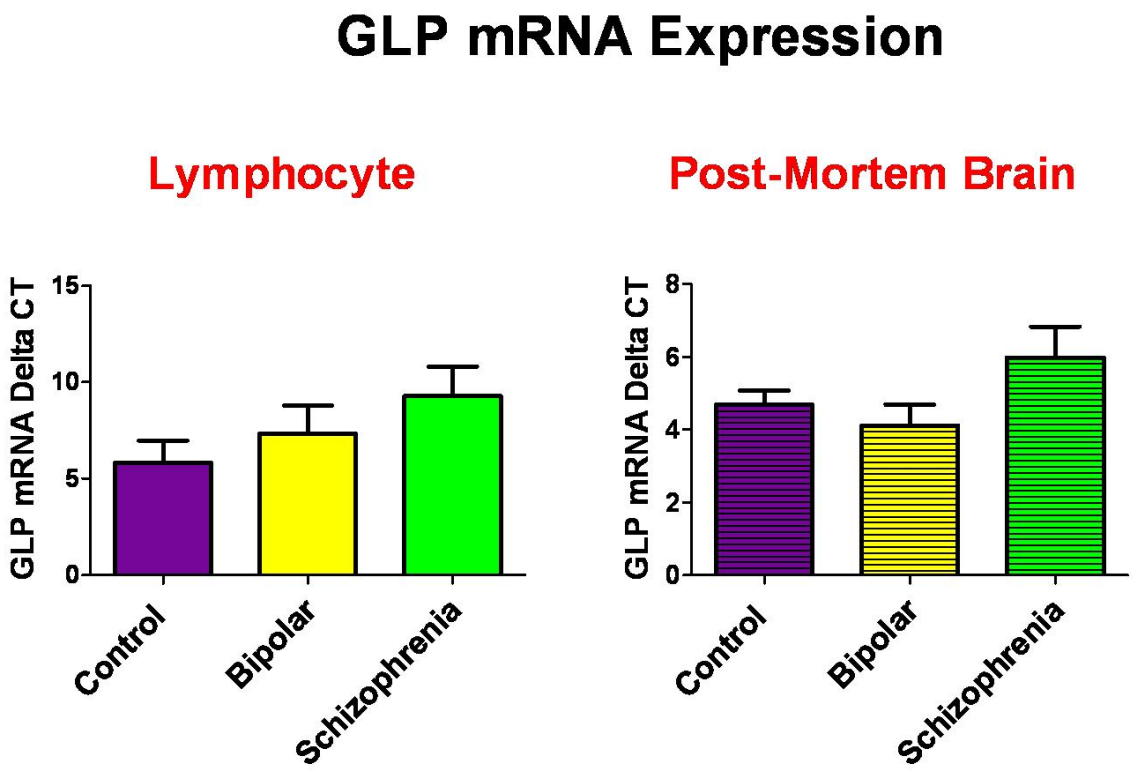

Figure 7 – Histone methyltransferase GLP mRNA expression in lymphocytes and post-mortem brain tissue. mRNA levels showed no significant differences between diagnosis group. Data is shown as means for independent experiments ± standard error. *p=ns,* as determined by Tukey post hoc.

However, the ANOVA revealed that based on diagnosis, participants' G9a mRNA levels did significantly vary in lymphocytes (ANOVA, $F_{2,44}$=7.32, p<0.01) as well as in post-mortem brain samples (ANOVA, $F_{2,44}$=5.38, p<0.01) (Figure 8) Tukey post-hoc tests revealed that patients with schizophrenia had significantly higher G9a mRNA levels when compared to control participants. Patients with bipolar disorder showed no significant differences in G9a mRNA levels compared to normal controls or patients with schizophrenia.

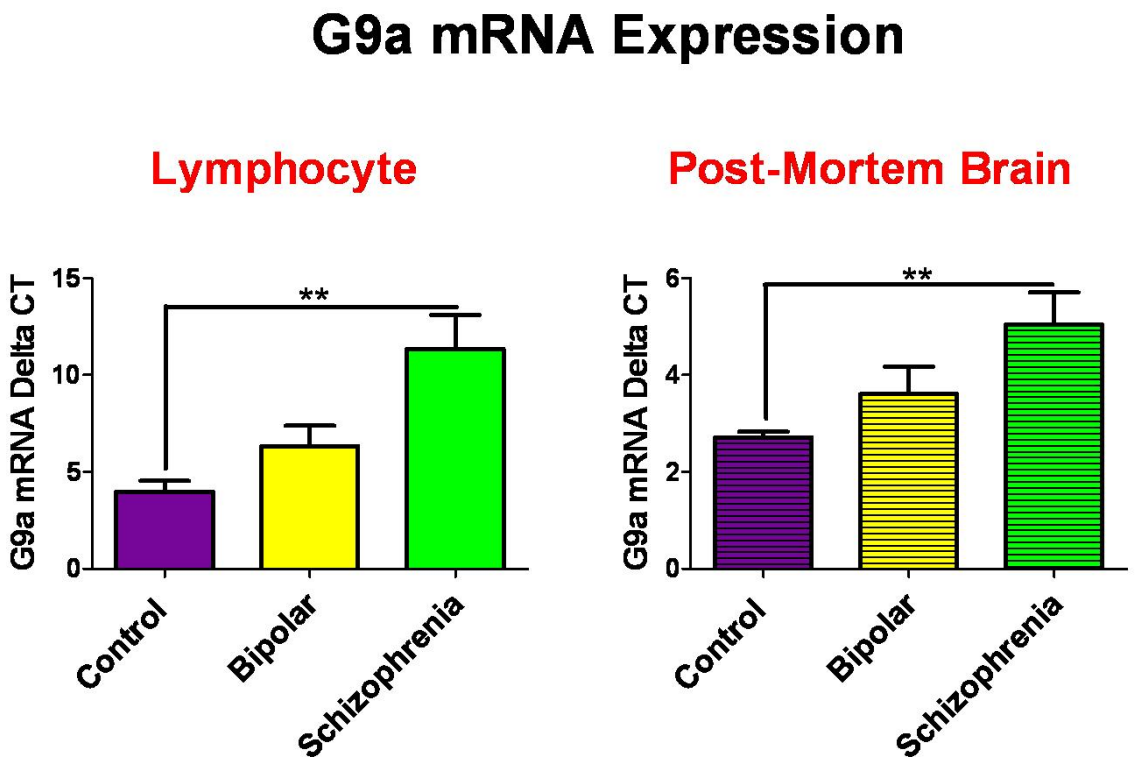

Figure 8 – Histone methyltransferase G9a mRNA expression in lymphocytes and post-mortem brain tissue. G9a mRNA levels showed significant increases in patients with schizophrenia compared to normal controls. Data is shown as means for independent experiments ± standard error. ** *p*<0.01, as determined by Tukey post hoc.

Furthermore, the ANOVA indicated Setdb1 mRNA levels did significantly vary in lymphocytes (ANOVA, $F_{2,44}$=4.87, p<0.01) and in post-mortem brain samples (ANOVA, $F_{2,44}$=5.23, p<0.01) dependent on diagnosis (Figure 9). Tukey post-hoc tests revealed that patients with schizophrenia had significantly higher Setdb1 mRNA levels when compared to control participants in both lymphocytes and post-mortem brain tissue. Patients with schizophrenia showed significant increases in Setdb1 mRNA levels compared to patients with bipolar disorder, only in post-mortem brain tissue. No significant differences were seen between bipolar patients and normal controls or patients with schizophrenia in lymphocyte samples.

In summary, both lymphocytes and post-mortem brain tissue show similar diagnosis related patterns of HMT mRNA expression. GLP mRNA was not significantly different between any diagnostic group in both lymphocytes and post-mortem brain tissue. G9a mRNA was significantly increased in patients with schizophrenia in both lymphocyte and post-mortem brain tissue. Both lymphocyte and post-mortem brain tissue exhibited significant increases in Setdb1 mRNA. These findings are important as through corresponding patterns in both lymphocytes and post-mortem brain tissue, they demonstrate the viability of the lymphocyte model for studying epigenetic mechanisms in mental illness. Additionally, these findings indicate a global dysregulation of restrictive epigenetic mechanisms in patients with schizophrenia, which perhaps may lead to the etiology of the disease.

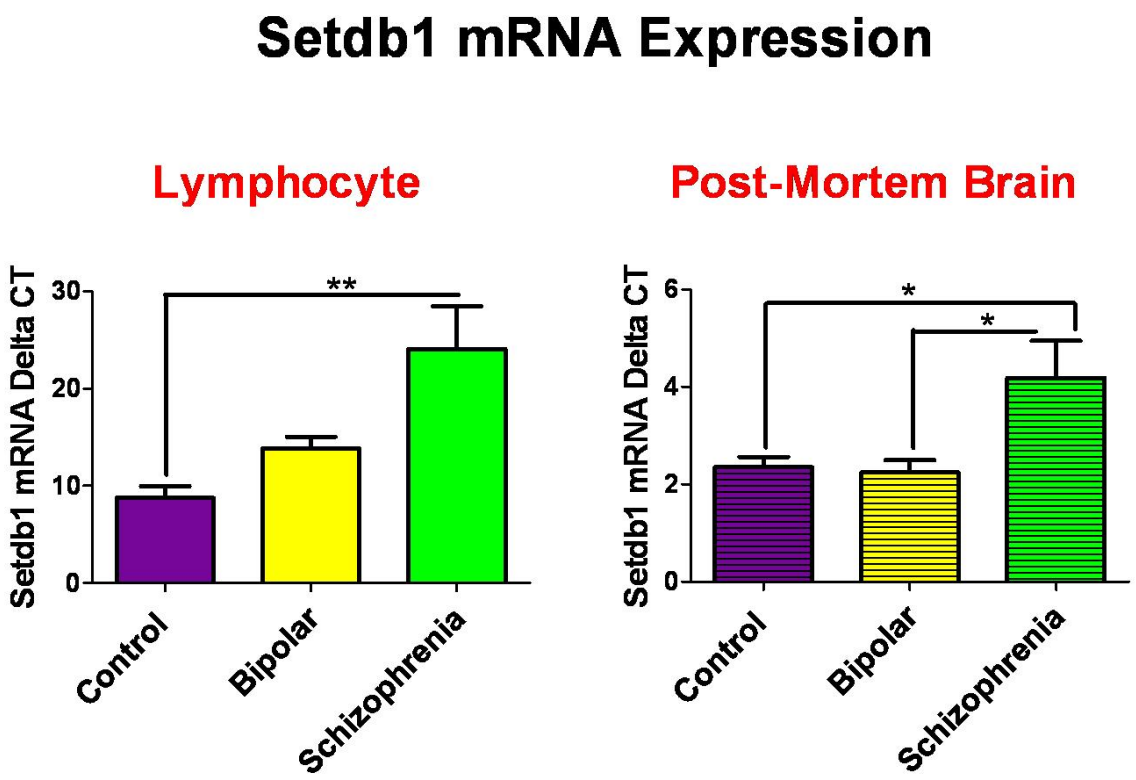

Figure 9 – Histone methyltransferase Setdb1 mRNA expression in lymphocytes and post-mortem brain tissue. Setdb1 mRNA levels showed significant increases in patients with schizophrenia compared to normal control participants. Data is shown as means for independent experiments ± standard error. * $p<0.05$, ** $p<0.01$, as determined by Tukey post hoc.

III.B.2 HMT Gene Expression Related to Clinical Measures

III.B.2.a History of Neurological Condition

A neurological condition is a broad term encompassing any type of damage to the central and peripheral nervous system as a result of illness or injury. Some conditions include stroke, traumatic brain injury, cerebral palsy, muscular dystrophy, epilepsy, multiple sclerosis, and meningococcal disease, but this list is far from all encompassing. As patients with schizophrenia display evidence of neurological impairment that seem to be inherent to the disease, not as a result of treatment, disease severity and outcome can perhaps be predicted from clinical measures (Browne *et al*., 2000; Salacz *et al*., 2011).

We compared ΔC_T (Experimental Gene C_T-Normalizing Gene C_T) values for GLP between patients with and without a neurological condition using an unpaired two-tailed t-test (Livak & Schmittgen, 2001). Patients with a history of a neurological condition had significantly increased levels of GLP mRNA in their lymphocytes (n=34, t=9.22, p<0.001). There were no significant differences in the other HMT mRNA (G9a, Setdb1) levels in relation to familial history (Figure 10).

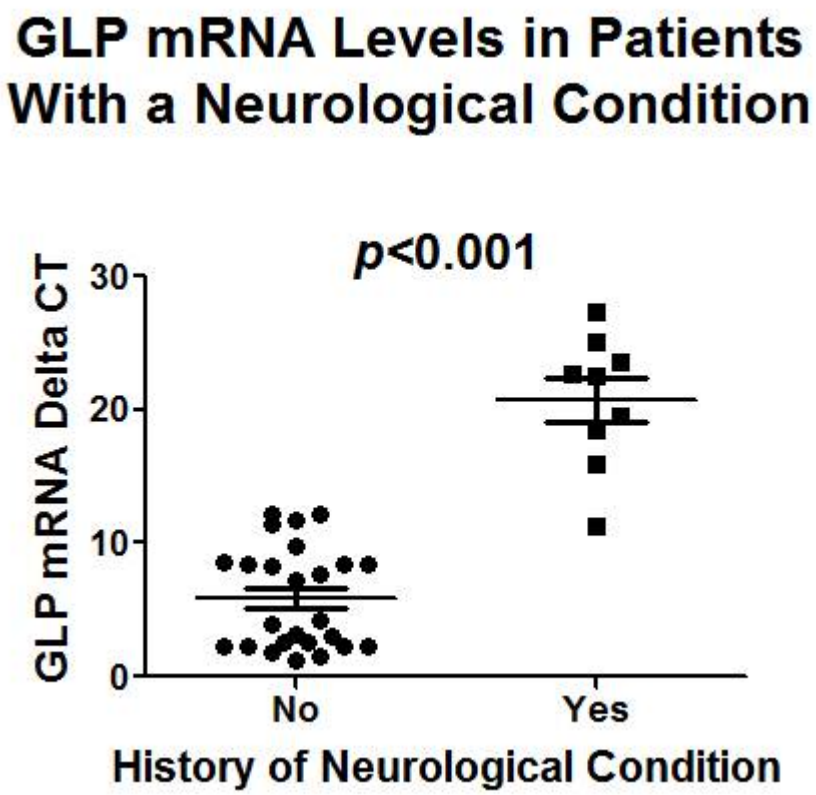

Figure 10 - GLP mRNA levels are increased in patients with a history of a neurological condition. *p<0.001, as determined by t-test.

III.B.2.b Familial History

All major psychiatric disorders aggregate in families, (Kendler, 1990) and there is strong evidence for a heritable component to schizophrenia, as demonstrated through twin and adoption studies (Gottesman & Bertelsen, 1989). Additionally, schizophrenia in a first degree relative is characterized as the highest risk factor for developing schizophrenia (Mortensen *et al*., 1999).

We compared ΔC_T (Experimental Gene C_T-Normalizing Gene C_T) values for Setdb1 between patients with and without a family history of schizophrenia using an unpaired two-tailed t-test (Livak & Schmittgen, 2001). Patients with a family history of schizophrenia had significantly increased levels of Setdb1 mRNA in their lymphocytes (n=20, t=2.52, p<0.05). There were no significant differences in the other HMT mRNA (GLP, G9a) levels in relation to familial history (Figure 11)

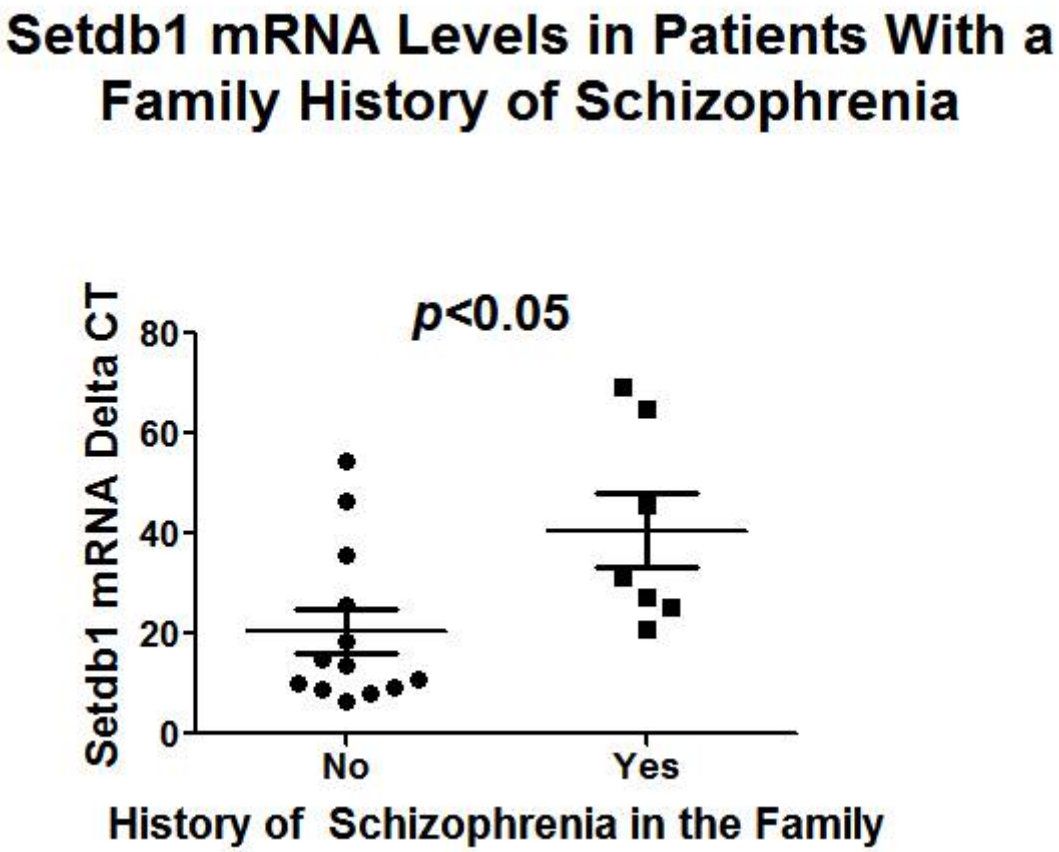

Figure 11 – Setdb1 mRNA levels are significantly increased in patients with a family history of schizophrenia. * *p*<0.05, as determined by t-test.

III.B.2.c Chronicity of Illness

The term 'first episode psychosis' applies to patients with schizophrenia who are experiencing positive symptoms, such as hallucinations and delusions, for the first time. The first three to five years are believed to be a critical period, (Birchwood *et al*., 1998) with effective and efficient treatment believed to prevent relapses and reduce the long-term impact of the illness (Craig *et al*., 2004; Grawe *et al*., 2006). We compared ΔC_T (Experimental Gene C_T-Normalizing Gene C_T) values for Setdb1 between patient illness chronicity using an unpaired two-tailed t-test. Patients with a more chronic form of the illness exhibit higher levels of Setdb1 mRNA compared to normals (ANOVA, $F(2,32)=3.65$, $p<0.01$). There were no significant differences in the other HMT mRNA (GLP, G9a) levels in relation on chronicity of illness (Sharma *et al*., 2012) (Figure 12)

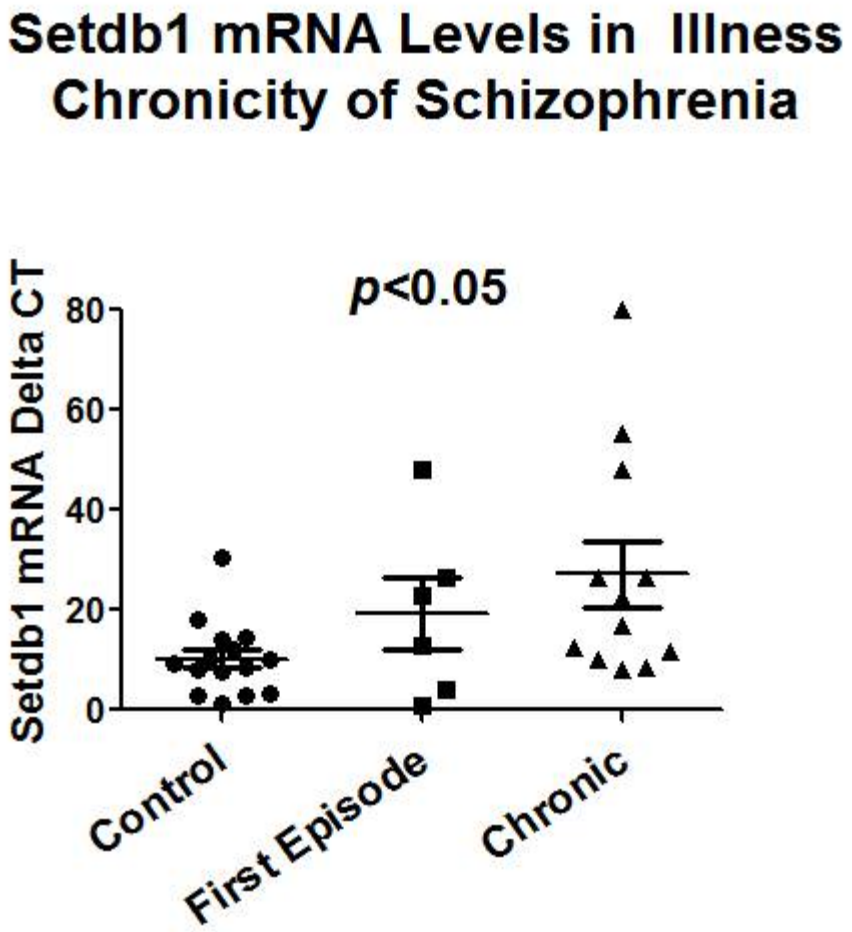

Figure 12 – Setdb1 mRNA levels are significantly increased with chronicity of schizophrenia. Chronic patient with schizophrenia have significantly higher levels of Setdb1 mRNA compared to normal subjects. Patients with first episode psychosis show no significant differences in Setdb1 mRNA levels compared to either chronic schizophrenic patients or normals. * *p*<0.05, as determined by Tukey post hoc.

III.B.2.d Negative Subscale (PANSS)

The heterogeneity of schizophrenia has been a colossal obstacle to understanding the etiology of the disease (Buchanan & Carpenter, 1994). As such, examining schizophrenia as a binary measurement of illness when researching biological relevancy and cause is archaic (Arango *et al*., 2000). The Positive and Negative Syndrome Scale is a behavioral inventory used for measuring severity of symptoms in patients with schizophrenia. The scale is broken into three main sections, a positive subscale, a negative subscale and a general psychopathology subscale, (Kay *et al*., 1987) and is discussed in more depth in section II.B.2.b. Negative symptoms of schizophrenia include emotional withdrawal, blunted affect, anhedonia and poverty of speech, as discussed in the introduction of this thesis. Antipsychotic treatment specifically targets and seeks to rectify positive symptoms, while treatment options for negative symptoms are unmet (Jiawan *et al*., 2010). Negative symptoms in schizophrenia have been shown to be associated with poor premorbid functioning, and increased chronicity of illness, leading to permanent disability (Fenton & McGlashan, 1991).

