NEUROBIOLOGICAL AND GENETIC MARKERS IN SCHIZOPHRENIA: A THEORETICAL REPLICATION USING THE ALLEN INSTITUTE FOR BRAIN SCIENCES ONLINE DATABASE

By

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ABSTRACT

Neurobiological and Genetic Markers in Schizophrenia: A Theoretical Replication Using The Allen Institute for Brain Sciences Online Atlas

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Schizophrenia is a psychiatric disease that affects 1% of the human population, and is characterized as a strongly heritable neurodevelopmental disorder. Genome wide association studies have identified altered expression of dopamine-related genes such as Regulator of G-Protein Signaling 4 and Catechol-O-Methyltransferase to be linked with the occurrence of schizophrenia. Furthermore, altered neuronal organization and neural connectivity within the Dorsolateral Prefrontal Cortex has been associated with the disease, however no specific biomarker has been identified, and pathogenesis of the disease remains extremely obscure.

The Allen Institute for Brain Science’s Human Brain Atlas is an online resource that provided Colorimetric in situ hybridization and Nissl stained images, for the current analysis. The current study used the Human Brain Atlas to compare schizophrenic and control tissue specimen on the schizophrenia-linked characteristics: neuronal cell density, Catechol-O-Methlytransferase, and Regulator of G-Protein signaling 4 expression in the Dorsolateral Prefrontal Cortex.

The current analysis hypothesized that there would be decreased RGS4, COMT and neuronal density within the DLPFC of patients with schizophrenia. It was also
hypothesized that there would be differences in gene expression and neuronal density when comparing between the subgroups: Gender, Ethnicity, and Smoking status.

The analysis of these schizophrenia-linked characteristics did not yield significant results, and patterns in gene expression or neuronal density between or within subgroups was not found. Due to the inconsistent findings of past literature in combination with this study, it is strongly warranted that new techniques are run in parallel with postmortem analysis in order to delineate the complex etiology of schizophrenia.
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CHAPTER I

INTRODUCTION

Schizophrenia is a debilitating disorder that affects 1% of the world population, and is estimated to cost 62.7 billion to the US annually (McEvoy, 2007). Schizophrenia is a complex neurodevelopmental disorder with roughly 70-90% heritability, and a high amount of heterogeneity (Gladys Mulle, 2012). The complexity of symptoms and etiology of the disorder creates many roadblocks for researchers; therefore, it is necessary to examine multiple genes and histopathological characteristics of schizophrenia.

Researchers are slowly coming to agreement that there are observable anatomical characteristics associated with the disorder (Pantelis et al., 2005). Through many different scanning procedures, researchers have continually identified structural and connectivity changes particular to patients with schizophrenia (Calicott et al., 2000). These physical abnormalities include alterations in brain structure, loss of gray matter volume, white matter connectivity aberrations, increased ventricular size and decreased executive performance (Pantelis et al., 2007; Spence et al., 2000; Zhou et al., 2007). Many of these changes have been specifically related to the prefrontal cortex (PFC), a region that is believed to have a strong association with the behavior found in schizophrenia (Barch, 2005). Observable characteristics via cell body Nissl staining, which utilizes cresyl violet staining to reveal neurons and glial cells, has also revealed patterns in neuronal organization, decreased dendritic growth, and decreased neuronal density that correlate
with schizophrenia (Harrison & Weinberger, 2005). Evidence across many studies have supported that schizophrenia has distinguishable neurobiological features.

Given the large amount of evidence finding that schizophrenia can be up to 80% heritable, much of the current research aims to identify specific genetic variations that may indicate increased risk or susceptibility for schizophrenia (Allen et al., 2008; Barch, 2005; Bray et al., 2003; Harrison & Weinberger, 2005). Many studies have shown increased risk of inheriting schizophrenia within families, including twin studies, which have continually shown a higher concordance rate of schizophrenia in monozygotic twins at 46% with dizygotic twins at a 14% concordance rate (Cardno et al., 1999). Due to the high heritability of schizophrenia, identifying risk factor genes are extremely important, and finding specific genes that indicate increased risk for the disease could lead to earlier targeting for medication or a new form of clinical diagnostics (Harrison & Weinberger, 2005). By observing patterns of single nucleotide polymorphisms (SNPs) across genes, genome wide association studies (GWAS) are able to detect genetic associations for many different disease types. After a GWAS identifies a susceptibility gene associated with the disease, candidate gene studies are performed in order to observe the function and expression patterns of a specific gene. Many GWAS studies have identified susceptibility genes associated with schizophrenia. Two genes of interest, the Regulator of G protein signaling 4 (RGS4), and the Catechol-O-methyltransferase (COMT) gene, have continually been identified as susceptibility genes for schizophrenia. Many candidate gene studies have been performed looking at functional patterns of RGS4 and
COMT, and have found that there is altered expression of these genes in patients with schizophrenia (Chen, Wang, O’Neil, Walsh, & Kendler, 2004; Chowdari et al., 2002; Lewitt, Ebert, Mirnics, Nimgaonkar, & Lewis, 2006; Shifman et al., 2002; Williams et al., 2004).