Spearman's rank-order correlation coefficient (Spearman's Rho) was used to measure the size and statistical significance of the association between PANSS negative subscale total and HMT gene expression. Only the HMT G9a demonstrated a correlation with PANSS negative subscale total, (Spearman's rho=0.44, $p<0.05$) in that as PANSS negative subscale total increased, G9a mRNA levels also increased. Thus, the more severe a patients negative symptoms are, the more elevated their mRNA G9a levels are (Figure 13)

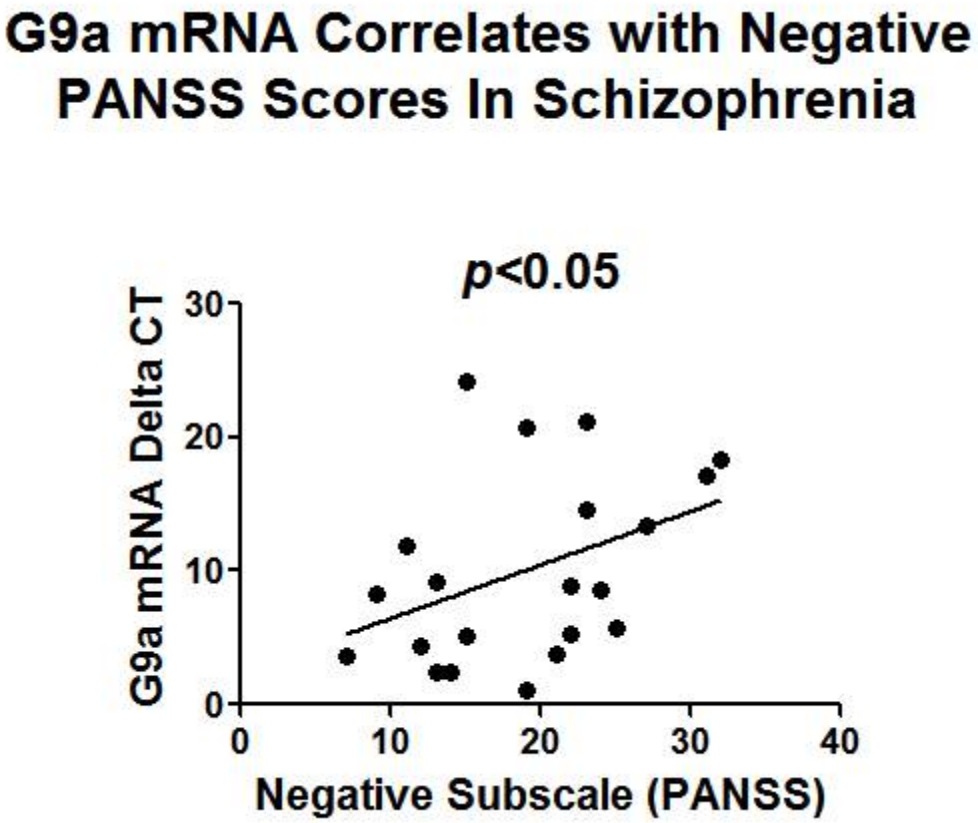

Figure 13 – G9a mRNA levels are significantly increased with increasing PANSS negative subscale totals in patients with schizophrenia. * *p*<0.05, as determined by Spearmans Rho.

III.B.2.e Blunted Affect (PANSS)

As previously mentioned, the clinical measure of a blunted affect is located on the PANSS negative scale (Kay *et al*., 1987). Blunted affect is characterized by diminished expressed or observed emotional responses, such as a reduction in facial expressions, and explicit expression of feelings.

Spearman's rank-order correlation coefficient (Spearman's Rho) was used to measure the size and statistical significance of the association between PANSS blunted affect score and HMT gene expression. Only the HMT G9a demonstrated a correlation with PANSS blunted affect score (Spearman's rho=0.46, $p<0.05$) in that as emotional communicability decreased, G9a mRNA levels increased. Thus, the more severe a patients negative symptoms are, specifically a blunted affect, the more elevated their mRNA G9a levels are (Figure 14).

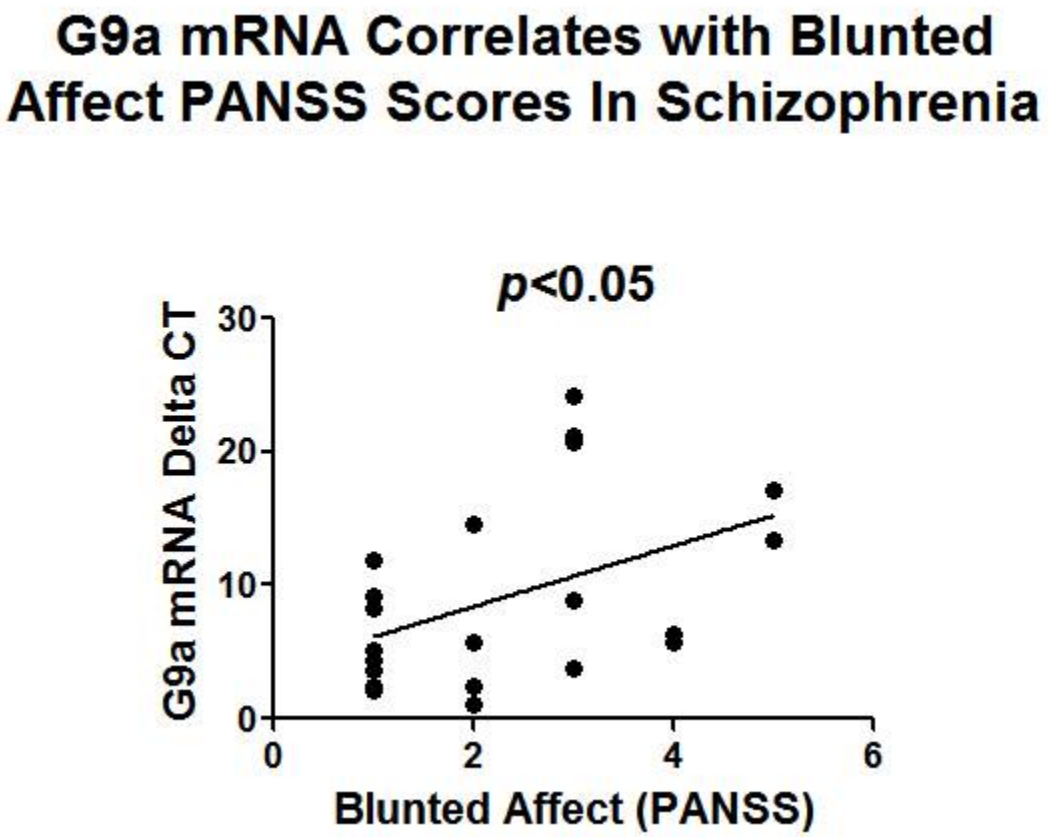

Figure 14 – G9a mRNA levels are significantly increased with increasing Blunted Affect totals. * *p*<0.05, as determined by Spearmans Rho.

In summary, increases in histone methyltransferase mRNA, as demonstrated by this thesis, may be playing a specific critical role in disease etiology. Increases in GLP mRNA are positively correlated with paternal age and history of a neurological condition. Increases in Setdb1 mRNA are positively correlated in both chronicity of illness and familial history of illness, and increases in G9a mRNA are positively correlated with negative symptoms in patients with schizophrenia.

III.C. Aim III: Pharmacological Manipulations of HMT Gene Expression and Comparative Alterations in H3K9me2

III.C.1. Nicotine

III.C.1.a Primary Cortical Neuronal Cell Culture

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and SETDB1 in primary cortical neuronal cultures. For HMT mRNA analysis, nine biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression between vehicle and nicotine treated cells were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance. In primary cortical neuronal cultures treated with nicotine for six hours, ANOVA revealed an effect of nicotine on mRNA expression for GLP (ANOVA, $F_{3,23}=12.8$, $p<0.001$), G9a (ANOVA, $F_{3,23}=46.1$, $p<0.001$) and Setdb1 (ANOVA, $F_{3,23}=18.4$, $p<0.001$) relative to vehicle. Tukey post hoc comparisons indicated that there was a concentration-dependent decrease in mRNA expression for GLP, G9a and Setdb1 in response to nicotine treatment (Figure 15). In summary, nicotine induced concentration dependent decreases in GLP, G9a and Setdb1 mRNA.

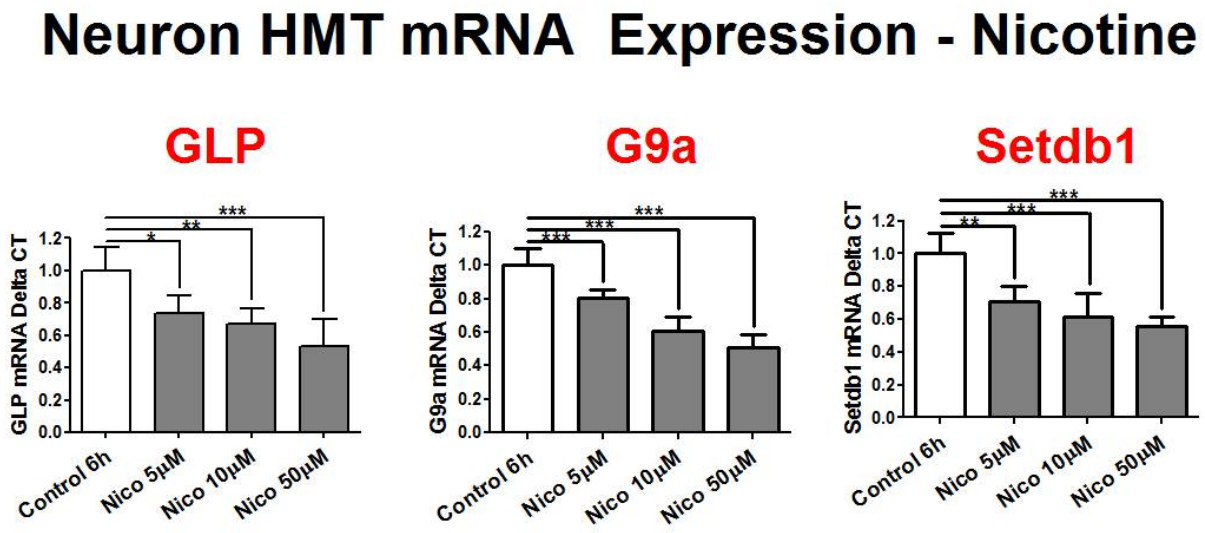

Figure 15 – Histone methyltransferases GLP, G9a and Setdb1 mRNA expression in post-mitotic primary cortical neuronal cell culture after nicotine treatment for six hours. mRNA levels showed a dose dependent decrease, as determined by Tukey post hoc. Shown are the means for 9 independent experiments ± standard error. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H39me2) in primary cortical neuronal cultures. For global H3K9me2 protein analysis, seven biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. Optical density levels of H3K9me2 protein levels between vehicle and nicotine treated cells were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as percent change from vehicle ± standard error of the mean. In primary cortical neuronal cultures treated with nicotine for six hours, data revealed a nicotine dependent decrease in H3K9me2 protein levels (ANOVA, $F_{2,20}$=3.31, p<0.05) relative to vehicle (Figure 16). Tukey post hoc comparisons indicated that there was a concentration-dependent decrease in H3K9me2 protein expression in response to nicotine treatment. Figure 17 is a representative western blot of H3K9me2 in post-mitotic neuronal cell culture treated with 50µm of nicotine. As expected from the nicotine-dependent decreases in HMT mRNA, nicotine treatment results in an H3K9me2 global protein decrease.

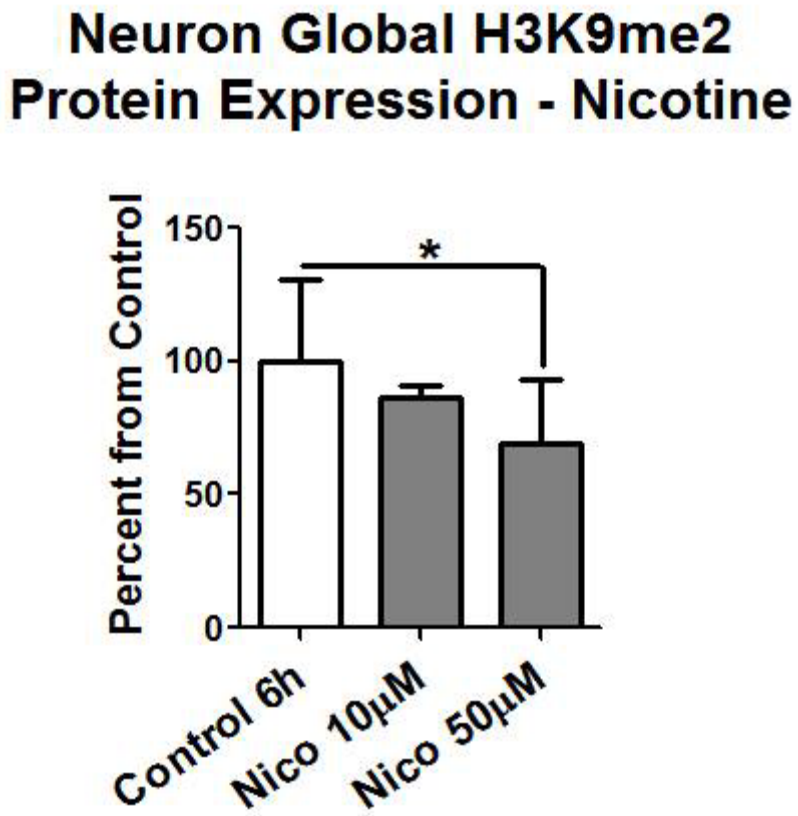

Figure 16 – Global histone post-translational modification H3K9me2 protein expression in post-mitotic primary cortical neuronal cell culture after nicotine treatment for six hours. Protein levels showed a significant decrease. Shown are the means for seven independent experiments ± standard error. *p<0.05, as determined by Tukey post hoc comparison.

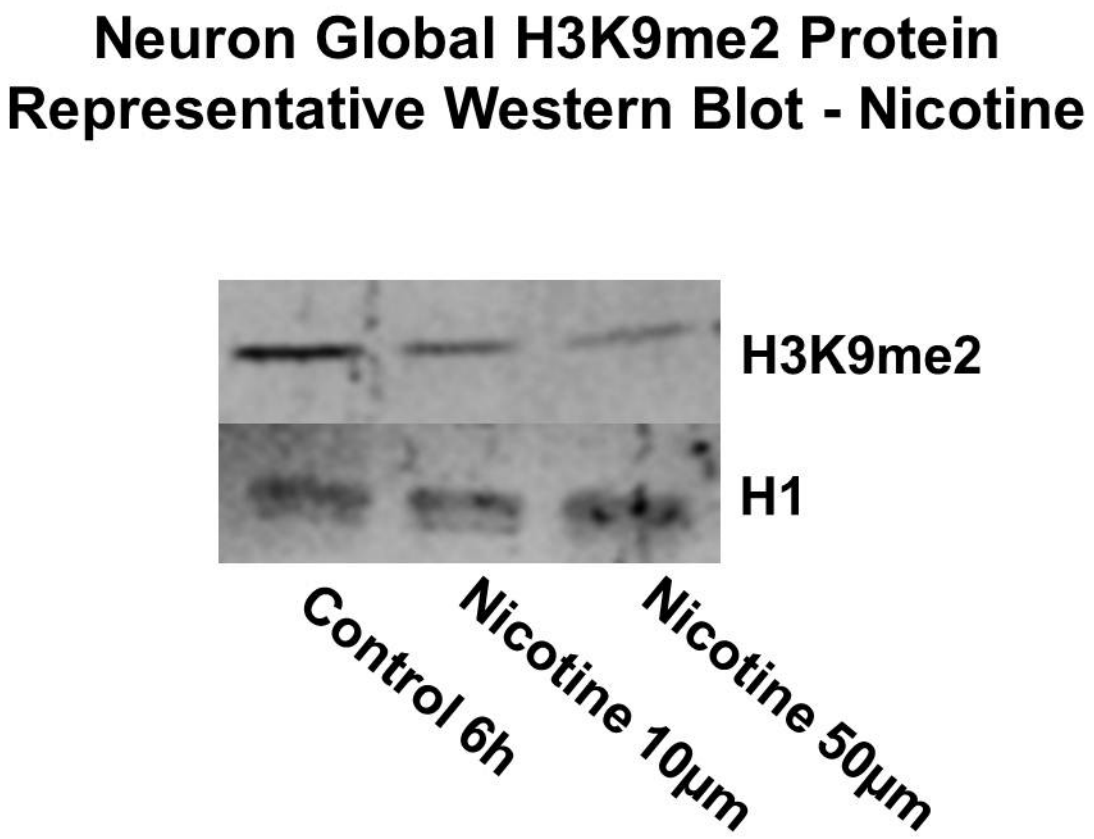

Figure 17 – Representative western blot for H3K9me2 in post-mitotic primary cortical neuronal culture treated with various concentrations of nicotine normalized to H1.

Chromatin Immunoprecipitation (ChIP) was used to investigate interactions between chromatin and specific DNA sequences. Through methods described in section II.A.9, chromatin associated DNA fragments are purified from whole cell extracts, interaction levels can be quantified. The amount of DNA isolated from precipitated H3K9me2 chromatin modification was analyzed using real-time PCR. For Chromatin Immunoprecipitation (ChIP) analysis, all data was analyzed using two controls, an 'input' sample, indicative of the presence and amount of total DNA bound to all chromatin used in the ChIP reaction, and a 'no-antibody' sample, which was treated the same way as the antibody treated sample, and indicates any background signal generated by the actual ChIP procedure. The PCR data was then normalized for differences in amount of total chromatin (input) and background noise (no-antibody) (Haring *et al*., 2007). This normalization was calculated using a 'percent of input' and 'background subtraction' method. For ChIP analysis, three biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. Two-tailed t-tests were performed for each individual transcript between nicotine and vehicle treated cell culture. Using ChIP, H3K9me2 binding at specific Brain Derived Neurotrophic Factor (Bdnf) promoter regions were examined. In primary cortical neurons exposed to 50μM of nicotine for six hours, there was a significant decrease in H3K9me2 binding at promoter I of Bdnf (t=5.38, p<0.01), Bdnf promoter IV (t=8.9, p<0.001), and Bdnf promoter IXa (t=3.9, p<0.05) (Figure 18A). Nicotine dependent decreases in Bdnf promoter specific binding of H3K9me2 are reflective of the global decreases in H3K9me2 protein levels demonstrated by western blot.

We next confirmed that decreased promoter occupancy by a restrictive H3K9me2 (through ChIP analysis) resulted in increased Bdnf transcript (I, IV, IXa) mRNA expression. For

ChIP-specific transcript mRNA analysis, nine biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. We compared ΔC_T (Experimental Gene C_T-Normalizing Gene C_T) values for each individual Bdnf transcript for nicotine or vehicle treated samples using a two-tailed t-test. In primary cortical neurons exposed to 50μM of nicotine for six hours, there was a significant increase in Bdnf transcript I mRNA (t=7.01, p<0.001), Bdnf transcript IV (t=6.5, p<0.001) and Bdnf transcript IXa (t=5.0, p<0.001) (Figure 18B). Collectively we conclude that nicotine is epigenetically modifying histone methyltransferase mRNA levels, resulting in a global and promoter specific decrease in the restrictive chromatin mark H3K9me2. Through this mechanism, nicotine is resulting in a more open chromatin state, allowing for increased gene transcription, as demonstrated through increases in Bdnf mRNA levels.

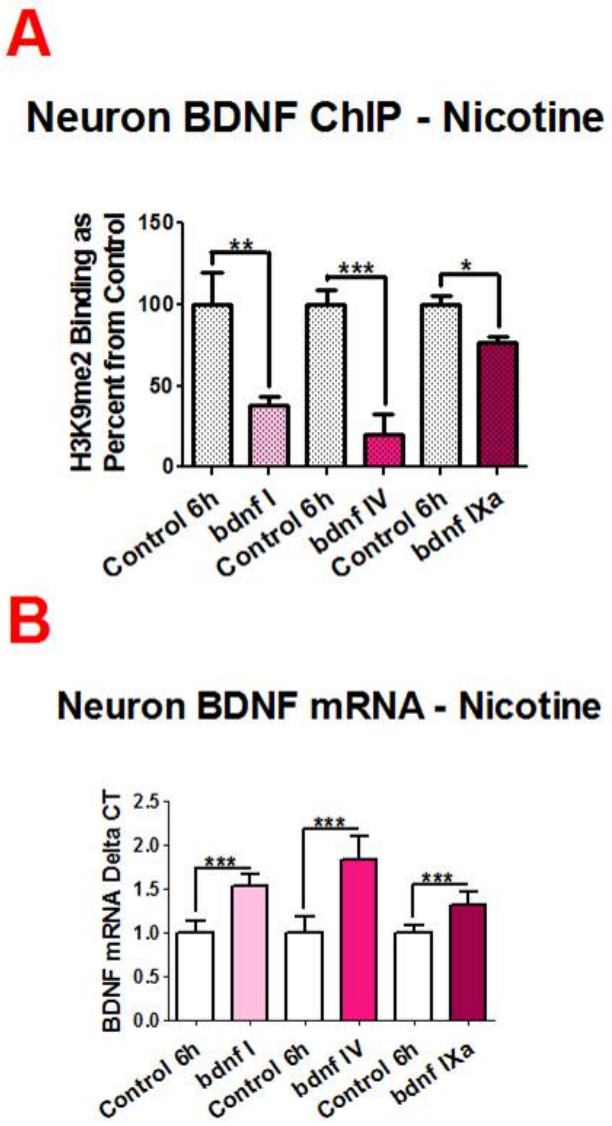

Figure 18 – Neuron Chromatin Immunoprecipitation and Bdnf mRNA levels - Nicotine
A. <u>Chromatin Immunoprecipitation</u>: H3K9me2 binding at Bdnf promoter regions I, V and IXa in post-mitotic primary cortical neuronal cell culture after six hours of 50µM nicotine treatment. Shown is the percent from vehicle for six independent experiments ± standard error. * *p*<0.05, ** *p*<0.01, * *p*<0.001, as determined by two-tailed t-test.**
B. <u>Bdnf mRNA:</u> Increases in Bdnf transcript I, V and IXa mRNA expression in post-mitotic primary cortical neuronal cell culture after nicotine treatment for six hours. Shown are the means for nine independent experiments ± standard error. * *p*<0.001, as determined by two-tailed t-test.**

III.C.1.b Mouse Cortex Extracts

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and Setdb1 in mouse cortex extracts. For HMT mRNA analysis, five biological replicates were used. A biological replicate was defined as single mouse injected IP. Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression between vehicle and nicotine treated mice were analyzed using a two-tailed t-test. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance. In the mouse cortex, 3mg/kg IP injections of nicotine significantly decreased mRNA expression for GLP ($t_8=5.823$, $p<0.001$), G9a ($t_8=4.23$, $p<0.01$) and Setdb1 ($t_8=8.06$, $p<0.001$) relative to vehicle treated mice (Figure 19). In summary, nicotine induced decreases in GLP, G9a and Setdb1 mRNA.

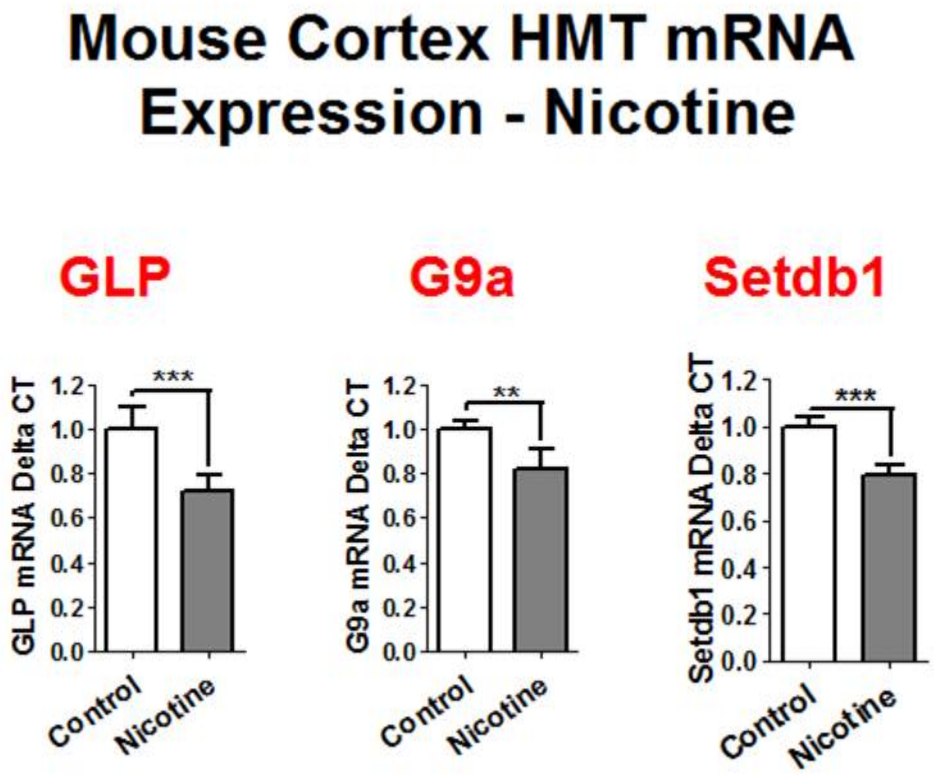

Figure 19 – Histone methyltransferases GLP, G9a and Setdb1 mRNA expression in mouse cortex extracts after nicotine treatment. mRNA levels showed a significant decrease, as determined by two-tailed T Test. Shown are the means for 5 independent experiments ± standard error. ** *p*<0.01, * *p*<0.001.**

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H39me2) in mouse cortex extracts. For global H3K9me2 protein analysis, five biological replicates were used. A biological replicate was defined as single mouse injected IP. Optical density levels of H3K9me2 protein levels between vehicle and nicotine treated cells were analyzed using a two-tailed student t-test. All data is presented as percent change from vehicle ± standard error of the mean. In mouse cortex extracts treated with nicotine, data revealed a nicotine dependent decrease in H3K9me2 protein levels (t_{10}=3.10, p<0.05) relative to vehicle (Figure 20). There was a decrease in H3K9me2 protein expression in response to nicotine treatment. Figure 21 is a representative western blot of H3K9me2 in mouse cortex extracts treated with 3 mg/kg of nicotine. As expected from the nicotine-dependent decreases in HMT mRNA, nicotine treatment results in an H3K9me2 global protein decrease.

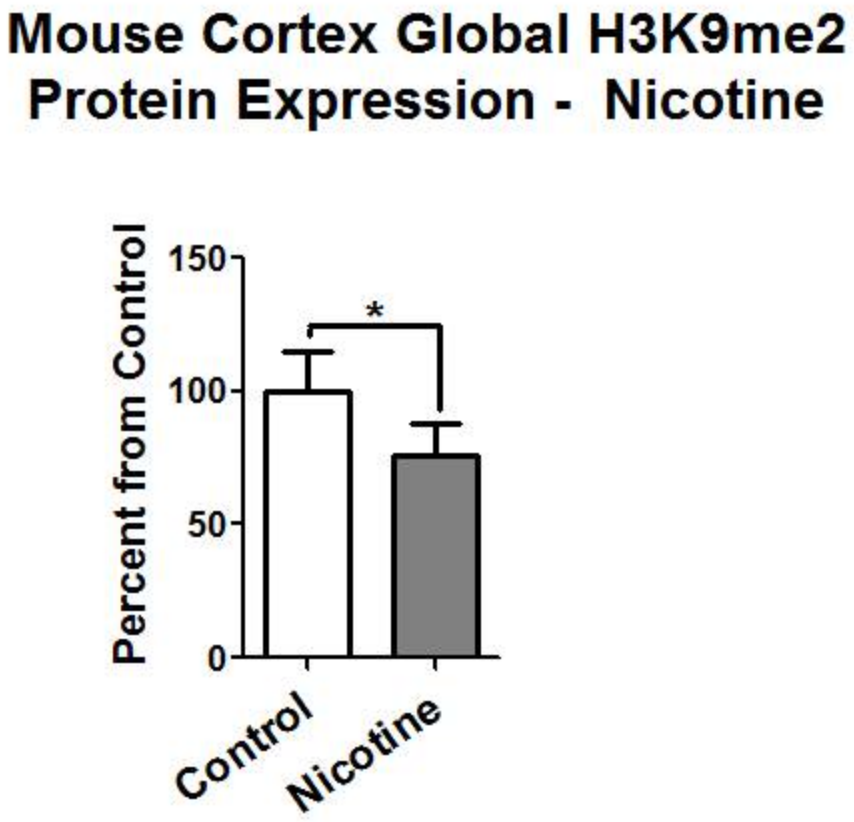

Figure 20 – Global histone post-translational modification H3K9me2 protein expression in mouse cortex extracts after nicotine treatment. Protein levels showed a significant decrease. Shown are the means for seven independent experiments ± standard error. *p<0.05, as determined by t-test.

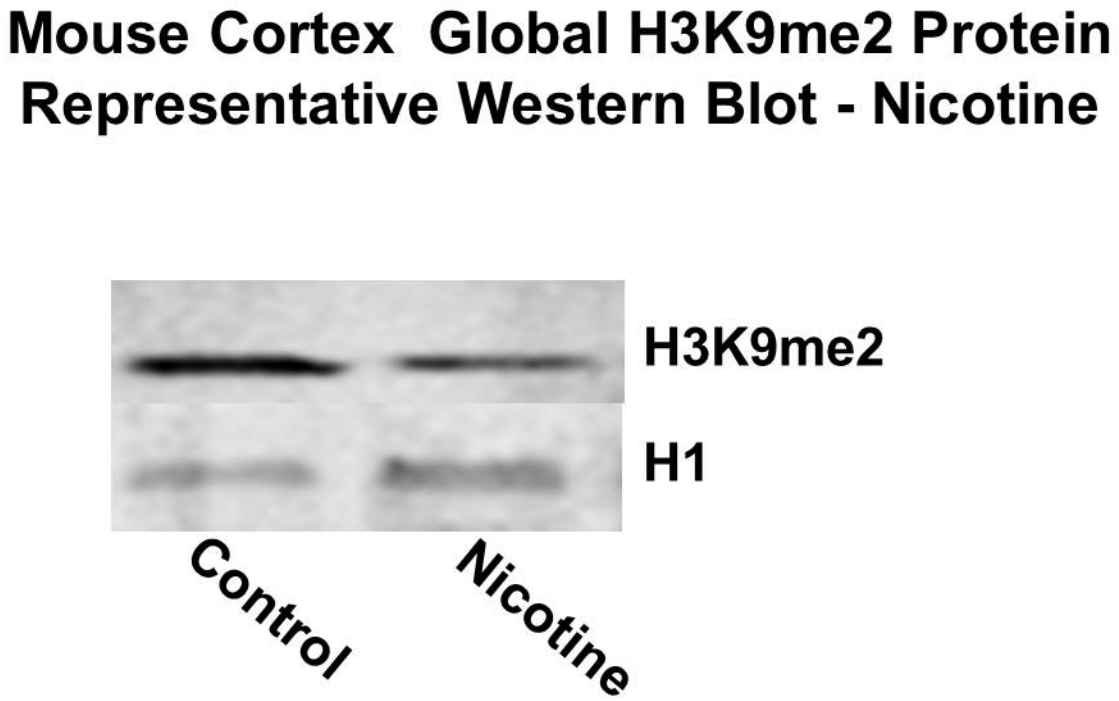

Figure 21 – Representative western blot for H3K9me2 in mouse cortex extracts treated with nicotine, normalized to H1.

Chromatin Immunoprecipitation (ChIP) is used to investigate interactions between protein and specific DNA sequences. For ChIP analysis, five biological replicates were used. A biological replicate was defined as single mouse injected IP. Two-tailed t-tests were performed for the individual transcript between nicotine and vehicle treated mice. Using ChIP, H3K9me2 binding at specific Brain Derived Neurotrophic Factor (Bdnf) promoter regions were examined. In mice two hours after nicotine treatment, there was a significant decrease in H3K9me2 binding at promoter I of Bdnf (t_{10}=25.03 p<0.001), IV of Bdnf (t_{10}=25.03 p<0.001), IXa of Bdnf (t_{10}=25.03 p<0.001) (Figure 22A). Nicotine dependent decreases in Bdnf promoter specific binding of H3K9me2 are reflective of the global decreases in H3K9me2 protein levels demonstrated by western blot.

We confirmed that decreased promoter occupancy by a restrictive H3K9me2 (through ChIP analysis) resulted in increased Bdnf transcript mRNA expression. For Bdnf mRNA analysis, five biological replicates were used. A biological replicate was defined as single mouse injected IP. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. We compared ΔC_T (Experimental Gene C_T-Normalizing Gene C_T) values for the individual Bdnf transcript between nicotine and vehicle treated mice using a two-tailed t-test. In mice two hours after nicotine treatment, there was a significant increase in Bdnf transcript I (t_8=5.04 p<0.01), IV (t_8=13.48 p<0.001), IXa (t_8=3.91 p<0.001) (Figure 22B). Collectively we conclude that nicotine is epigenetically modifying HMT mRNA levels, resulting in a global and promoter specific decrease in the restrictive chromatin mark H3K9me2. Through this mechanism, nicotine is resulting in a more open chromatin state, allowing for increased gene transcription, as demonstrated through increases in Bdnf mRNA levels.

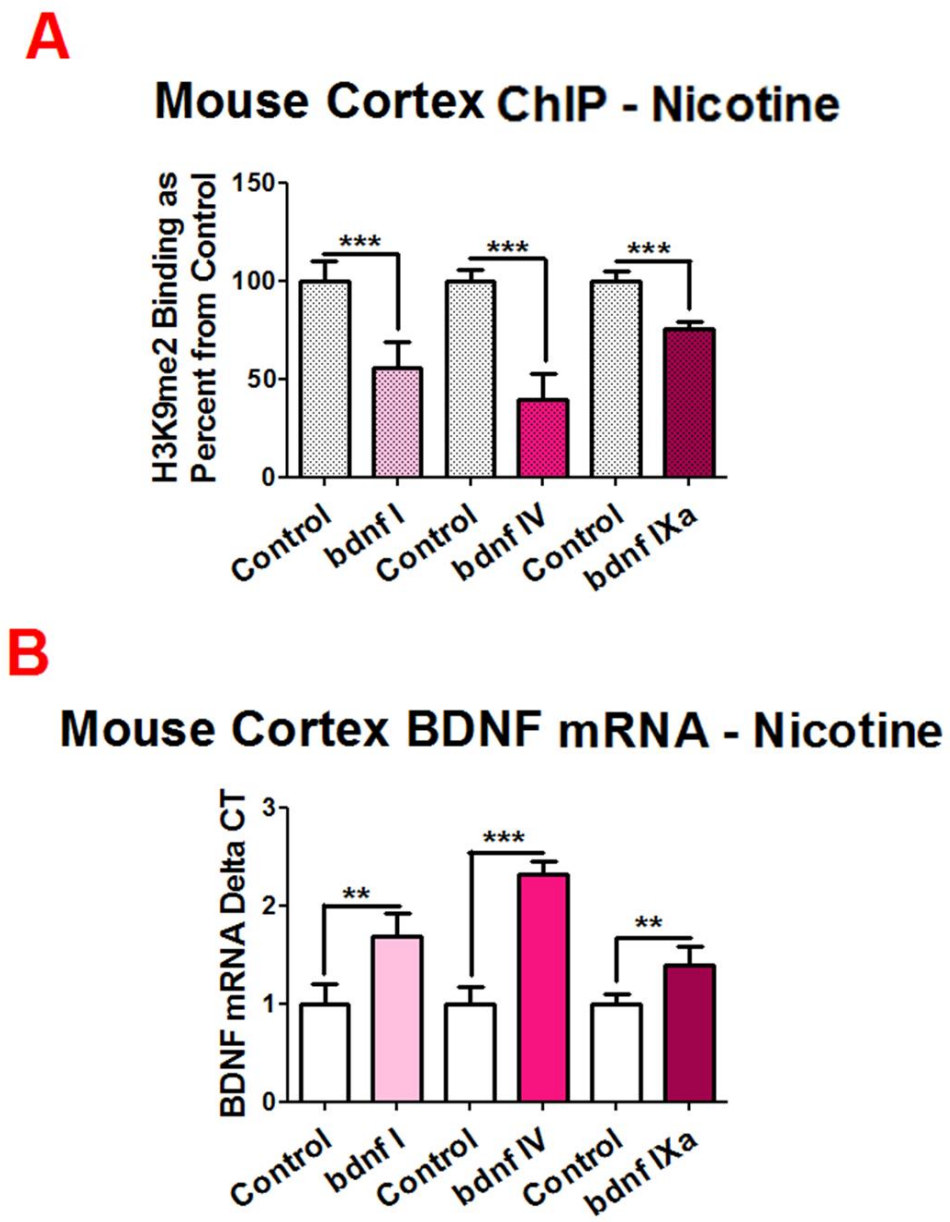

Figure 22 – Mouse Cortex Chromatin Immunoprecipitation and Bdnf mRNA levels - Nicotine

A <u>Chromatin Immunoprecipitation</u>: H3K9me2 binding at Bdnf promoter regions in mouse cortex extracts after nicotine treatment. Shown is the percent from vehicle for five independent experiments ± standard error. * *p*<0.001, as determined by two-tailed t-test.**

B <u>Bdnf mRNA:</u> Increases in Bdnf transcript mRNA expression in mouse cortex extracts after nicotine treatment. Shown are the means for five independent experiments ± standard error. ** *p*<0.01, * *p*<0.001, as determined by two-tailed t-test.**

III.C.1.c. Lymphocyte Cell Culture

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and SETDB1 in lymphocyte cultures. For HMT mRNA analysis, nine biological replicates were used. A biological replicate was defined as a blood draw from a single participant. Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression between vehicle and nicotine treated cells were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance. In lymphocyte cultures treated with nicotine for six hours, ANOVA revealed an effect of nicotine on mRNA expression for GLP (ANOVA, $F_{3,35}=16.40$, $p<0.001$), G9a (ANOVA, $F_{3,35}=7.61$, $p<0.001$) and Setdb1 (ANOVA, $F_{3,35}=5.87$, $p<0.01$) relative to vehicle. Tukey post hoc comparisons indicated that there was a concentration-dependent decrease in mRNA expression for GLP, G9a and Setdb1 in response to nicotine treatment (Figure 23). In summary, nicotine induced concentration dependent decreases in GLP, G9a and Setdb1 mRNA.

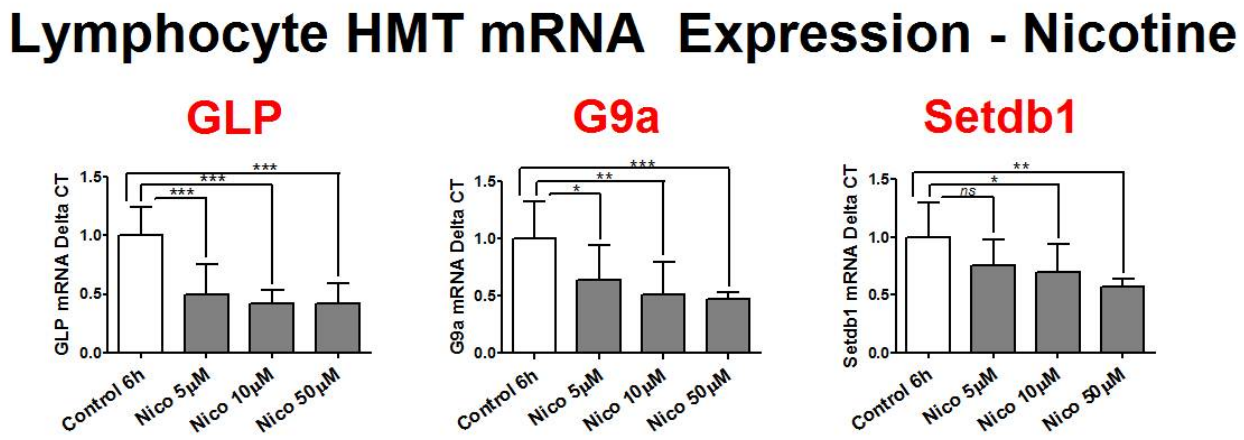

Figure 23 – Histone methyltransferases GLP, G9a and Setdb1 mRNA expression in lymphocyte cell culture after nicotine treatment for six hours. mRNA levels showed a dose dependent decrease, as determined by Tukey post hoc. Shown are the means for 9 independent experiments ± standard error. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H39me2) in lymphocyte cultures. For global H3K9me2 protein analysis, four biological replicates were used. Optical density levels between vehicle and nicotine treated lymphocytes demonstrated significant decreases in H3K9me2 levels using (ANOVA, $F_{3,15}$=63.25, p<0.001) relative to vehicle (Figure 24). Tukey post hoc comparisons indicated that there was a concentration-dependent decrease in H3K9me2 protein expression in response to nicotine treatment. Figure 25 is a representative western blot of H3K9me2 in lymphocyte culture treated with nicotine. As expected from the nicotine-dependent decreases in HMT mRNA, nicotine treatment results in an H3K9me2 global protein decrease.

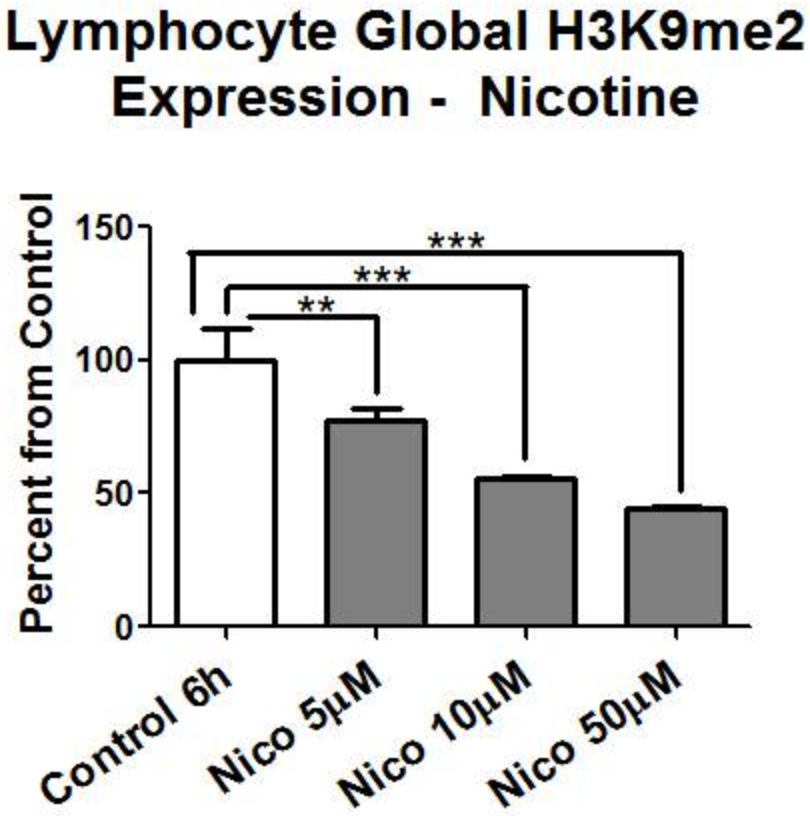

Figure 24 – Global histone post-translational modification H3K9me2 protein expression in lymphocyte cell culture after nicotine treatment for six hours. Protein levels showed a significant decrease. Shown are the means for four independent experiments ± standard error. **p<0.01, ***p<0.001 as determined by t-test.

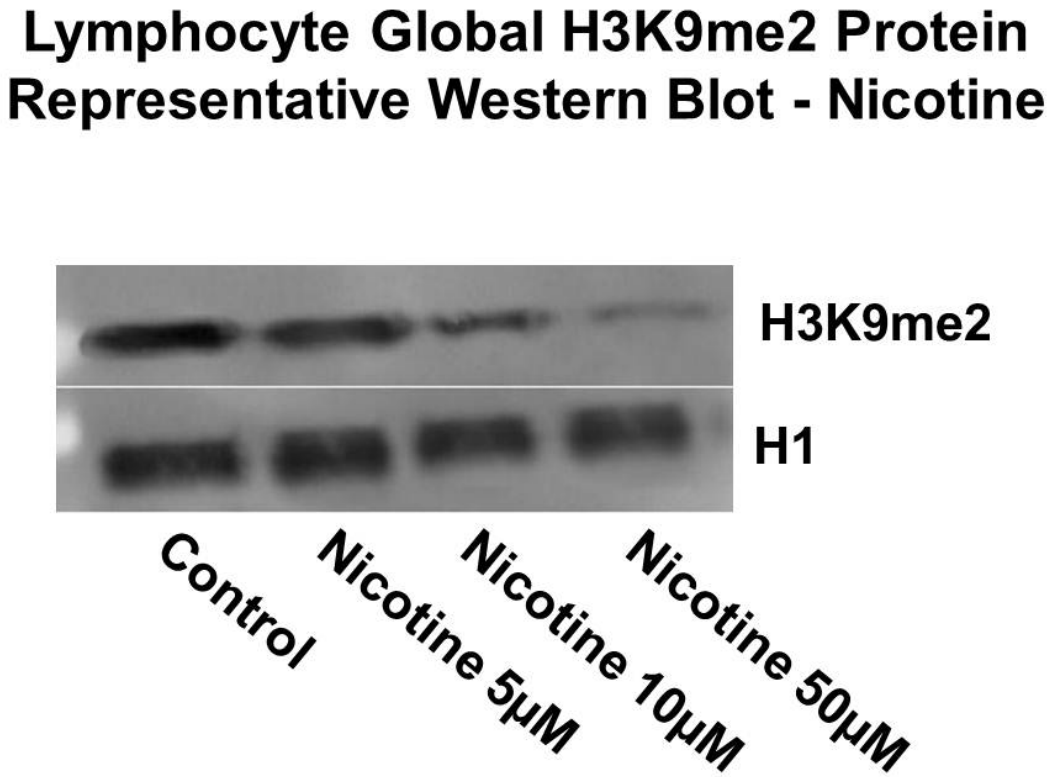

Figure 25 – Representative western blot for H3K9me2 lymphocyte culture treated with various concentrations of nicotine, normalized to H1.

Chromatin Immunoprecipitation (ChIP) is used to investigate interactions between protein and specific DNA sequences. For ChIP analysis, six biological replicates were used. A biological replicate was defined as a blood draw from a single participant. Two-tailed t-tests were performed for the individual transcript between nicotine and vehicle treated cell culture. Using ChIP, H3K9me2 binding at specific BDNF promoter regions were examined. In lymphocyte culture exposed to 50μM of nicotine for six hours, there was a significant decrease in H3K9me2 binding at promoter IXabcd of BDNF (t_{10}=25.03 p<0.001) (Figure 26A). Nicotine dependent decreases in BDNF promoter specific binding of H3K9me2 are reflective of the total decreases in H3K9me2 protein levels demonstrated by western blot.

We next confirmed that decreased promoter occupancy by a restrictive H3K9me2 (through ChIP analysis) resulted in increased BDNF transcript IXabcd mRNA expression. For ChIP-specific transcript mRNA analysis, four biological replicates were used. A biological replicate was defined as a blood draw from a single participant. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. We compared ΔC_T (Experimental Gene C_T-Normalizing Gene C_T) values for the individual BDNF transcript between nicotine and vehicle treated samples using a two-tailed t-test. In lymphocyte culture exposed to 50μM of nicotine for six hours, there was a significant increase in mRNA of BDNF transcript IXabcd (t_6=11.81 p<0.001) (Figure 26B). Collectively we conclude that nicotine is epigenetically modifying histone methyltransferase mRNA levels, resulting in a global and promoter specific decrease in the restrictive chromatin mark H3K9me2. Through this mechanism, nicotine is resulting in a more open chromatin state, allowing for increased gene transcription, as demonstrated through increases in BDNF mRNA levels.

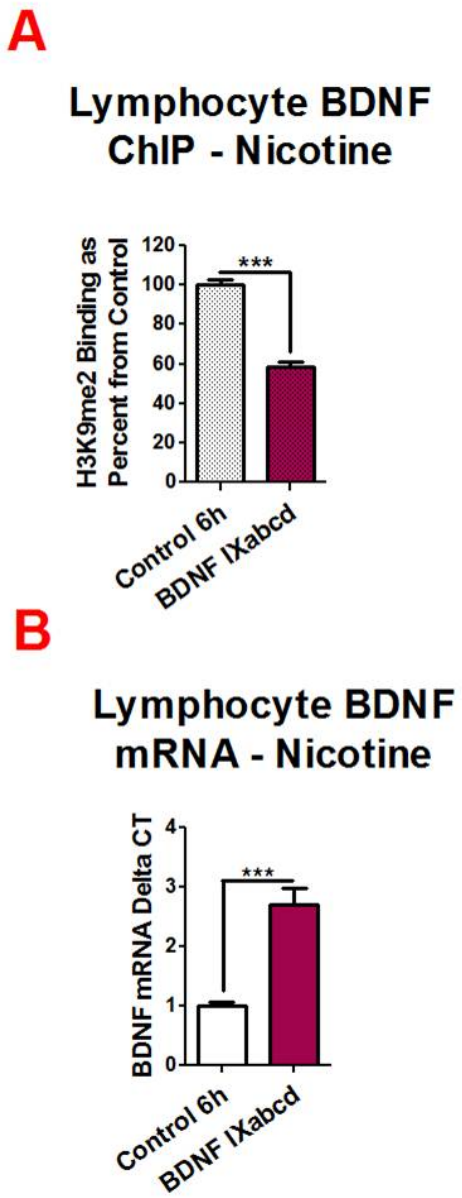

Figure 26 – Lymphocyte Chromatin Immunoprecipitation and BDNF mRNA levels - Nicotine

A <u>Chromatin Immunoprecipitation</u>: H3K9me2 binding at BDNF promoter regions IXabcd in lymphocyte cell culture after six hours of 50µM nicotine treatment. Shown is the percent from control condition for six independent experiments ± standard error. * *p*<0.001, as determined by two-tailed t-test.**

B <u>BDNF mRNA:</u> Increases in BDNF transcript IXabcd mRNA expression in lymphocyte cell culture after nicotine treatment for six hours. Shown are the means for four independent experiments ± standard error. * *p*<0.001, as determined by two-tailed t-test.**

III.C.2 Valproic Acid

III.C.2.a Primary Cortical Neuronal Cell Culture

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and SETDB1 in primary cortical neuronal cultures. For HMT mRNA analysis, four biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression between vehicle VPA treated cells were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance. In primary cortical neuronal cultures treated with VPA for 24 hours, ANOVA revealed an effect of nicotine on mRNA expression for GLP (ANOVA, $F_{2,11}=6.95$, $p<0.05$), G9a (ANOVA, $F_{2,11}=5.13$, $p<0.05$) and Setdb1 (ANOVA, $F_{2,11}=10.5$, $p<0.01$) relative to control. Tukey post hoc comparisons indicated that there was a concentration-dependent decrease in mRNA expression for GLP, G9a and Setdb1 in response to VPA treatment (Figure 27). In summary, Valproic acid induced concentration dependent decreases in GLP, G9a and Setdb1 mRNA.

Figure 27 – Histone methyltransferases GLP, G9a and Setdb1 mRNA expression in lymphocyte cell culture after VPA treatment for 24 hours. mRNA levels showed a dose dependent decrease, as determined by Tukey post hoc. Shown are the means for 4 independent experiments ± standard error. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H39me2) in primary cortical neuronal cultures. For global H3K9me2 protein analysis, four biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. Optical density levels of H3K9me2 protein levels in non-VPA, vehicle treated control samples and VPA treated cells were analyzed using two-way student t-test. All data is presented as percent change from control ± standard error of the mean. In primary cortical neuronal cultures treated with VPA for 24 hours, data revealed a VPA dependent decrease in H3K9me2 protein levels (t_6=7.04, p<0.001) relative to control (Figure 28). Figure 29 is a representative western blot of H3K9me2 in post-mitotic neuronal cell culture treated with 2mM of VPA. As expected from the VPA-dependent decreases in HMT mRNA, Valproic acid treatment results in an H3K9me2 global protein decrease.

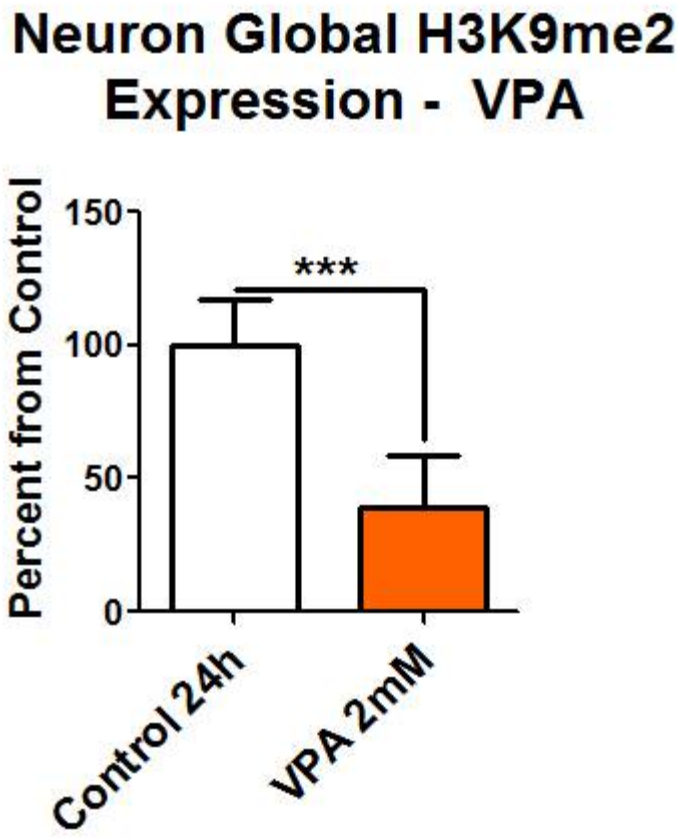

Figure 28 – Global histone post-translational modification H3K9me2 protein expression in post-mitotic primary cortical neuronal cell culture after VPA treatment for 24 hours. Protein levels showed a significant decrease. Shown are the means for four independent experiments ± standard error. ******p*<0.001, as determined by t-test.

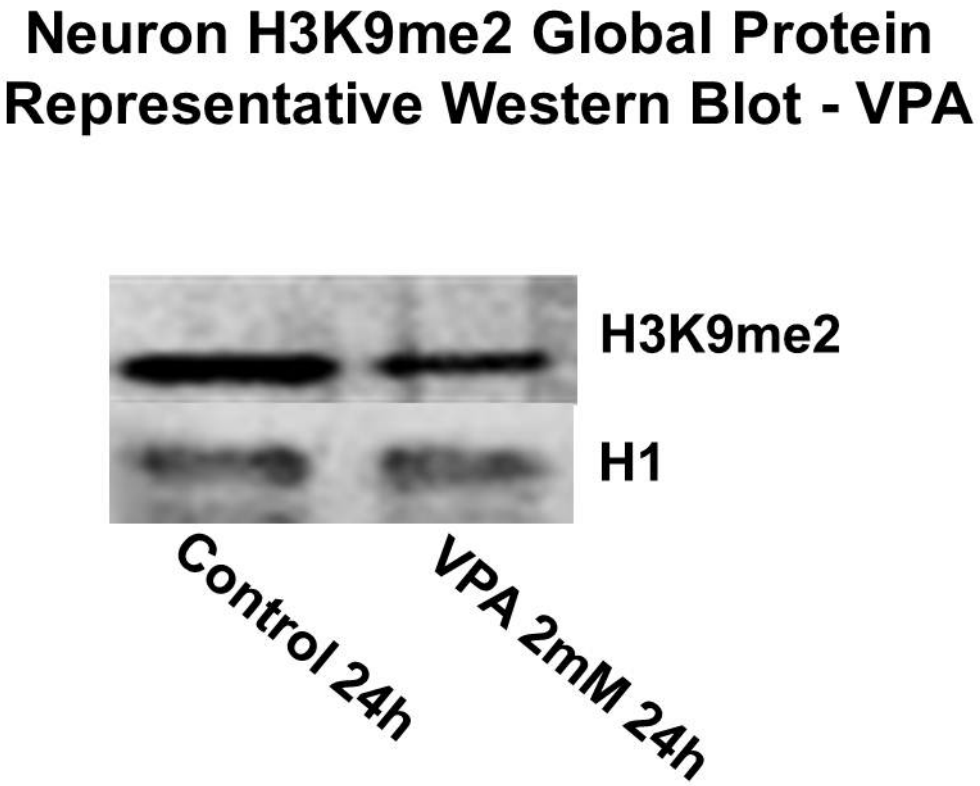

Figure 29 – Representative western blot for H3K9me2 in post-mitotic primary cortical neuronal culture treated with 2mM of Valproic acid for 24 hours, normalized to H1

Chromatin Immunoprecipitation (ChIP) was used to investigate interactions between protein and specific DNA sequences. For ChIP analysis, three biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. Two-tailed t-tests were performed for the individual transcript between VPA and vehicle treated cell culture. Using ChIP, H3K9me2 binding at specific Brain Derived Neurotrophic Factor (Bdnf) promoter regions were examined. In primary cortical neurons exposed to 2mM of VPA for 24 hours, there was a significant decrease in H3K9me2 binding at promoter I of Bdnf (t_4=4.55 p<0.05), IV of Bdnf (t_5=5.47 p<0.01), and IXa of Bdnf (t_4=3.1 p<0.01) (Figure 30A). Valproic acid dependent decreases in Bdnf promoter specific binding of H3K9me2 are reflective of the global decreases in H3K9me2 protein levels demonstrated by western blot.

Decreased promoter occupancy by a restrictive H3K9me2 resulted in increased Bdnf transcript mRNA expression. For ChIP-specific transcript mRNA analysis, four biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. We compared ΔC_T values for the individual Bdnf transcript for VPA treatment and vehicle treated control samples using a two-tailed t-test. In primary cortical neurons exposed to 2mM VPA for 24 hours, there was a significant increase in mRNA of promoter I of Bdnf (t_4=15.93 p<0.001), IV of Bdnf (t_4=6.21 p<0.01), and IXa of Bdnf (t_4=6.34 p<0.01) (Figure 30B). Collectively we conclude that Valproic acid is epigenetically modifying HMT mRNA levels, resulting in global and promoter specific decreases in the restrictive chromatin mark H3K9me2. Through this mechanism, VPA 'opens' the epigenome, thus allowing for increased gene transcription, as demonstrated by increases in Bdnf mRNA levels.

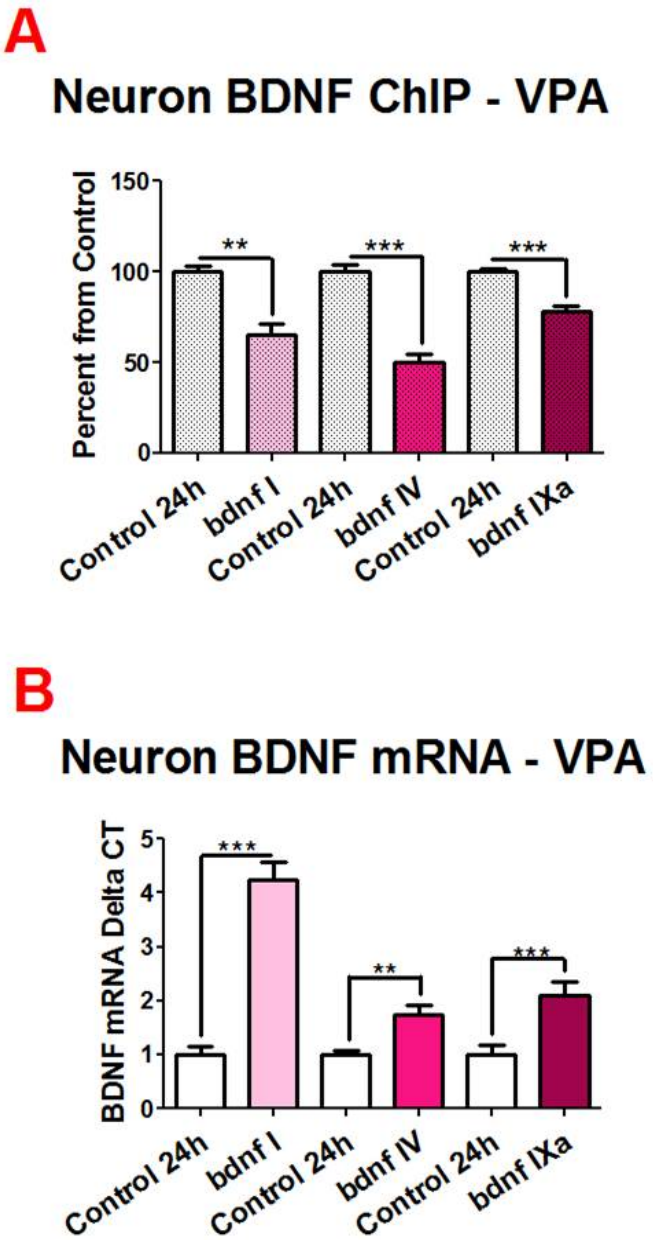

Figure 30 – Neuron Chromatin Immunoprecipitation and Bdnf mRNA levels - VPA
A <u>Chromatin Immunoprecipitation</u>: H3K9me2 binding at Bdnf promoter regions I, V and IXa in post-mitotic primary cortical neuronal cell culture after 24 hours of 2mM Valproic acid treatment. Shown is the percent from control condition for three independent experiments ± standard error. ** *p*<0.01, * *p*<0.001, as determined by two-tailed t-test.**
B <u>Bdnf mRNA:</u> Increases in Bdnf transcript I, V and IXa mRNA expression in post-mitotic primary cortical neuronal cell culture after VPA treatment for 24 hours. Shown are the means for four independent experiments ± standard error. ** *p*<0.01, * *p*<0.001, as determined by two-tailed t-test.**

III.C.2.b Mouse Cortex Extracts

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and SETDB1 in mouse cortex extracts. For HMT mRNA analysis, five biological replicates were used. A biological replicate was defined as single mouse injected IP. Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression between vehicle and Valproic acid treated mice were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance. In mouse cortex extracts, 3mg/kg IP injections of nicotine significantly decreased mRNA expression for GLP (ANOVA, $F_{3,19}=8.56$, $p<0.01$), G9a (ANOVA, $F_{3,19}=10.47$, $p<0.001$) and Setdb1 (ANOVA, $F_{3,19}=10.27$, $p<0.001$) relative to control. Tukey post hoc comparisons indicated that there was a time-dependent decrease in mRNA expression for GLP, G9a and Setdb1 in response to VPA treatment (Figure 31). In summary, Valproic acid induced decreases in GLP, G9a and Setdb1 mRNA.

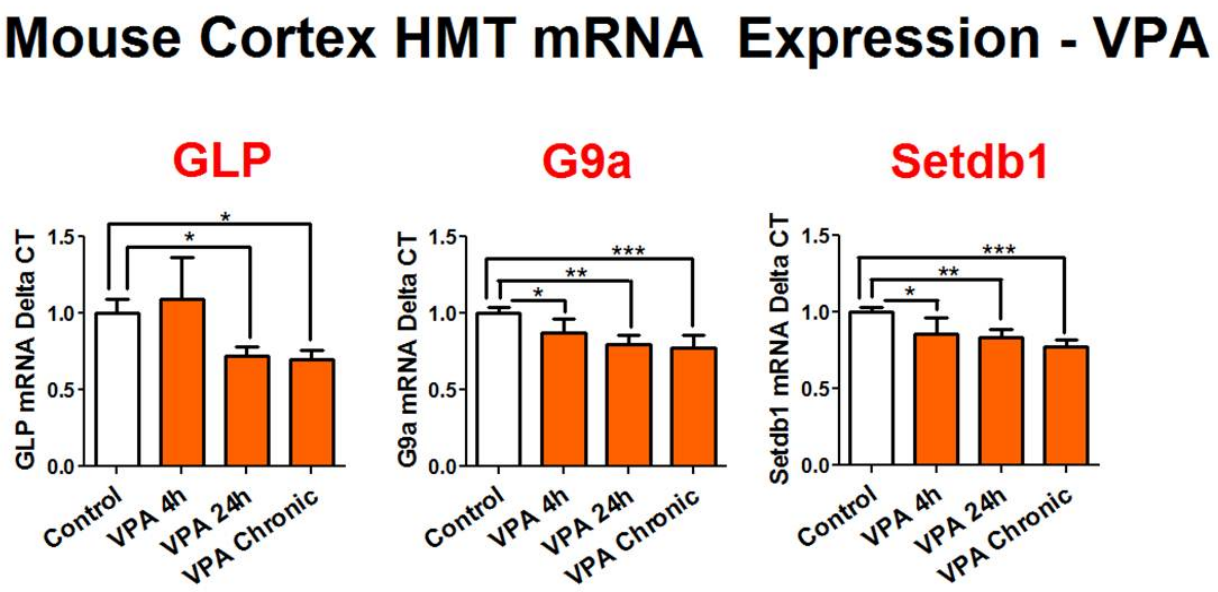

Figure 31 – Histone methyltransferases GLP, G9a and Setdb1 mRNA expression in mouse cortex extracts after VPA treatment. mRNA levels showed a significant decrease, as determined by Tukey post hoc test. Shown are the means for 5 independent experiments ± standard error. * *p*<0.05, ** *p*<0.01, * *p*<0.001.**

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H39me2) in mouse cortex extracts. For global H3K9me2 protein analysis, five biological replicates were used. A biological replicate was defined as single mouse injected IP. Optical density levels of H3K9me2 protein levels in non-VPA, vehicle treated control samples and VPA treated mice were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as percent change from control ± standard error of the mean. In mice treated with Valproic for, data revealed a VPA dependent decrease in H3K9me2 protein levels (ANOVA, $F_{3,19}$=24.18, p<0.001) relative to control (Figure 32). Tukey post hoc comparisons indicated that there was a time-dependent decrease in H3K9me2 protein expression in response to Valproic acid treatment treatment. Figure 33 is a representative western blot of H3K9me2 in mice treated with 150 mg/kg of Valproic acid for various time points. As expected from the Valproic acid-dependent decreases in HMT mRNA, VPA treatment results in an H3K9me2 global protein decrease.

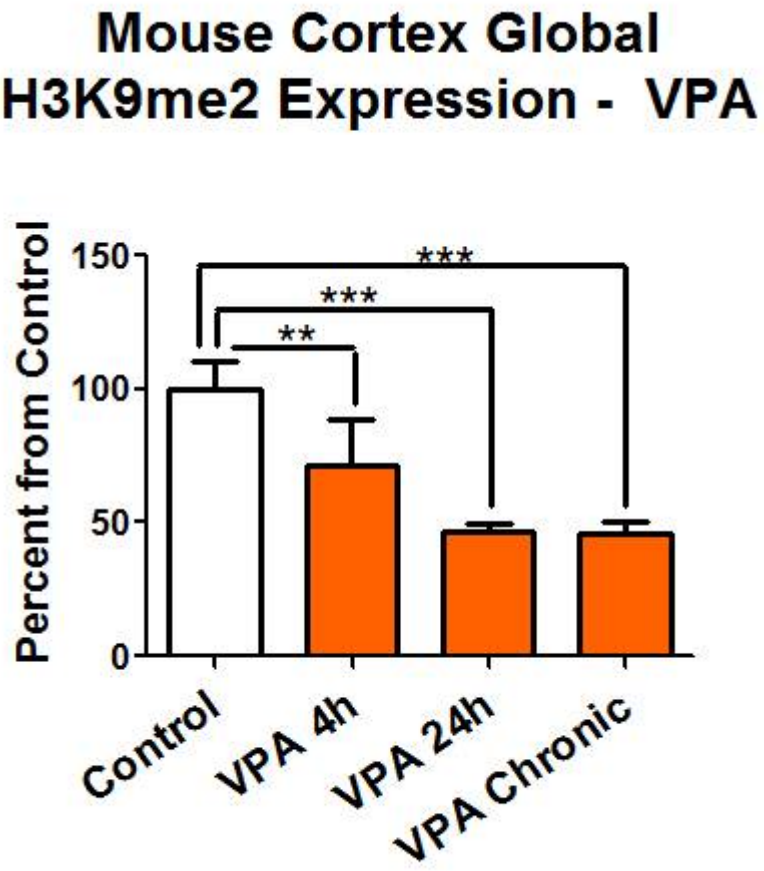

Figure 32 – Global histone post-translational modification H3K9me2 protein expression in mice brain tissue after Valproic Acid treatment. Protein levels showed a time-dependent significant decrease. Shown are the means for five independent experiments ± standard error. *****p*<0.01, ******p*<0.001, as determined by Tukey post-hoc test.

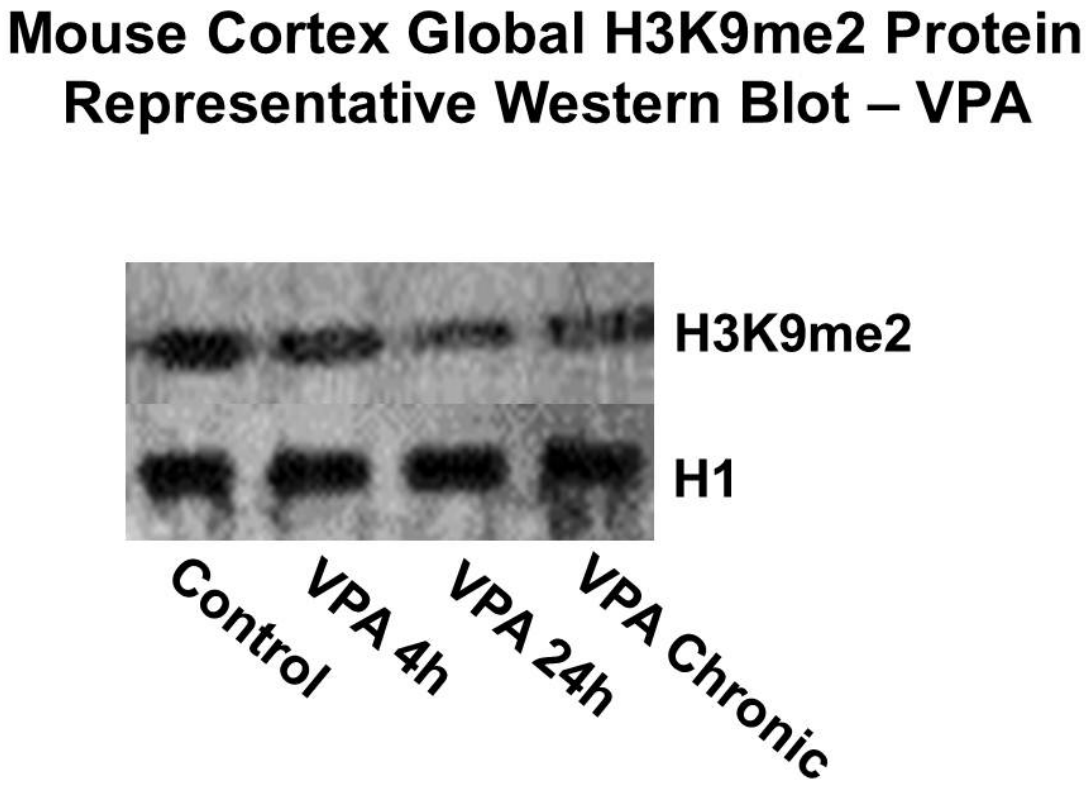

Figure 33 – Representative western blot for H3K9me2 in mouse cortex tissue treated with Valproic acid for various time lengths, normalized to H1

Chromatin Immunoprecipitation (ChIP) is used to investigate interactions between protein and specific DNA sequences. For ChIP analysis, five biological replicates were used. A biological replicate was defined as single mouse injected IP. Two-tailed t-tests were performed for the individual transcript between VPA and saline treated mice. Using ChIP, H3K9me2 binding at specific Bdnf promoter regions were examined. In mice 24 hours after treatment with 150 mg/kg of VPA, there was a significant decrease in H3K9me2 binding at promoters I of Bdnf (t_8=3.02 p<0.05), IV of Bdnf (t_8=6.33 p<0.001), IXa of Bdnf (t_8=4.29 p<0.01) (Figure 34A). Valproic acid dependent decreases in Bdnf promoter specific binding of H3K9me2 are reflective of the global decreases in H3K9me2 protein levels demonstrated by western blot.

Decreased promoter occupancy by a restrictive H3K9me2 (through ChIP analysis) resulted in increased Bdnf transcript mRNA expression. For ChIP-specific transcript mRNA analysis, five biological replicates were used. A biological replicate was defined as single mouse injected IP. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. We compared ΔC_T (Experimental Gene C_T-Normalizing Gene C_T) values for the individual Bdnf transcript for VPA and saline treated mice using a two-tailed t-test. In mice 24 hours after treatment with Valproic acid, there was a significant increase in Bdnf transcript I of Bdnf (t_8=5.17 p<0.001), IV of Bdnf (t_8=3.52 p<0.01), IXa of Bdnf (t_8=3.63 p<0.01) (Figure 34B). Collectively we conclude that Valproic acid is epigenetically modifying HMT mRNA levels, resulting in a global and promoter specific decrease in the restrictive chromatin mark H3K9me2. Through this mechanism, VPA is resulting in an open chromatin state, allowing for increased gene transcription, as shown through increases in Bdnf mRNA.

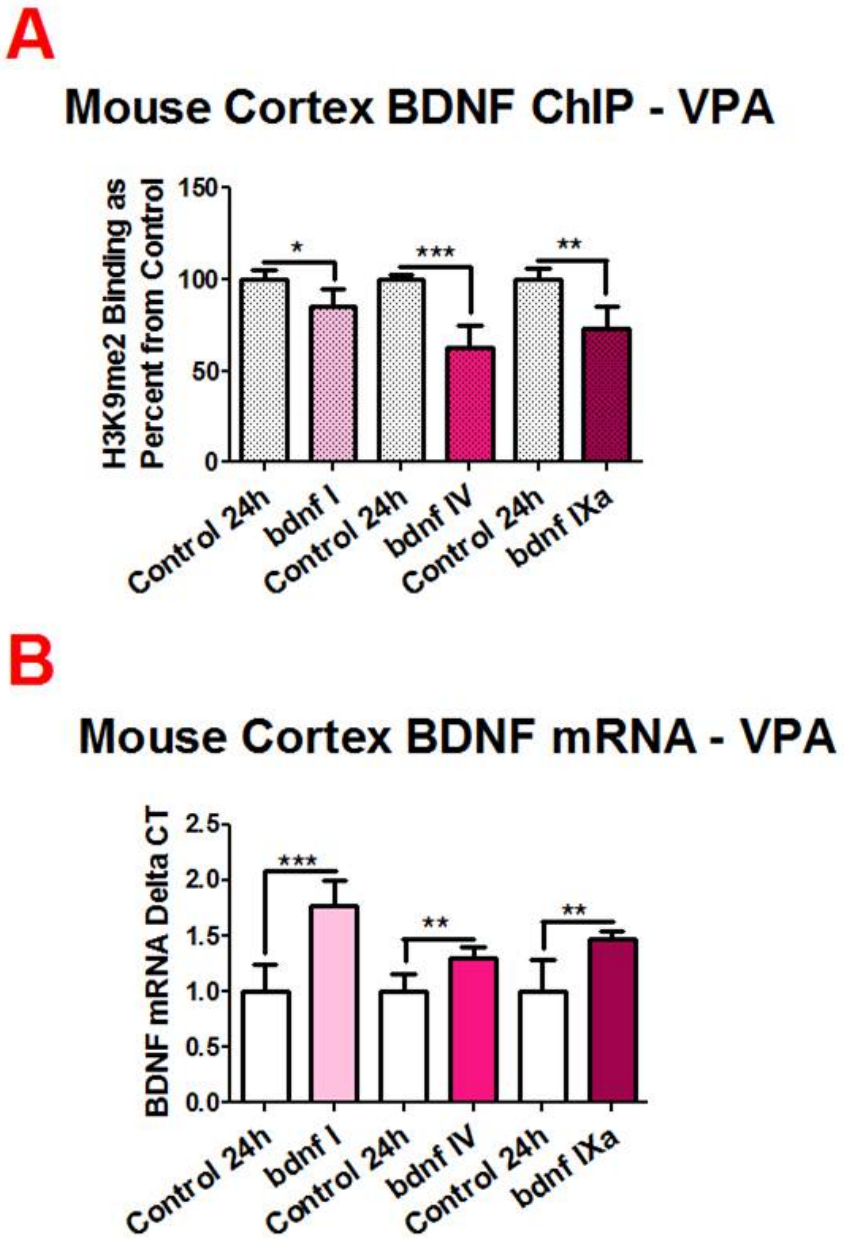

Figure 34 – Mouse Cortex Chromatin Immunoprecipitation and Bdnf mRNA Levels - VPA
A <u>Chromatin Immunoprecipitation</u>: H3K9me2 binding at Bdnf promoter regions I, V and IXa in brain tissue from mice after 24 hours of Valproic acid treatment. Shown is the percent from control condition for three independent experiments ± standard error. * *p*<0.05, ** *p*<0.01, * *p*<0.001, as determined by two-tailed t-test.**
B <u>Bdnf mRNA:</u> Increases in Bdnf transcript I, V and IXa mRNA expression in brain tissue from mice after 24 hours of Valproic acid treatment. Shown are the means for four independent experiments ± standard error. ** *p*<0.01, * *p*<0.001, as determined by two-tailed t-test.**

III.C.2.c Lymphocyte Cell Culture

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and SETDB1 in lymphocyte cultures. For HMT mRNA analysis, nine biological replicates were used. A biological replicate was defined as a blood draw from a single participant. Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression between vehicle and VPA treated cells were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance. In lymphocyte cultures treated with nicotine for 24 hours, ANOVA revealed an effect of nicotine on mRNA expression for GLP (ANOVA, $F_{3,11}=18.72$, $p<0.001$), G9a (ANOVA, $F_{3,11}=8.67$, $p<0.01$) and Setdb1 (ANOVA, $F_{3,11}=8.42$, $p<0.01$) relative to control. Tukey post hoc comparisons indicated that there was a concentration-dependent decrease in mRNA expression for GLP, G9a and Setdb1 in response to VPA treatment (Figure 35). In summary, Valproic acid induced concentration dependent decreases in GLP, G9a and Setdb1 mRNA.

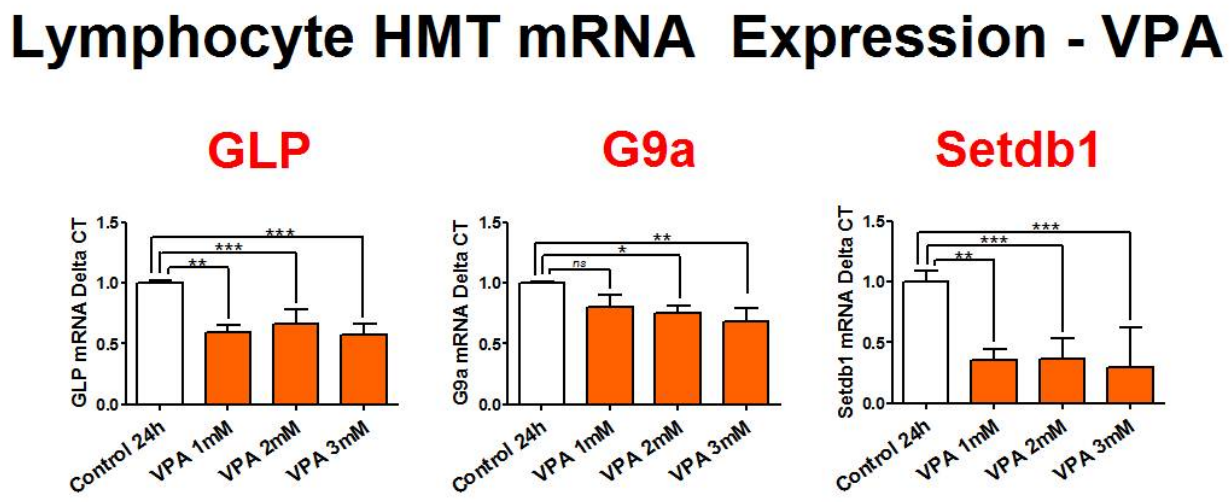

Figure 35 – Histone methyltransferases GLP, G9a and Setdb1 mRNA expression in lymphocyte cell culture after VPA treatment for 24 hours. mRNA levels showed a dose dependent decrease, as determined by Tukey post hoc. Shown are the means for 4 independent experiments ± standard error. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H39me2) lymphocyte cultures. For global H3K9me2 protein analysis, nine biological replicates were used. A biological replicate was defined as a blood draw from a single participant. Optical density levels of H3K9me2 protein levels in non-nicotine, vehicle treated control samples and nicotine treated cells were analyzed using two-tailed students t-test. All data is presented as percent change from control ± standard error of the mean. In lymphocyte cultures treated with VPA for 24 hours, data revealed a VPA dependent decrease in H3K9me2 protein levels (t_{16}=3.30, p<0.001) relative to control (Figure 36). Figure 37 is a representative western blot of H3K9me2 in post-mitotic neuronal cell culture treated with 2mM of VPA. As expected from the VPA-dependent decreases in HMT mRNA, Valproic acid treatment results in an H3K9me2 global protein decrease.

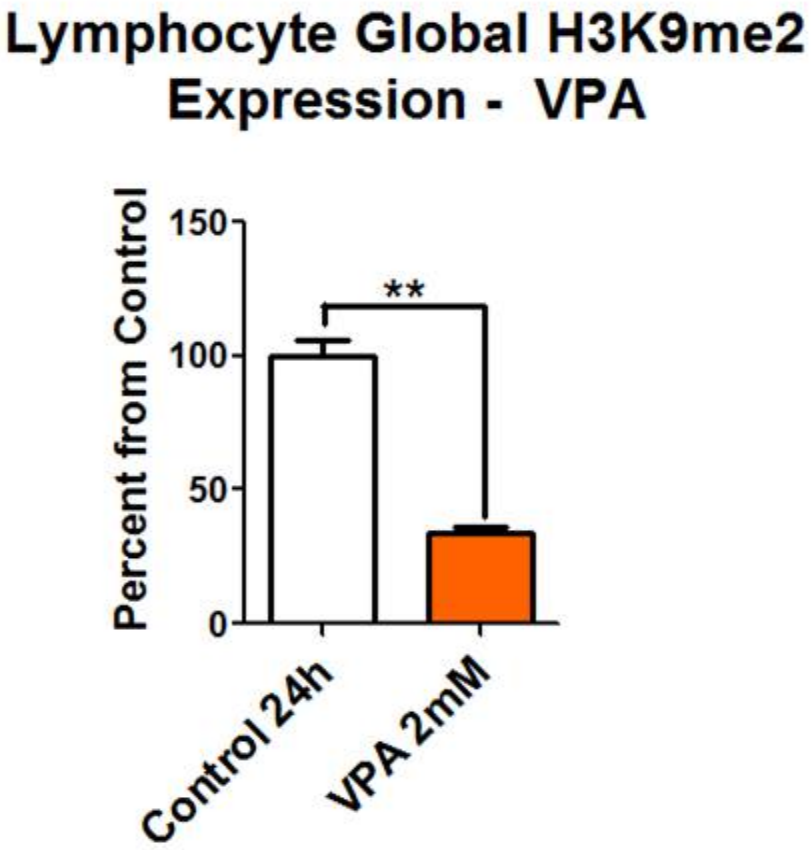

Figure 36 – Global histone post-translational modification H3K9me2 protein expression in lymphocyte cell culture after VPA treatment for 24 hours. Protein levels showed a significant decrease. Shown are the means for nine independent experiments ± standard error. *****p*<0.01, as determined by t-test.

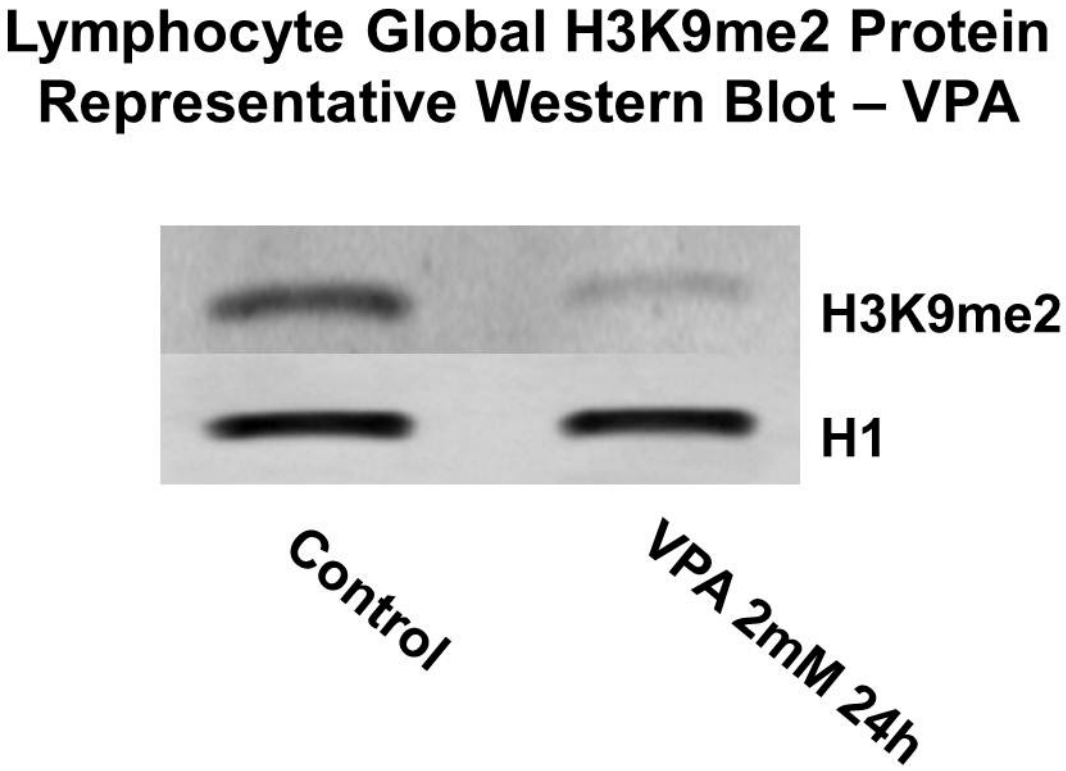

Figure 37 – Representative western blot for H3K9me2 I lymphocyte culture treated with 2mM of Valproic acid for 24 hours, normalized to H1

Chromatin Immunoprecipitation (ChIP) is used to investigate interactions between protein and specific DNA sequences. For ChIP analysis, six biological replicates were used. A biological replicate was defined as a blood draw from a single participant. Two-tailed t-tests were performed for the individual transcript between VPA and vehicle treated cell culture. Using ChIP, H3K9me2 binding at specific BDNF promoter regions were examined. In lymphocytes exposed to 2mM of VPA for 24 hours, there was a significant decrease in H3K9me2 binding at promoter IXabcd of BDNF (t_{10}=5.29 p<0.001) (Figure 38A). Valproic acid dependent decreases in BDNF promoter specific binding of H3K9me2 are reflective of the global decreases in H3K9me2 protein levels demonstrated by western blot.

We next confirmed that decreased promoter occupancy by a restrictive H3K9me2 (through ChIP analysis) resulted in increased BDNF transcript mRNA expression. For ChIP-specific transcript mRNA analysis, six biological replicates were used. A biological replicate was defined as a blood draw from a single participant. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. We compared ΔC_T values for the individual BDNF transcript for VPA treatment and vehicle treated control samples using a two-tailed t-test. In primary cortical neurons exposed to 2mM of VPA for 24 hours, there was a significant increase in promoter mRNA IXabcd of BDNF (t_{10}=16.42 p<0.001) (Figure 38B). Collectively we conclude that VPA is epigenetically modifying HMT mRNA levels, resulting in a global and promoter specific decrease in the restrictive chromatin mark H3K9me2. Through this mechanism, Valproic Acid is resulting in a more open chromatin state, allowing for increased gene transcription, as demonstrated through increases in BDNF mRNA levels.

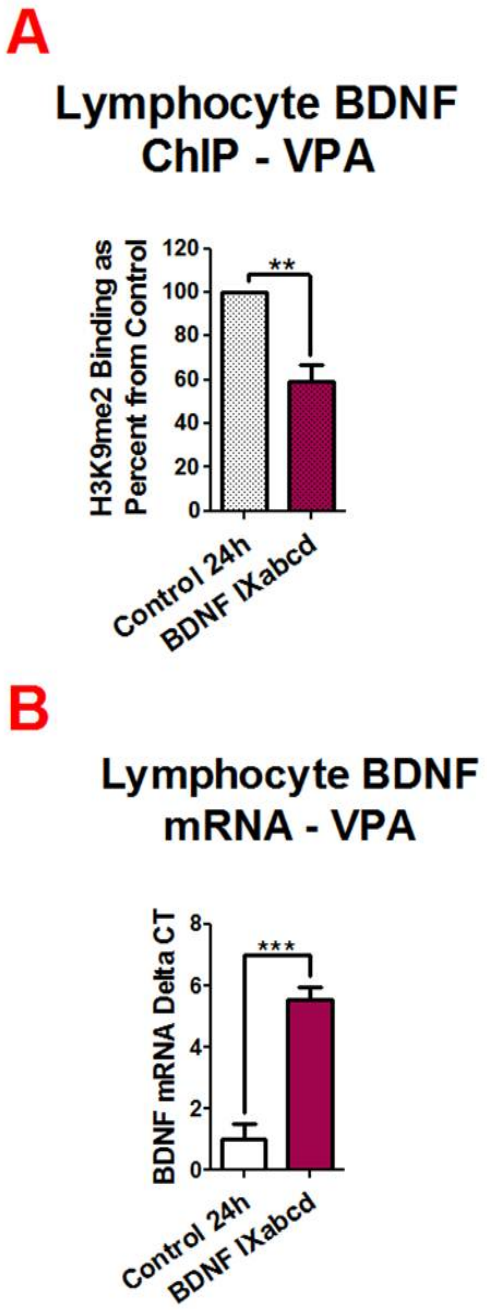

Figure 38 – Lymphocyte Global H3K9me2 Protein Representative Western Blot – VPA
A <u>Chromatin Immunoprecipitation</u>: H3K9me2 binding at BDNF promoter regions IXabcd in lymphocyte cell culture after 24 hours of 2mM Valproic acid treatment. Shown is the percent from control condition for six independent experiments ± standard error. ** *p*<0.01, as determined by two-tailed t-test.
B <u>BDNF mRNA:</u> Increases in BDNF transcript IXabcd mRNA expression in lymphocyte cell culture after VPA treatment for 24 hours. Shown are the means for six independent experiments ± standard error. * *p*<0.001, as determined by two-tailed t-test.**

III.C.3 Trichostatin A

III.C.3.a Primary Cortical Neuronal Cell Culture

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and SETDB1 in primary cortical neuronal cultures. For HMT mRNA analysis, four biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression between vehicle and TSA treated cells were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance. In lymphocyte cultures treated with TSA for 24 hours, ANOVA revealed an effect of nicotine on mRNA expression for GLP (ANOVA, $F_{3,11}=11.33$, $p<0.01$), G9a (ANOVA, $F_{3,11}=8.54$, $p<0.01$) and Setdb1 (ANOVA, $F_{3,11}=11.86$, $p<0.01$) relative to control. Tukey post hoc comparisons indicated that there was a concentration-dependent decrease in mRNA expression for GLP, G9a and Setdb1 in response to TSA treatment (Figure 39). In summary, Trichostatin A induced concentration dependent decreases in GLP, G9a and Setdb1 mRNA.

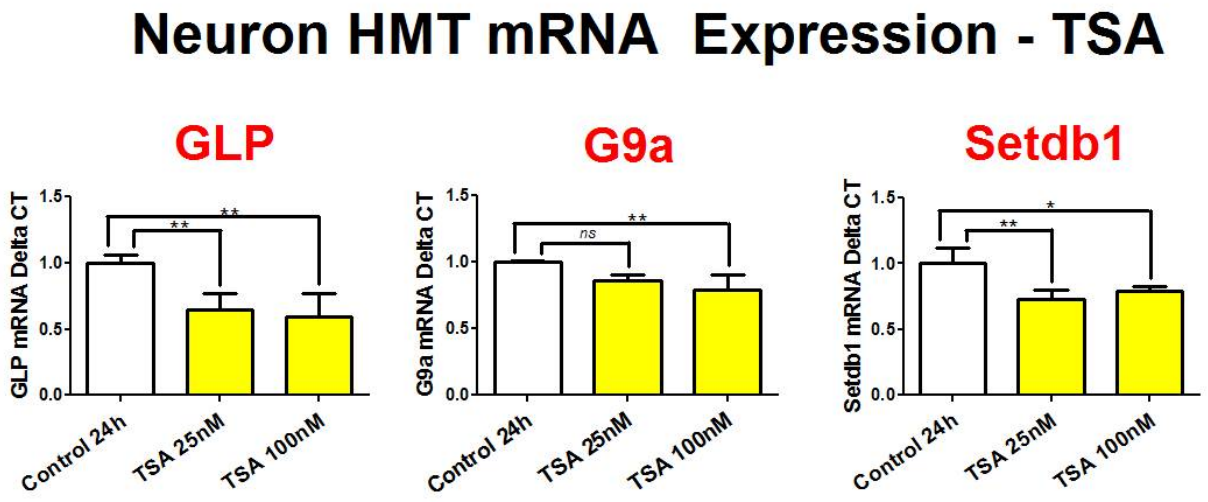

Figure 39 – Histone methyltransferases GLP, G9a and Setdb1 mRNA expression in lymphocyte cell culture after TSA treatment for 24 hours. mRNA levels showed a dose dependent decrease, as determined by Tukey post hoc. Shown are the means for 4 independent experiments ± standard error. * *p*<0.05, ** *p*<0.01.

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H39me2) in primary cortical neuronal cultures. For global H3K9me2 protein analysis, four biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. Optical density levels of H3K9me2 protein levels in non-TSA, vehicle treated control samples and TSA treated cells were analyzed using two-tailed t-test. All data is presented as percent change from control ± standard error of the mean. In primary cortical neuronal cultures treated with TSA for 24 hours, data revealed a TSA dependent decrease in H3K9me2 protein levels (t_6=6.30, p<0.01) relative to control (Figure 40). Figure 41 is a representative western blot of H3K9me2 in post-mitotic neuronal cell culture treated with 100nM of TSA. As expected from the TSA-dependent decreases in HMT mRNA, Trichostatin A treatment results in an H3K9me2 global protein decrease.

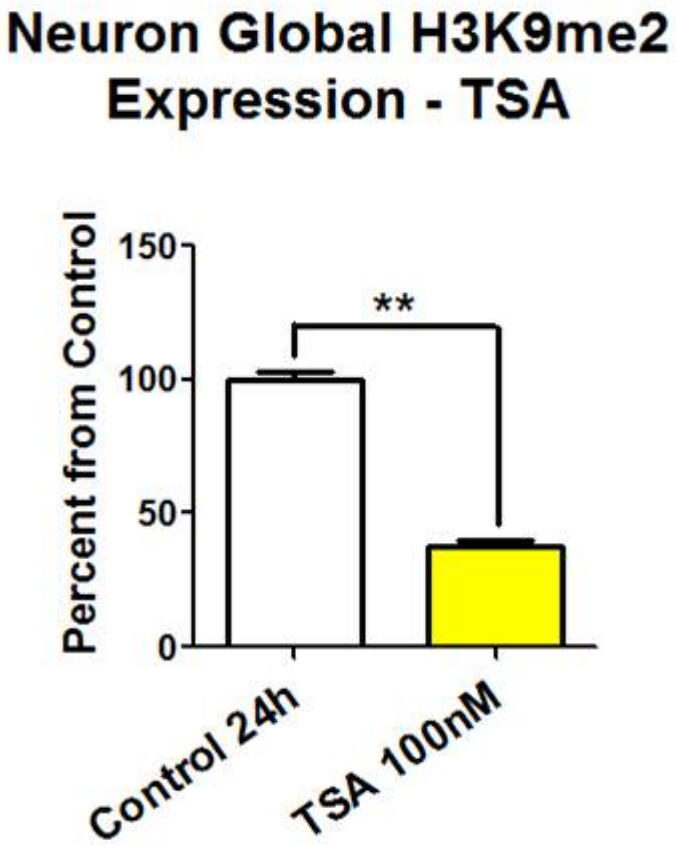

Figure 40 – Global histone post-translational modification H3K9me2 protein expression in post-mitotic primary cortical neuronal cell culture after TSA treatment for 24 hours. Protein levels showed a significant decrease. Shown are the means for seven independent experiments ± standard error. **p<0.01, as determined by t-test.

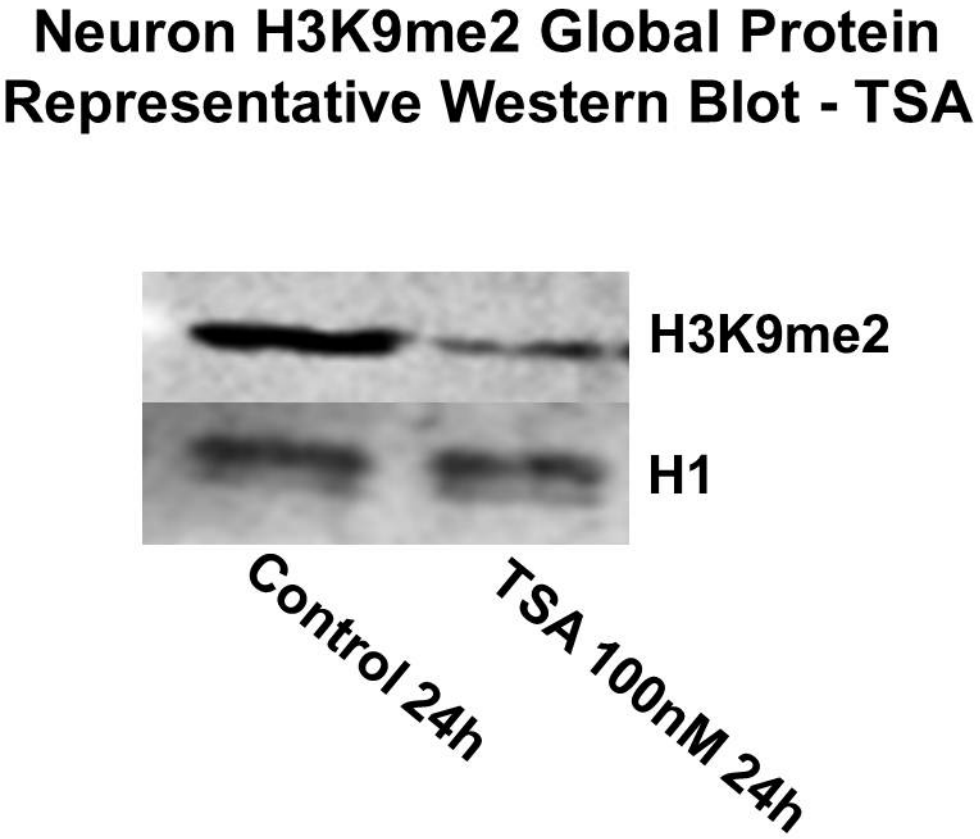

Figure 41 – Representative western blot for H3K9me2 in post-mitotic primary cortical neuronal culture treated with 100nM of Trichostatin A for 24 hours, normalized to H1

Chromatin Immunoprecipitation (ChIP) is used to investigate interactions between protein and specific DNA sequences. For ChIP analysis, four biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. -tailed t-tests were performed for the individual transcript between nicotine and vehicle treated cell culture. Using ChIP, H3K9me2 binding at specific Bdnf promoter regions were examined. In primary cortical neurons exposed to 100nM of TSA for 24 hours, there was a significant decrease in H3K9me2 binding at promoter I of Bdnf (t_6=4.30 p<0.001), IV of Bdnf (t_6=4.23 p<0.001) and IXa of Bdnf (t_6=4.143 p<0.001) (Figure 42A). Trichostatin A dependent decreases in Bdnf promoter specific binding of H3K9me2 are reflective of the global decreases in H3K9me2 protein levels demonstrated by western blot.

Decreased promoter occupancy by a restrictive H3K9me2 (through ChIP analysis) resulted in increased Bdnf transcript mRNA expression. For ChIP-specific transcript mRNA analysis, four biological replicates were used. A biological replicate was defined as surgery/cultures from a single pregnant mouse dam. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. We compared ΔC_T values for the individual Bdnf transcript for TSA treatment and vehicle treated control samples using a two-tailed t-test. In primary cortical neurons exposed to 100nM of TSA for 24 hours, there was a significant increase in Bdnf transcript I (t_6=8.33 p<0.01), IV (t_6=14.22 p<0.01) and IXa (t_6=7.116 p<0.01) (Figure 42B). Collectively we conclude that Trichostatin A is epigenetically modifying HMT mRNA levels, resulting in a global and promoter specific decrease in the restrictive chromatin. Through this mechanism, TSA is resulting in a more open chromatin state, allowing for increased gene transcription, as demonstrated through increases in Bdnf mRNA levels.

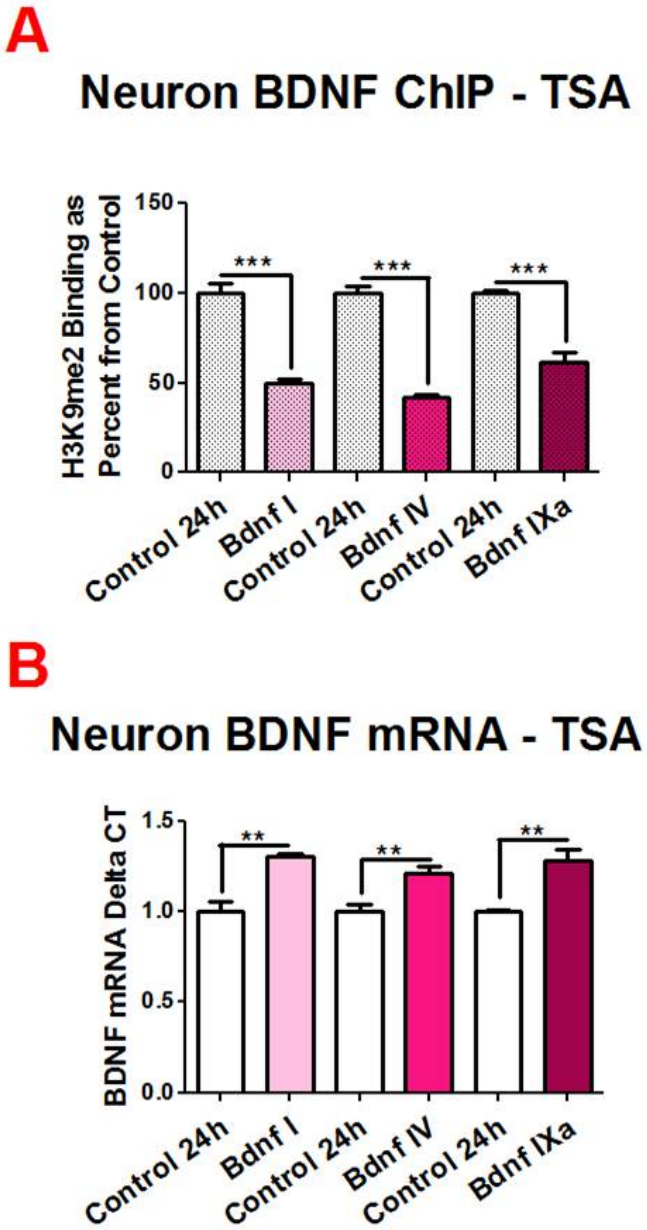

Figure 42 – Neuron Chromatin Immunoprecipitation and Bdnf mRNA Levels – TSA
A <u>Chromatin Immunoprecipitation</u>: H3K9me2 binding at Bdnf promoter regions I, V and IXa in post-mitotic primary cortical neuronal cell culture after 24 hours of 100nM Trichostatin A treatment. Shown is the percent from control condition for four independent experiments ± standard error. ** *p*<0.01, as determined by two-tailed t-test.
B <u>Bdnf mRNA:</u> Increases in Bdnf transcript I, V and IXa mRNA expression in post-mitotic primary cortical neuronal cell culture after TSA treatment for 24 hours. Shown are the means for four independent experiments ± standard error. ** *p*<0.01, as determined by two-tailed t-test.

III.C.3.b Lymphocyte Cell Culture

Real-time RT-PCR was used to examine mRNA expression for the histone methyltransferases GLP, G9a and SETDB1 in lymphocyte cultures. For HMT mRNA analysis, nine biological replicates were used. A biological replicate was defined as a blood draw from a single participant. Delta C_T (ΔC_T) (Experimental Gene C_T-Normalizing Gene C_T) values of HMT mRNA expression between vehicle and TSA treated cells were analyzed using one-way analysis of variance (ANOVA). Group differences were further elucidated by Tukey post hoc comparison. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. A probability level of $p<0.05$ was the criterion to achieve statistical significance. In lymphocyte cultures treated with TSA for 24 hours, ANOVA revealed an effect of nicotine on mRNA expression for GLP (ANOVA, $F_{3,11}=19.46$, $p<0.001$), G9a (ANOVA, $F_{3,11}=8.60$, $p<0.01$) and Setdb1 (ANOVA, $F_{3,11}=11.96$, $p<0.01$) relative to control. Tukey post hoc comparisons indicated that there was a concentration-dependent decrease in mRNA expression for GLP, G9a and Setdb1 in response to TSA treatment (Figure 43). In summary, Trichostatin A induced concentration dependent decreases in GLP, G9a and Setdb1 mRNA.

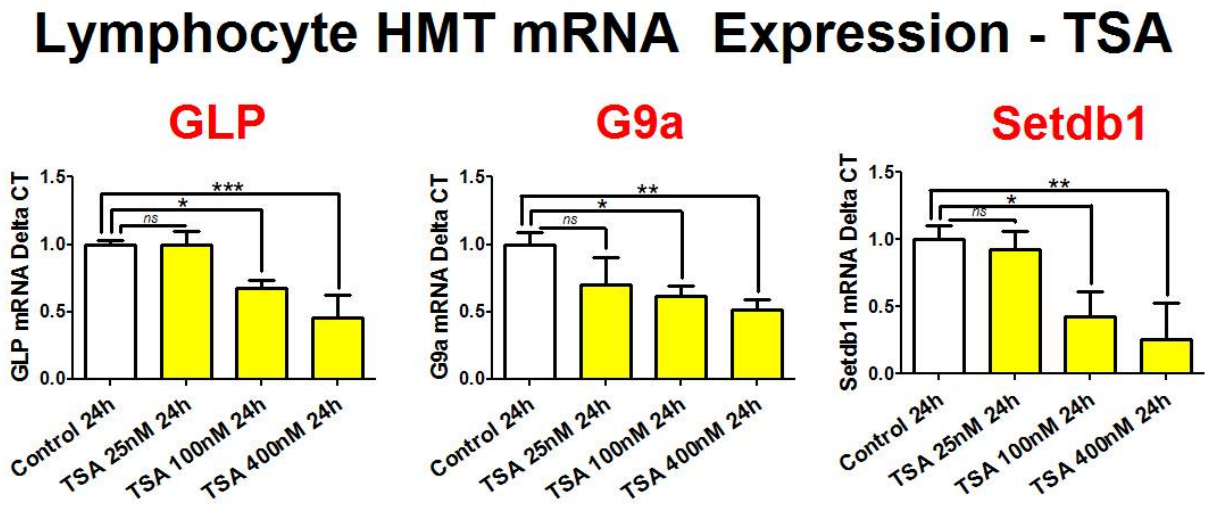

Figure 43 – Histone methyltransferases GLP, G9a and Setdb1 mRNA expression in lymphocyte cell culture after TSA treatment for 24 hours. mRNA levels showed a dose dependent decrease, as determined by Tukey post hoc. Shown are the means for 4 independent experiments ± standard error. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Western blot analysis was used to examine global protein levels of di-methylated lysine 9, histone 3 (H39me2) in lymphocyte cultures. For global H3K9me2 protein analysis, nine biological replicates were used. A biological replicate was defined as a blood draw from a single participant. Optical density levels of H3K9me2 protein levels in non-TSA, vehicle treated control samples and TSA treated cells were analyzed using twi-tailed students t-test. All data is presented as percent change from control ± standard error of the mean. In lymphocyte cultures treated with TSA for 24 hours, data revealed a TSA dependent decrease in H3K9me2 protein levels (t_{16}=3.10, p<0.01) relative to control (Figure 44). Figure 45 is a representative western blot of H3K9me2 in lymphocyte cell culture treated with 100nM of nicotine. As expected from the TSA-dependent decreases in HMT mRNA, Trichostatin A treatment results in an H3K9me2 global protein decrease.

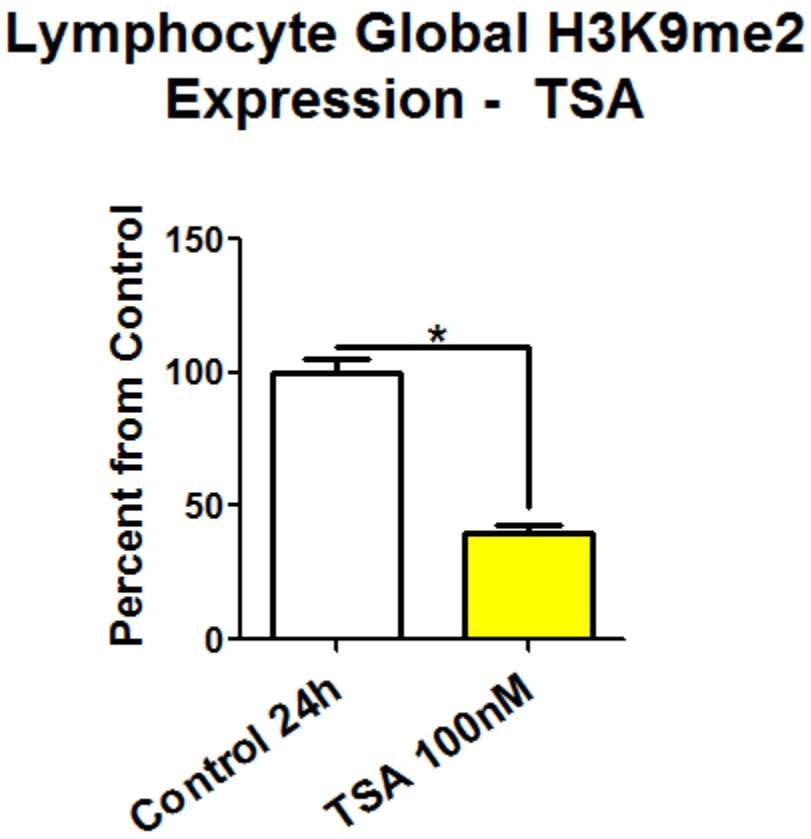

Figure 44 – Global histone post-translational modification H3K9me2 protein expression in lymphocyte cell culture after TSA treatment for 24 hours. Protein levels showed a significant decrease. Shown are the means for nine independent experiments ± standard error. *p<0.05, as determined by t-test.

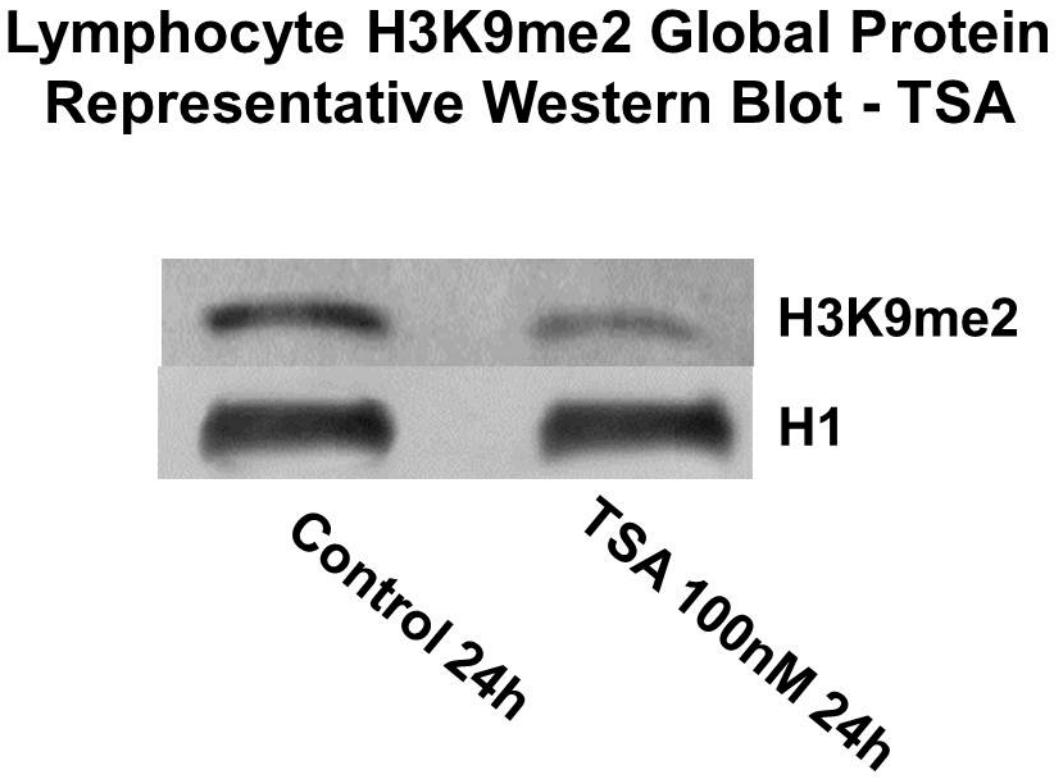

Figure 45 – Representative western blot for H3K9me2 in lymphocyte culture treated with 100nM of Trichostatin A for 24 hours, normalized to H1

Chromatin Immunoprecipitation (ChIP) is used to investigate interactions between protein and specific DNA sequences. For ChIP analysis, six biological replicates were used. A biological replicate was defined as a blood draw from a single participant. Two-tailed t-tests were performed for the individual transcript between nicotine and vehicle treated cell culture. Using ChIP, H3K9me2 binding at specific BDNF promoter regions were examined. In primary cortical neurons exposed to 100nM of TSA for 24 hours, there was a significant decrease in H3K9me2 binding at promoter IXabcd of BDNF (t_{10}=23.12 p<0.001) (Figure 46A). Trichostatin A dependent decreases in BDNF promoter specific binding of H3K9me2 are reflective of the global decreases in H3K9me2 protein levels demonstrated by western blot.

Decreased promoter occupancy by a restrictive H3K9me2 (through ChIP analysis) resulted in increased BDNF transcript IXabcd mRNA expression. For ChIP-specific transcript mRNA analysis, three biological replicates were used. A biological replicate was defined as a blood draw from a single participant. All data is presented as mean $\Delta\Delta C_T$ values ± standard error of the mean. We compared ΔC_T (Experimental Gene C_T-Normalizing Gene C_T) values for the individual BDNF transcript for nicotine and vehicle treated samples using a two-tailed t-test. In primary cortical neurons exposed to 100nM of TSA for 24 hours, there was a significant increase in BDNF transcript IXabcd (t_4=4.529 p<0.05) (Figure 46B). Collectively we conclude that Trichostatin A is epigenetically modifying HMT mRNA levels, resulting in a global and promoter specific decrease in the restrictive chromatin mark H3K9me2. Through this mechanism, TSA is resulting in a more open chromatin state, allowing for increased gene transcription, as demonstrated through increases in BDNF mRNA levels.

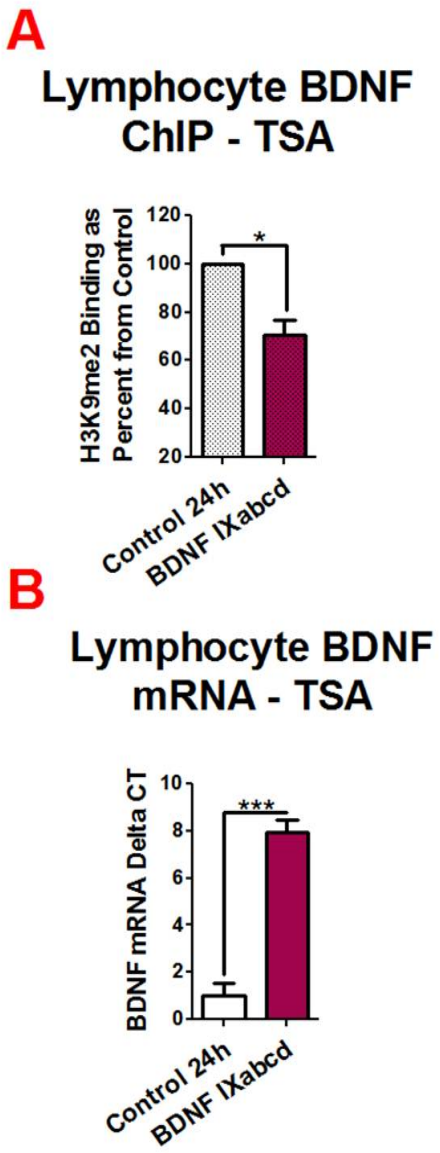

Figure 46 – Lymphocyte Chromatin Immunoprecipitation and BDNF mRNA Levels - TSA
A <u>Chromatin Immunoprecipitation</u>: H3K9me2 binding at BDNF promoter regions IXabcd in lymphocyte cell culture after 24 hours of 100nM Trichostatin A treatment. Shown is the percent from control condition for six independent experiments ± standard error. * *p*<0.05, as determined by two-tailed t-test.
B <u>BDNF mRNA:</u> Increases in BDNF transcript IXabcd mRNA expression in lymphocyte cell culture after TSA treatment for 24 hours. Shown are the means for three independent experiments ± standard error. * *p*<0.001, as determined by two-tailed t-test.**

IV. DISCUSSION

IV.A. Restrictive Chromatin in Schizophrenia

This thesis assessed levels of three histone methyltransferases (HMT), GLP, G9a and Setdb1 mRNA, and the resulting restrictive epigenetic modification, H3K9me2, in post-mortem brain tissue and lymphocytes from patients with schizophrenia, patients with bipolar disorder and normal controls. In patients with schizophrenia, G9a and Setdb1 mRNA were significantly increased in patients with schizophrenia compared to normal controls. Furthermore, patterns of HMT mRNA expression were similar in both lymphocytes and post-mortem brain tissue. These increases in HMT mRNA also resulted in similar increases in the restrictive chromatin modification Di-methylated lysine 9 of histone 3 (H3K9me2) of post-mortem brains of patients with schizophrenia. Additionally, pharmacological inhibition of these methyltransferases, and resulting H3K9me2 levels were examined in lymphocyte cell culture from normal controls. To further demonstrate the translatability of the lymphocyte to the neuron, pharmacological repression of these epigenetic markers were also examined in post-mitotic primary cortical neurons, and cortex extracts from mice. Levels of HMT mRNA and H3K9me2 global and promoter-specific binding were tested in response to the nAChR agonist nicotine and the HDAC inhibitors Valproic acid and Trichostatin A. At the concentrations tested, these compounds significantly decreased selected HMTs GLP, G9a and Setdb1 mRNA expression, as well as global and site-specific H3K9me2 binding. H3K9me2 has been previously shown to be significantly increased in lymphocytes of patients with schizophrenia, (Gavin *et al*., 2009) and this thesis aimed to explain the underlying mechanism behind these findings. The results suggest that patients with schizophrenia have increased levels of restrictive chromatin, which are

modifiable through pharmacological manipulation, implicating their usefulness in developing better treatment outcomes.

Schizophrenia is a chronic and debilitating mental disorder that affects 0.4 to 1% of the total world population (American Psychiatric Association & American Psychiatric Association. Task Force on DSM-IV, 2000; Saha *et al*., 2005). Schizophrenia-like illnesses have been reported and studied as early as the 19th century, (Palha & Esteves, 1997) with research to the biological underpinnings of the disease beginning shortly after. Neurotransmitter dysregulation and dysfunction have been highly characterized in schizophrenia, and arose from the initial finding that antipsychotics exhibit dopamine-receptor D2 receptor blockade effects (Seeman *et al*., 1975; Stone *et al*., 2007). Since this finding, attempts have been made to localize the site of schizophrenia pathophysiology in the brain. Dysfunction has been identified as brain area specific; the hippocampus, (Chung *et al*., 2003; Harrison, 2004) amygdala, (Lawrie *et al*., 2003) prefrontal, (Cohen & Servan-Schreiber, 1992; Manoach, 2003) temporal (Pearlson, 1997; Seidman *et al*., 2003) and parietal (Torrey, 2007) corticies. Additionally, dysfunction has also been identified to be cell type and neurotransmitter specific, implicating dopamine, (Lee *et al*., 1978; Seeman *et al*., 1975; Toda & Abi-Dargham, 2007) GABA, (Akbarian *et al*., 1995; Guidotti *et al*., 2000; Impagnatiello *et al*., 1998; Veldic *et al*., 2007) and glutamate (Olney & Farber, 1995). Given that almost the entire computing brain has been implicated in schizophrenia, it seems that a global dysfunction is at the root of this mental disorder.

In the first aim of this study, fresh frozen post-mortem parietal cortex from patients with schizophrenia, bipolar and normal controls was examined. Although this brain area is believed to play a critical role in schizophrenia, very little research and attention has been paid to it, with

research tending to favor the prefrontal cortex, or the hippocampus (Maruff *et al*., 2005). This brain region tends to be overlooked in schizophrenia research, but could provide some unique insight into the disease for a variety of reasons. The parietal cortex is one of the last areas of the human brain to mature, (Geschwind, 1965) and as a result this area can have high amount of variability. Early life environmental insults could thereby have a profound effect on this brain region, perhaps more so than other areas. Additionally, the parietal cortex shows sexual dimorphism, with the left hemisphere being larger in males than females (Frederikse *et al*., 1999).

The major role of the parietal cortex is to integrate and evaluate sensory information into a coherent pattern (Andersen & Buneo, 2003; Cohen & Andersen, 2002). In fact, the most common symptom, disordered thought, is most likely explainable through disruption of this system (Torrey, 2007). Patients with schizophrenia report either acute (for example colors looking brighter) (McGhie & Chapman, 1961) or blunted (Freedman, 1974) sensitivity to sensory stimuli, and demonstrate overall impairment of sensory integration, such as the ability to tactually identify objects without visual assistance (Manschreck & Ames, 1984; Torrey, 1980). Furthermore, while executive functioning is primarily associated with the frontal cortex, its activity is not possible without connections with other structures, which include the parietal cortex. Tests of executive functioning, including the Wisconsin Card Sorting Test, activate both the prefrontal and parietal cortices (Buchsbaum *et al*., 2005). Imaging studies of structural differences in patients with schizophrenia are varied, with no clear relation demonstrated between cortex size and diagnosis (Buchanan *et al*., 2004; Zhou *et al*., 2007).

Identifying causative factors in the development of schizophrenia have been shortcoming,

even with years of targeted research and effort. As mentioned, a global molecular underpinning seems to be more feasible as a disease incubator rather than dysfunction in specific area or cell-type. Epigenetic regulation is the study of environmentally induced changes in gene regulation that can be observed in the post-mitotic neuron, and can endure for the lifespan of the organism (Wolffe & Hayes, 1999). As such, epigenetics is the ideal scaffold to examine underlying non-mendelian etiology of schizophrenia, as it is the interface between underlying genetic code and environmental impact. Epigenetics has been shown to play a role in schizophrenia endophenotype through increases in DNA methylation patterns of various candidate genes, as well as increases in restrictive chromatin modifications and decreases in open chromatin marks (Gavin *et al*., 2009). Specifically, dimethylated lysine 9 of histone 3 (H3K9me2) has been previously shown to be significantly increased in lymphocytes of patients with schizophrenia (Gavin *et al*., 2009). H3K9me2 has been shown to receive the diametrically opposed functional groups of acetylation and methylation post-translational modifications, thus serving as a molecular 'switch' for gene activation. Methylation of H3K9 is a highly conserved post-translational modification of both transcriptionally facultative and 'closed' chromatin, known as heterochromatin (Barski *et al*., 2007). H3K9 modifications are found specifically in gene-rich areas of the genome, and are inversely associated with gene expression (Wen *et al*., 2009). H3K9me2 is dynamically regulated, and can be altered by environmental stimuli, such as cocaine.

The data shown in this thesis indicates that H3K9me2 protein levels are significantly increased in parietal cortex of patients with schizophrenia compared to normal controls, similarly to what has been demonstrated in lymphocytes (Gavin *et al*., 2009). The similarity in findings

between post-mortem brain tissue and lymphocytes further demonstrates a more global dysregulation of this epigenetic marker. Interestingly, this restrictive chromatin mark seems to be psychosis-specific, as patients with bipolar disorder do not show significant increases in H3K9me2 protein levels compared to normal controls.

The restrictive mark H3K9me2 is specifically catalyzed by histone methyltransferases (HMT), which include GLP, G9a and Setdb1. G9a and GLP form heteromeric complexes, and are responsible for the bulk of euchromatic H3K9me2 modifications across the genome (Shinkai & Tachibana, 2011; Tachibana *et al*., 2005). GLP and G9a conditional knockouts produce a significant decrease in global H3K9me2 levels, and inappropriate gene expression, which result in deficits in learning, reduction in exploratory behaviors, motivation and environmental adaption such as contextual fear conditioning (Schaefer *et al*., 2009; Shinkai & Tachibana, 2011; Tachibana *et al*., 2005; Tzeng *et al*., 2007). These genes, and thus H3K9me2 are dynamically regulated by environmental influence. Upon chronic cocaine administration in rats, G9a and GLP mRNA are decreased, resulting in a decrease in H3K9me2 protein levels (Maze *et al*., 2010). SETDB1 is a histone methyltransferase that specifically di- and tri-methylates lysine 9 of histone H3, effectively repressing gene transcription (Harte *et al*., 1999). SETDB1 acts in a progressive manner, binding to a previously di-methylated residue and remaining bound until subsequent methylation occurs (Wang *et al*., 2003; Zee *et al*., 2010). Di- and tri-methylation of H3K9, acts as a platform for recruitment of HP1 proteins, previously discussed, and proteins associated with DNA methylation, such as MBD1 and DNMTs, thus further decreasing gene transcription (Li *et al*., 2006; Schultz *et al*., 2002).

In this thesis, G9a, GLP and Setdb1 mRNA were examined in human lymphocytes and

post-mortem parietal cortex tissue of normal controls, patients with schizophrenia and patients with bipolar disorder via Real-time RT-PCR. Using Primary human lymphocyte culture to identify abnormalities and dysregulation in chromatin structure is both a viable and advantageous model for studying mental illness and was examined for possible use in future clinical studies. Lymphocytes can be obtained from living patients, thus their use as a biomarker to characterize subsets of patients with schizophrenia could be established to lead to targeted pharmacological interventions (Gavin & Sharma, 2009). The full complement of epigenetic enzymes and machinery that exists in neurons is also present in lymphocytes, and requires no a-priori assumptions of neuroanatomy, neuronal connectivity or synaptic events. Whole blood shares a similar gene expression profile with the brain, specifically around 50% of schizophrenia candidate genes are comparably expressed in both tissues (Sullivan *et al*., 2006). Additionally, lymphocytes share much of the non-synaptic biochemical environment of neurons, such as neurohormones, (Heagy *et al*., 1990; Maestroni, 2000) neuropeptides, chemo/cytokines, metabolites, (Costa *et al*., 1990; Vose *et al*., 2007) and medication blood levels, (Tsankova *et al*., 2007) thus epigenetic responses to pharmacological, metabolic and environmental events can demonstrate shared effects. The present study hypothesized that schizophrenia may be due to abnormal regulation of more fundamental epigenetic mechanisms, thus demonstrating significant and replicable patterns of gene expression changes across multiple species and cell types is more appropriate.

As reported in this thesis, both lymphocytes and post-mortem brain tissue show similar patterns of HMT mRNA expression that is diagnosis dependent. GLP mRNA was not significantly different between any diagnostic group in both lymphocytes and post-mortem brain

tissue. Conversely, G9a mRNA was significantly increased in patients with schizophrenia in both lymphocyte and post-mortem brain tissue. While G9a/GLP can both function as a dimer, and are responsible for the bulk of H3K9me2 modifications across the genome; they do not substitute for each other and their function is not redundant (Shinkai & Tachibana, 2011). Thus, while surprising, different regulation of these two HMT mRNAs is certainly plausible. Both lymphocyte and post-mortem brain tissue also exhibited significant increases in Setdb1 mRNA in patients with schizophrenia. These findings are important as through corresponding patterns in both lymphocytes and post-mortem brain tissue, they demonstrate the viability of the lymphocyte model for studying epigenetic mechanisms in mental illness. Additionally, these findings indicate a global dysregulation of epigenetic mechanisms in patients with schizophrenia, which perhaps may lead to understanding the etiology of the disease. When considered in conjunction, increases in histone methyltransferase mRNA and H3K9me2 protein levels, as demonstrated by this thesis, may be playing a specific critical role in disease etiology.

IV.A.1 Clinical Measures

The heterogeneity of schizophrenia has been a colossal obstacle to understanding the etiology of the disease (Buchanan & Carpenter, 1994). Examining schizophrenia as a binary measurement of illness is archaic, especially when researching biological relevancy and cause (Arango *et al*., 2000). Due to this, various clinical measures were used in conjunction with lymphocyte HMT mRNA levels. We discovered that increases in GLP mRNA are significantly positively correlated with a history of a neurological condition. A neurological condition is a broad term encompassing any type of damage to the central and peripheral nervous system as a result of illness or injury. Some conditions include stroke, traumatic brain injury, cerebral palsy,

muscular dystrophy, epilepsy, multiple sclerosis, and meningococcal disease, but this list is far from all encompassing. As GLP mRNA levels were not significant in either lymphocytes or post-mortem brain tissue in terms of diagnosis, perhaps this HMT is acting more as a precursor to schizophrenia development. As patients with schizophrenia display evidence of neurological impairment that seem to be inherent to the disease, not as a result of treatment, increases in HMT mRNA may rather be working to predispose patients to mental illness, acting more in prodromal dysregulation, rather than playing a significant role in the disease state (Browne *et al*., 2000; Salacz *et al*., 2011).

This thesis also identified increases in Setdb1 mRNA in lymphocytes to be correlated with both chronicity of illness and familial history of illness. All major psychiatric disorders aggregate in families, (Kendler, 1990) and there is strong evidence for a heritable component to schizophrenia, as demonstrated through twin and adoption studies (Gottesman & Bertelsen, 1989). Additionally, schizophrenia in a first degree relative is characterized as the highest risk factor for developing schizophrenia (Mortensen *et al*., 1999). Patients with a family history of mental illness demonstrated a significant increase in Setdb1 mRNA. Increases in Setdb1 mRNA, and by extension increases in restrictive chromatin, seem to be an incubator of pathology for schizophrenia, demonstrating the heritability aspect. Various environmental factors may act as the 'final straw' on this increased restrictive chromatin, explaining the disconcordant development of schizophrenia in monozygotic twins. Additionally, patients with chronic schizophrenia also demonstrated increases in Setdb1 mRNA compared to normal controls and patients with first episode psychosis. This effect is not an artifact of prolonged antipsychotic treatment, as no significant differences were demonstrated between HMT mRNA and

antipsychotic treatment. Therefore, increases in restrictive chromatin may be contributing specifically to the severity of the illness. Additionally, these Setdb1 mRNA levels may act as a biomarker, indicating which patients, upon first appearance of florid psychotic symptoms, may end up having a more chronic form of the illness, and consequently be less susceptible to antipsychotic treatment.

The Positive and Negative Syndrome Scale (PANSS) is a likert scale used for measuring severity of symptoms in patients with schizophrenia (Kay *et al*., 1987). Using the PANSS, correlations between gene expression and specific symptom severity can be examined, thereby reducing the overall heterogeneity of the disease. The scale is broken into three main sections, a positive subscale, a negative subscale and a general psychopathology subscale. Negative symptoms of schizophrenia include emotional withdrawal, blunted affect, anhedonia and poverty of speech, as discussed in the introduction of this thesis. Antipsychotic treatment specifically targets and seeks to rectify positive symptoms, while treatment options for negative symptoms are unmet (Jiawan *et al*., 2010). The clinical measure of a blunted affect is located on the PANSS negative scale, (Kay *et al*., 1987) and is characterized by diminished expressed or observed emotional responses, such as a reduction in facial expressions, and explicit expression of feelings. This thesis discovered a significant positive correlation between G9a mRNA expression and the total negative symptom scale in patients with schizophrenia, in that the more negative symptoms patients with schizophrenia exhibit, the higher G9a mRNA levels that are present in lymphocyte samples. As previously mentioned, antipsychotic treatment does not ameliorate negative symptoms, implicating a gap in truly efficacious patient care. Increased severity of negative symptoms are correlated with reduced disease prognosis (Ho *et al*., 1998;

Wieselgren *et al*., 1996).

These findings coincide perfectly with the central hypothesis of this thesis, stating that overall, chromatin is restrictive in schizophrenia. Through increases in H3K9me2, and the methyltransferases GLP, G9a and Setdb1, this hyper-restrictive insult in psychosis significantly contributes to disease pathology, specifically the negative symptoms that our current regimen of antipsychotics do not alleviate. As such, further research needs to examine pharmacology that can be used in conjunction with current psychotropic medication to enhance treatment outcomes, a process deemed "genome softening."

IV.B <u>Pharmacological Manipulation of Restrictive Chromatin</u>

Levels of HMT mRNA and H3K9me2 global and promoter-specific binding were tested in response to the nAChR agonist nicotine and the HDAC inhibitors Valproic acid and Trichostatin A. At the concentrations tested, these compounds significantly decreased selected HMTs GLP, G9a and Setdb1 mRNA expression, both global and promoter specific H9K9me2 protein levels as well as increased BDNF/Bdnf mRNA. Pharmacological inhibition of these methyltransferases, and resulting H3K9me2 levels were examined in lymphocyte cell culture from normal controls. To further demonstrate the translatability of the lymphocyte to the neuron, pharmacological repression of these epigenetic markers were also examined in post-mitotic primary cortical neurons, and cortex extracts from mice.

In order to further elucidate the epigenetic regulation of the three methyltransferases GLP, G9a and Setdb1, various *in-vitro* and *in-vivo* models were used. One advantage to using *in-vitro* culture models is the absence of drug-drug interactions. Anything ingested by a clinical population during the testing period, including vitamins and diet, may interact with any of the

drugs examined in this thesis. Additionally, drugs which are impossible to test in a clinical population due to toxicity or extreme dose can be examined in cell culture without risk. Only through cell culture exist the ability to segregate a homogeneous population of cells, be it inactivated t-lymphocytes or GABAergic neurons.

These *in-vitro* culture models do come with certain limitations. In culture, these cells are disengaged from their natural environment and have lost the support of trophic factors and proteins found in circulation. As schizophrenia is a human brain disorder, using primary lymphocytes obtained from blood samples has its limitations. Lymphocytes cultures are not neuronal cells, thus no assumptions of the effects of pharmacological inhibition of restrictive chromatin schizophrenia treatment outcomes, illness course, symptom severity or drug responsiveness can be ascertained from these findings. However, the goal of this study was to identify basic epigenetic modifications made in response to these drugs not consequent to synaptic activity. Our lab and others have previously demonstrated the reliability of measuring epigenetic parameters in lymphocytes, thus demonstrating lymphocytes as useful as a means to study overall epigenetic machinery (Fraga *et al*., 2005). Primary cortical neuronal cultures are primarily GABAergic cells (Noh *et al*., 2005). Additionally, testing pharmacological agents *in-vitro* does not account for physiological and physicochemical factors of drug absorption, all of which ultimately determine bioavailability (Lin, 1998). The *in-vivo* techniques used in this thesis also have specific limitations. As the entire cortex was used to examine mRNA and histone changes in response to drug administration, not one specific cell type was identified and implicated in the changes examined. Thus changes could not be identified specific to a particular neuron type. Regardless, as similar patterns of decreased HMT mRNA and H3K9me2 protein

levels were demonstrated between all models utilized in this thesis, the basic epigenetic changes in response to these pharmacological manipulations was adequately demonstrated.

IV.B.1 Nicotine

Nicotine is the primary psychoactive component of tobacco and acts as an agonist at ligand-gated ionotropic nicotinic acetylcholine receptors (nAChR). Nicotinic AChRs have a pentameric structure which includes a heteromeric stoichiometric arrangement of five different subunits (α2-α7, α9, α10, and β2-β4), or homomeric receptors predominately composed of the α7 subunit. Data from binding studies reveal the existence of a high-affinity nicotine-binding site in brain, predominantly composed of α4 and β2 subunits, and a low-affinity binding site that is mostly the homomeric α7 subunit (Barik & Wonnacott, 2009). Stimulation of nAChRs induce Ca^{+2}, K^{+} and Na^{+} influx, and in the nervous system has been shown to facilitate GABAergic, glutamatergic, serotonergic, cholinergic and dopaminergic transmission (Dani & De Biasi, 2001; Wonnacott *et al*., 2005).

Patients with schizophrenia have higher rates of cigarette smoking compared to other clinical diagnoses and normal controls, (Bobes *et al*., 2010; Lohr & Flynn, 1992; McClave *et al*., 2010; Sagud *et al*., 2009) and there is considerable research implicating nAChR activation as clinically beneficial in patients with schizophrenia. Nicotine administration has been shown to ameliorate some attention and cognitive deficits demonstrated in patients with schizophrenia and their first-degree relatives (Adler *et al*., 1992; Adler *et al*., 1993; Avila *et al*., 2003; Griffith *et al*., 1998; Olincy *et al*., 1998; Ross *et al*., 1998). Most importantly to this thesis, patients with schizophrenia who smoke show higher plasma BDNF levels and fewer negative symptoms than non-smokers (Zhang *et al*., 2010). The exact mechanism of the beneficial effects of nicotine is

unknown, thus leaving the field open to investigation.

With data indicating patients with schizophrenia who smoke have fewer negative symptoms than non-smokers, and negative symptoms seem to be a result of an overly restrictive epigenome, specifically through increases in HMT mRNA, patients with schizophrenia may be smoking in an attempt to target dysregulated epigenetic mechanisms. Studies examining the epigenetic effects of nicotine are limited. From the few studies conducted, nicotine seems to relax the epigenome, through increases in acetylated histones, and decreases in DNA methylation (Landais *et al*., 2005; Satta *et al*., 2008; Smith *et al*., 2010). As stated previously, schizophrenia is characterized by increases in global restrictive chromatin marks, thus suggesting a potential medium for pharmacological manipulation. Through acting on epigenetic mechanisms, patients may be smoking as a form of self-medication to relax the restrictive chromatin insult. Furthermore, as demonstrated, these epigenetic modifications are correlated with increases in negative symptoms. As patients with schizophrenia who smoke demonstrate less negative symptoms, acting through these epigenetic mechanisms may be the technique in which patients are self-medicating.

At the concentrations tested in this thesis, (Calabresi *et al*., 1989; Fang *et al*., 1991; Misbahuddin *et al*., 1985; Schreiber *et al*., 2002; Schreiber *et al*., 2002; Stolerman *et al*., 1973) the nAChR agonist nicotine significantly decreased mRNA expression of the HMTs GLP, G9a and Setdb1, as well as global and site-specific H3K9me2 binding. Through decreases in H3K9me2 promoter specific binding, Brain Derived Neurotrophic factor (BDNF/Bdnf) mRNA was also increased upon nicotine treatment. We hypothesized that nicotine influences gene expression by modifying levels of various HMTs, thereby decreasing H3K9me2 levels. This

down-regulation results in an increase in mRNA expression of various schizophrenia candidate genes, including Bdnf (Lu & Martinowich, 2008). Down-regulation of BDNF has been demonstrated in patients with schizophrenia, and up-regulation demonstrated in chronic nicotine exposure, thus we selected BDNF/Bdnf as an epigenetically regulated 'readout' (Buckley *et al*., 2007; Jindal *et al*., 2010; Kenny *et al*., 2000; Rizos *et al*., 2010; Son & Winzer-Serhan, 2009; Zhang *et al*., 2010).

We tested our hypothesis in three cell tissues, human primary lymphocyte culture, a post-mitotic primary cortical neuron culture from mice, and cortex extracts from mice treated intraperitoneally with nicotine. As previously described in this thesis, lymphocytes from patients with schizophrenia demonstrate increases in HMT mRNA and H3K9me2 protein levels. The ability of nicotine to manipulate this restrictive epigenetic mechanism was examined in lymphocytes from control participants as a basis for future clinical studies in lymphocytes from patients with schizophrenia. Studying the effect of nicotine on epigenetic regulation in the lymphocyte is possible through identified nAChRs on the cell surface, (Fujii *et al*., 2008; Hiemke *et al*., 1996) specifically the homomeric α7 nAChR, which is activated by nicotine, (De Rosa, *et al*., 2005; Sato *et al*., 1999) and α4β2 (Benhammou *et al*., 2000). As this thesis found that nicotine was able to significantly decrease HMT mRNA expression, thereby decreases in H3K9me2 global and promoter specific protein levels, perhaps smoking is acting as a form of self-medication in patients with schizophrenia. To examine the applicability of nicotine treatment effect on epigenetic machinery in neurons, mouse primary cortical neuronal culture and cortex from mice treated intraperitoneally with nicotine were also used. Neuronal cultured cells are primarily GABAergic, (Noh *et al*., 2005) thus express α7 nAChRs (Burli *et al*., 2010).

Similarly to the effects seen in lymphocytes, nicotine administration significantly decreased HMT mRNA, thereby decreasing global and promoter specific H3K9me2 protein levels. As schizophrenia is a disorder of the brain, similarities seen in both neuronal cells and lymphocyte culture can be better applied to this mental illness.

We also selected BDNF/Bdnf as an epigenetically regulated 'readout' of chromatin remodeling, with down-regulation being demonstrated in patients with schizophrenia and up-regulation demonstrated in chronic nicotine exposure (Buckley *et al*., 2007; Jindal *et al*., 2010; Kenny *et al*., 2000; Rizos *et al*., 2010; Son & Winzer-Serhan, 2009; Zhang *et al*., 2010). Furthermore, both mouse Bdnf exon I, IV and IXa and human BDNF IXabcd have been demonstrated to be epigenetically modified through both *in-vivo* and *in-vitro* depolarization models, indicating these transcripts are inducible upon normal neuronal functioning (Aid *et al*., 2007; Chen *et al*., 2003; Koppel *et al*., 2009; Pruunsild *et al*., 2007; Pruunsild *et al*., 2011; Timmusk *et al*., 1993; Tsankova *et al*., 2004).

Our finding that nicotine induced epigenetic modifications in human lymphocytes, mouse primary cortical neuronal culture and mouse cortex extracts has important implications for the understanding of nicotine consumption in patients with schizophrenia. We observed that nicotine decreases the HMTs GLP, G9a and SETDB1 mRNA levels in a concentration dependent manner. Decreases were also observed in global and promoter specific binding of the restrictive chromatin mark H3K9me2, which was then correlated with increases in BDNF/Bdnf mRNA expression, a schizophrenia candidate gene. We believe this lymphocyte model could be translated to clinical studies. The conclusion of this study is that patients with schizophrenia may be smoking to relieve the burden of preexisting restrictive chromatin via down-regulation of

various HMTs, thus alleviating negative symptoms.

IV.B.2 HDACi Compounds – VPA and TSA

Histone deacetylase inhibitors are pharmacological agents that work to block the enzymatic activity of histone deacetylases, and through this inhibition, histone acetylation, or 'open' chromatin is effectively increased (Finnin *et al*., 1999). Treatment with HDAC inhibitors result in highly specific and targeted changes in gene expression, increasing mRNA expression of only around 2% of genes expressed in mammalian cells, without affecting generalized function (Mishra *et al*., 2001; Nambiar *et al*., 2002; Van Lint *et al*., 1996). In this thesis, the effects of two HDAC inhibitors, Valproic acid (VPA) and Trichostatin A (TSA), were examined on HMT gene expression, and resulting histone methylation patterns. TSA is the most potent reversible HDACi, and broadly inhibits class I, II and IV HDAC enzymes, and VPA, although not as efficient, inhibits Class I HDAC activity (Gottlicher *et al*., 2001).

Patients with schizophrenia have increased in restrictive chromatin, specifically histone deacetylases, (Benes *et al*., 2007; Sharma *et al*., 2008) and thus a reduction in 'open' or acetylated chromatin (Gavin *et al*., 2008; Gavin *et al*., 2009). HDACi have been shown to increase acetylated histones, (Gottlicher *et al*., 2001; Phiel *et al*., 2001) although this effect is blunted in patents with schizophrenia (Gavin *et al*., 2009). Interestingly, levels of the restrictive chromatin mark, H3K9me2 are also significantly decreased upon treatment with HDACi, (Lawrence & Volpe, 2009; Luo *et al*., 2010; VerMilyea *et al*., 2009; Zhang *et al*., 2007) VPA and TSA are known to act through inhibiting the enzymatic activity of histone deacetylases, thus creating a more open chromatin environment. The hypothesis of this thesis was that decreases demonstrated in methylated H3K9 upon VPA and TSA treatment were not a secondary effect

due to overwhelming increases in histone acetylation, and thus blocking of histone methylation, but rather consequent of a larger mechanism of action. From the data in this thesis, it seems that these pharmacological agents open chromatin through dual action, by decreasing deacetylation and decreasing methylation.

At the concentrations tested, the histone deacetylase inhibitors (HDACi) Valproic acid and Trichostatin A (Kaiser *et al*., 2006; Tremolizzo *et al*., 2002; Yildirim *et al*., 2003; Yoshida *et al*., 1990) significantly decreased selected HMTs GLP, G9a and Setdb1 mRNA expression, as well as global and site-specific H3K9me2 binding. Through decreases in H3K9me2 promoter specific binding, BDNF/Bdnf mRNA was also increased upon HDACi treatment. We hypothesized that HDACis can influence gene expression by modifying levels of various HMTs and thus decrease H3K9me2 levels. This down-regulation results in an increase in mRNA expression of various schizophrenia candidate genes, including Bdnf (Lu & Martinowich, 2008). We selected BDNF/Bdnf as an epigenetically regulated 'readout' with down-regulation being demonstrated in patients with schizophrenia and an up-regulation demonstrated upon HDACi treatment (Buckley *et al*., 2007; Jindal *et al*., 2010; Rizos *et al*., 2010). We tested our hypothesis using a similar three-tissue model as described in the previous nicotine study. The three tissues studied included: the clinically relevant human primary lymphocyte culture, mouse post-mitotic primary cortical neuron culture, and cortex extracts from mice treated intraperitoneally with Valproic acid to examine neuron-specific effects of HDACi on epigenetic machinery. As previously mentioned BDNF is down-regulated in patients with schizophrenia (Buckley *et al*., 2007; Jindal *et al*., 2010; Rizos *et al*., 2010), and Bdnf expression has been shown to be inducible upon VPA (Bredy *et al*., 2007; Chen *et al*., 2006) and TSA (Aid *et al*., 2007; Yasuda *et*

al., Chuang, 2009) treatment, with increases in promoter-associated acetylated histone H3 (Wu *et al*., 2008).

Our finding that the histone deacetylases Valproic acid and Trichostatin induced epigenetic modifications in human lymphocytes, mouse primary cortical neuronal culture and mouse cortex extracts has important implications for the function of these HDACis. Once believed to only inhibit decreases of histone acetylation, these pharmacological agents have shown to 'relax' chromatin through decreases in histone methylation. Through demonstrating the ability of VPA and TSA to target enzymes responsible for histone methylation, this thesis demonstrated a never before seen function of these HDACi. The data indicate that VPA and TSA decreased GLP, G9a and SETDB1 mRNA levels, as well as a decrease in global and promoter specific binding of the restrictive chromatin mark H3K9me2. Additionally, these decreases in restrictive chromatin marks were then correlated with increases in BDNF/Bdnf mRNA expression, a schizophrenia candidate gene. From this thesis, administration of HDACi may be beneficial to patient treatment outcomes, and work as a 'genome softener,' in conjunction to the current regime of antipsychotic treatment.

V. CONCLUSION

The central hypothesis of this thesis was that that chromatin is more restrictive in patients with schizophrenia. As a result, reducing restrictive chromatin modifications could be a therapeutic target for treatment. If, through pharmacological interventions, a reduction in the restrictive state of the chromatin can be relaxed, a process deemed "genome softening," then neuronal gene expression can be enhanced, thus allowing for increased treatment outcomes.

From the data presented, patients with schizophrenia indeed exhibit increases in restrictive chromatin. Through examining lymphocytes and post-mortem brain tissue in patients with schizophrenia, protein levels of the 'restrictive' chromatin mark H3K9me2 were significantly increased compared to normal controls in both models tested. Additionally, mRNA levels of the histone methyltransferase (HMT) enzymes which add this restrictive chromatin mark, G9a and SETDB1 were also significantly increased in both lymphocytes and post-mortem brain tissue compared to normal controls. Furthermore, increases in negative symptoms, which are characterized by blunted affect, anhedonia, and poverty of speech, to name a few, were positively correlated with increases in G9a mRNA levels in lymphocytes from patients with schizophrenia. Negative symptoms are not treated by our current regime of antipsychotic treatment, thus leaving this facet of the illness untreated by current clinical interventions.

From these findings, this thesis attempted to examine pharmacological agents which may target this specific restrictive epigenetic machinery, thus perhaps leading to better treatment outcomes. Three pharmacological agents were examined, nicotine, Valproic acid and Trichostatin A. The incidence of smoking in patients with schizophrenia is significantly higher than the general population, and this is believed to be a form of self-medication. Therefore, we

sought to determine whether there was an underlying epigenetic mechanism behind increased nicotine intake in patients with schizophrenia. The histone deacetylase inhibitors Valproic acid and Trichostatin A were chosen due to their applicability in 'relaxing' chromatin through decreasing histone deacetylation. This thesis demonstrated that all three agents chosen significantly decreased HMT mRNA, resulting in decreased global and promoter-specific H3K9me2 levels. By decreasing this restrictive epigenome, brain derived neurotrophic factor (BDNF/Bdnf) mRNA was significantly increased, indicating a more 'relaxed' chromatin structure. Targeting these restrictive histone modifications, specifically G9a and H3K9me2, in patients with schizophrenia, through the agents listed here, may result in decreases in negative symptomology, thus better treatment prognosis.

APPENDIX A

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Approval Notice
Initial Review
Response To Modifications

September 25, 2003

Rajiv P. Sharma, MD
Associate Professor, Department of Psychiatry
College of Medicine
1601 W. Taylor St.
475 P.I., M/C 912
Phone: (312) 413-4508 / Fax: (312) 355-1492

RE: Protocol # 2003-0490
"Epigenetic Effects of Depakote In Subjects with Schizophrenia or Bipolar Disorder"

Dear Dr. Sharma,

The Institutional Review Board (IRB) # 1 conducted an initial review of your research protocol at its convened meeting of August 13, 2003 and requested modifications. Members of the IRB # 1 reviewed your September 9, 2003 response under expedited review procedures [45 CFR 46.110(b)(2) and 21 CFR 56.110(b)(2)] on September 17, 2003 and determined that it is acceptable. You may now begin your research.

Please note the following information about your approved research protocol:

- Protocol Approval period: September 17, 2003 - August 11, 2004
- Informed Consent: UIC Adult Consent: "Epigenetic effects of depakote in subjects with schizophrenia or bipolar disorder – Patient consent form", version 2, 09/09/2003
- HIPAA Authorization: UIC "Epigenetic Effects of Depakote® in Subjects with Schizophrenia or Bipolar Disorder", Version # 1, 07/07/2003
- Recruiting Material: Recruitment Flyer: Epigenetic Effects of Depakote in Subjects with Schizophrenia or Bipolar Disorder, version dated 07/07/2003
- Research Protocol: Investigator Protocol: "Epigenetic Effects of Depakote In Subjects with Schizophrenia or Bipolar Disorder", PI – Rajiv R. Sharma, M.D., no version #, no date
- Sponsor: Abbott Pharmaceutical Laboratories
- Approved Enrollment: 20 Total (1-20 male, 1-20 female)
- Performance Sites: UIC
- Investigational Drug Brochure: N/A

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Approval Notice
Continuing Review
Convened Review

December 7, 2007

Rajiv P. Sharma, MD
Psychiatry
1601 W. Taylor St.
475 P.I., M/C 912
Chicago, IL 60612
Phone: (312) 413-4508 / Fax: (312) 355-1492

RE: Protocol # 2006-0583
"Chromatin Remodeling in Primary Lymphocyte Cultures Obtained from Schizophrenia and Bipolar Disorder Patients"

Dear Dr. Sharma:

The Institutional Review Board (IRB) # 1 reviewed and approved your Continuing Review by the Convened review process on December 5, 2007. You may now continue your research.

Please note the following information about your approved research protocol:

Protocol Approval Period: December 6, 2007 - December 4, 2008
Approved Subject Enrollment #: 120 Total (38 enrolled to date)
Additional Determinations for Research Involving Minors: These determinations have not been made for this study since it has not been approved for enrollment of minors.
Performance Sites: UIC
Sponsor: Department
PAF#: Unavailable
Grant/Contract No: N/A
Grant/Contract Title: N/A
Research Protocol(s):

a) Investigator Protocol: Chromatin Remodeling in Primary Lymphocyte Cultures Obtained from Schizophrenia and Bipolar Disorder Patients; as submitted September 5, 2006

Recruitment Material(s):

a) Flyer: Chromatin Remodeling (patient); V2, 12/18/2006
b) Flyer: Chromatin Remodeling (control); V2, 12/18/2006
c) Print Ad/Electronic Ad: Chromatin Remodeling (patient); V2, 12/18/2006
d) Print Ad/Electronic Ad: Chromatin Remodeling (control); V2, 12/18/2006

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Approval Notice
Initial Review (Response To Modifications)

August 18, 2009

Rajiv P. Sharma, MD
Psychiatry
1601 W. Taylor Street
475 P.I., M/C 912
Chicago, IL 60612
Phone: (312) 413-4508 / Fax: (312) 355-1492

RE: Protocol # 2009-0349
"Chromatin Remodeling in Schizophrenia"

Please note that when changes are made to documents, such as the protocol, Initial Review Application, appendices, consent/recruitment documents, etc., regardless of whether the changes are made as part of an amendment or in response to modifications requested by the IRB, a marked and clean version of the revised document should be submitted. A marked copy of the revised Initial Review Application was not submitted with the Initial Review - Response to Modifications. All changes should be visible on the marked copy where text that is ~~stricken through~~ is being deleted and text that is highlighted/shaded is being added. This allows the IRB to identify the changes that are being made. Footers should also be revised to update the version number and version date of the document if applicable. Please be reminded to submit a marked and clean copy of revised documents with future submissions.

Dear Dr. Sharma:

Your Initial Review (Response To Modifications) was reviewed and approved by the Expedited review process on July 31, 2009. You may now begin your research.

Please note the following information about your approved research protocol:

Protocol Approval Period: July 31, 2009 - July 13, 2010
Approved Subject Enrollment #: 250
Additional Determinations for Research Involving Minors: These determinations have not been made for this study since it has not been approved for enrollment of minors.
Performance Sites: UIC
Sponsor: National Institute of Mental Health

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

August 20, 2009

Alessandro Guidotti
Psychiatry
M/C 912

Dear Dr. Guidotti:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **8/18/2009**. *The protocol was not initiated until final clarifications were reviewed and approved on* **8/20/2009**. *The protocol is approved for a period of 3* years with annual continuation.

Title of Application: Mouse Models For GABA Epigenetic Dysfunction

ACC Number: 09-127

Initial Approval Period: 8/20/2009 to 8/18/2010

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Grant Title**			**Portion of Grant Matched**
NIH	*Mouse Models for GABA Epigenetic Dysfunction*			*Matched*
Grant Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Grant PI**
R01MH070855	*Funded*	*2005-05470*	*UIC*	*Alessandro Guidoti*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Richard D. Minshall

Richard D. Minshall, PhD
Chair, Animal Care Committee
RDM/*ss*
cc: BRL, ACC File, Roberta Baruch, Erbo Dong, Patricia Tueting, **PAF # 2005-05470**

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIBC) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

8/17/2011

Rajiv Sharma
Psychiatry
M/C 912

Dear Dr. Sharma:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 8/17/2011.**

Title of Application: **Epigenetic Modifications of Gaba Neurons in Psychosis**
ACC NO: **10-150**
Original Protocol Approval: **8/26/2010 (3 year approval with annual continuation required).**
Current Approval Period: **8/17/2011 to 8/17/2012**

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Grant Title**			**Portion of Grant Matched**
NIH	*Epigenetic Modifications of GABA Neurons in Psychosis*			*Matched*
Grant Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Grant PI**
KO1 MH069839	*Funded*	*2005-05597*	*UIC*	*Rajiv Sharma*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/kg
cc: BRL, ACC File

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

12/21/2011

Alessandro Guidotti
Psychiatry
M/C 912

Dear Dr. Guidotti:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 12/21/2011.**

Title of Application: **Nicotinic Receptor Stimulation and Epigenetic Regulation of GABAergic Function**
ACC NO: **10-212**
Original Protocol Approval: **01/14/2011 (3 year approval with annual continuation required).**
Current Approval Period: **12/21/2011 to 12/21/2012**

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Grant Title**			**Portion of Grant Matched**
NIH	*Nicotinic Receptor Stimulation and Epigenetic Regulation of GABAergic Function*			*Matched*
Grant Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Grant PI**
RO1 MH03348 (yrs 1-5) A1 version	*Funded*	*2011-02525*	*UIC*	*Alessandro Guidotti*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/kg
cc: BRL, ACC File, Patricia Tueting, Francesco Matriciano, Roberta Baruch

CITED LITERATURE

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PROFESSIONAL EXPERIENCE:

Research Assistant

6/2000 – 2012 University of Illinois Department of Psychiatry
Chicago, Illinois

Clinical data collection and development of statistical databases in SPSS; Statistical analysis of preliminary data; Participation in psychiatric diagnostic interviews; Administration of clinical ratings including: the Positive and Negative Syndrome Scale, Hamilton Depression Scale, the Young Mania Scale and the SCID; Lymphocyte and other nucleated blood cell extraction and culture from patient blood samples; DNA and mRNA isolation; Plasmid construction and transfection; Cell Culture of NT2, Primary Cortical Neurons, NIH-3T3 Cells, Conducting real-time PCR and Western blot analysis; and lab management.

Teaching Assistant

8/2010 - 06/2011 University of Illinois Department of Psychiatry
Chicago, Illinois

Taught several lectures pertaining to epigenetic modifications in schizophrenia; Graded papers; Designed and graded exams.

Undergraduate Mentor
7/2009 – 2012 University of Illinois Department of Psychiatry
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Taught undergraduate students the basics of wet laboratory techniques; assisted in the design, problem solving, execution, and data analysis of student-directed research culminating in the publication of several posters.

MEMBERSHIPS: Phi Theta Kappa: 2005-Current

Psy Chi: 2007-Current

Society for Neuroscience: 2008-Current

PROFESSIONAL PUBLICATIONS:

Chase K, Sharma RP. Nicotine Induces Chromatin Remodeling Through Decreases in the Methyltransferases GLP, G9a, Setdb1 and Levels of H3K9me2. Under Review.

Sharma RS, **Chase K**. Increasing Neuronal 'Stemness'; Chromatin Relaxation and the Expression of Reprogramming Genes in post-mitotic neurons. Medical Hypothesis. 2012 Apr;78(4):553-4

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PRESENTATIONS:

Chase K, Guidotti A, Gavin D, Sharma RP. Histone Methyltransferases (glp, g9a, setdb1) and h3k9me2; Regulation in Psychiatric Disorders. Poster presentation at the 41st annual meeting of the Society for Neuroscience, Washington DC, 2011.

Sharma RP, **Chase K**. Increasing Neuronal 'stemness'; Chromatin Relaxation and the Expression of Reprogramming Genes. Poster presentation at the 41st annual meeting of the Society for Neuroscience, Washington DC, 2011.

Gavin D, Grayson DR, Guidotti A, **Chase K,** Sharma RP. Growth arrest and DNA-damage-inducible, beta (GADD45b) in psychosis. Poster presentation at the 41st annual meeting of the Society for Neuroscience, Washington DC, 2011.

Gavin DP, **Chase K**, Matrisciano F, Grayson DR, Guidotti A, Sharma RP. Growth Arrest and DNA-Damage-Inducible, Beta (GADD45b) Expression and Function in the Brain: An examination of a putative member of a DNA demethylation pathway abnormally expressed in psychosis. Poster presentation at the 49th annual meeting of the American College of Neuropsychopharmacology, Miami Beach, FL, 2010.

Chase K, Gavin DP, Guidotti A, Sharma RP. The Role of Histone Methyltransferases (EHMT1, EHMT2, SETDB1) Expression in psychiatric disorders. Poster presentation at the 40th annual meeting of the Society for Neuroscience, San Diego, CA, 2010.

Gavin DP, **Chase K,** Matrisciano F, Grayson DR, Guidotti A, Sharma RP. Growth Arrest and DNA-Damage-Inducible, Beta (GADD45b) Expression and Function in the Brain: An examination of a putative member of a DNA demethylation pathway abnormally expressed in psychosis. Poster presentation at the 40th annual meeting of the Society for Neuroscience, San Diego, CA, 2010.

Gavin DP, Sharma RP, Dong E, **Chase** K, Guidotti A. GADD45b Expression and Function in the Brain: The regulation of a putative member of a DNA demethylase pathway in neuronal cultures and dysregulation in psychosis. Poster presentation at American Psychiatric Association 163rd Annual Meeting, New Orleans, LA, 2010.

Tun N, **Chase K**, Gavin D, Grayson D, Sharma RP. DNMT1 promoter is vigorously expressed in post-mitotic neurons. Poster presentation at the 39th annual meeting of the Society for Neuroscience, Chicago, IL, 2009.

Chase K, Sharma, RP: The Study of the Dnmt1 and Dnmt3a Promoter. Poster presentation at the 39th annual meeting of the Society for Neuroscience, Chicago, IL, 2009.

Sharma RP, Tun N, **Chase K**, Grayson D. The Study of the DNMT1 promoter in primary neurons. Poster presentation at the Society of Biological Psychiatry, Vancouver, Canada, May 2009.

Gavin DP, **Chase K**, Rosen C, Grayson DR, Sharma RP. Histone Modifications (H3k9k14ac and H3K9me2) in Schizophrenia; Evidence for an Excess of Restrictive Chromatin. Poster presentation at the International Congress on Schizophrenia Research, San Diego, CA, 2009.

Gavin D, **Chase K**, Rosen C, Grayson D, Sharma RP. Increased Acetyl H3 and Acetyl H4 in Schizophrenia; Evidence for an Excess of Restrictive Chromatin. Poster presentation at the International Congress for Schizophrenia Research. San Diego California, March, 2009

Rosen C, Gavin D, **Chase K**, Kartan S, Sharma RP. Restrictive chromatin modifications in patients with schizophrenia; implications for pathophysiology. Poster presentation at the Federation European Neuroscience Societies. July 12-16; Geneva 2008.

Chase K, Rosen C. A measure to evaluate the subjective experience of psychiatric research participants. Poster presentation at the American Psychiatric Association, May 2008.

Gavin DP, Rosen C, **Chase K**, Marvin R, Sharma RP: Levels of two independent histone modifications in lymphocytes suggest schizophrenia is associated with a restrictive chromatin state. Poster presentation at the 38th annual meeting of the Society for Neuroscience, Washington, D.C., 2008.

Gavin D, Rosen C, Kartan S, **Chase K**, Marvin R, Sharma R. PharmacoEPIgenetics: Therapeutic and diagnostic application of chromatin remodeling agents in mental illness. Poster presentation at the Scientific and Clinical Reports, American Psychiatric Association 161st Annual meeting, Washington, DC, May 5th, 2008.

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