Additional evidence supporting a role for RGS4 and COMT in schizophrenia relates to their regulatory properties within the dopaminergic signaling pathway. Due to the role of pharmaceutical treatment, hyperactivity of dopamine signaling is believed to play a role in psychosis. The leading medications for schizophrenia are responsible for the inhibition of dopamine receptors, and medications suppress many positive symptoms associated with schizophrenia (Carlsson, 1988; Durstewitz & Seamans, 2008).

Dopamine also plays a major role in the dorsolateral prefrontal cortex (DLPFC), which has also become keen interest to current researchers when studying schizophrenia. The DLPFC, also termed Brodmann’s area 46, is connected to the thalamus, hippocampus, basal ganglia, orbitofrontal cortex, and the neocortex (Figure 1). The DLPFC is responsible for executive thinking by integrating sensory information, and also plays an important role in working memory (Lewis & Moghaddam, 2006). Other studies have also found structural changes such as, enlarged ventricles and alterations in gray and white matter volume in the DLPFC (Bunney & Bunney, 2000; Fallon, Opole, & Potkin, 2003). Distinguishable anatomical abnormalities in the DLPFC have been identified across many studies, and research is now directed towards understanding the molecular mechanisms that may cause these anatomical changes.
Therefore, with past literature supporting the dopamine hypothesis, the current study looked at gene expression patterns that have a strong influence on the dopaminergic signaling pathway. The general area of interest was to see if there were changes in expression patterns of COMT and RGS4 within the DLPFC in schizophrenic brain tissue. The current study went further to observe if there were any correlations between altered gene expression and observable histological changes. Nissl stained image slices provided by the Allen Institute for Brain Science (AIBS), were analyzed in order to observe neuronal density. AIBS provided ISH and Nissl Staining data, which allowed for the visualization of gene expression patterns and neuronal density.
CHAPTER II

REVIEW OF LITERATURE

In this section, I will first introduce the background and utilization of the Allen Institute for Brain Science Atlas. I will also discuss past findings that support reasoning for the current analysis on RGS4 and COMT within the DLPC as biomarkers for schizophrenia, and review the pathology findings on schizophrenia, as well as address methodological procedures used to study neuropsychiatric disease.

The Allen Institute for Brain Science (AIBS)

The Allen Institute for Brain Science (AIBS) is an independent nonprofit research organization, founded in 2003 by Paul Allen with a 40 million dollar funding donation (AIBS, 2012). The goal of the Allen Institute is to provide scientists with vital information on the human brain. The Allen Atlas is a portal that gives researchers access to genomic and anatomic data, allowing researchers to analyze and observe patterns of gene activity throughout the brain (AIBS, 2012). The data is acquired in a facility in Seattle Washington, where researchers acquire genetic and anatomical information by performing microarray assays, CISH, Nissl staining, and taking tissue slice images for each specimen. The Atlas is able to provide detailed information on 20,000 genes that are active in the brain, as well as information on the level and location of gene expression (AIBS, 2012).
The Atlas provides five online public datasets, which include, the Mouse Brain Atlas, Adult Human Brain Atlas, Spinal Cord Atlas, Developing Mouse Brain Atlas, Mouse Brain Activity Atlas, and Non-Human Primate Brain Atlas (AIBS, 2012). The Allen Atlas provides high-resolution images and detailed genetic data that are free and publicly available online through the Allen Brain Atlas data portal (AIBS, 2012). The AIBS is able to provide these resources with funding from multiple avenues such as government, private donors, and foundation sources. With over 50 Pub Med hits, the Allen Brain Atlas is the largest and the most used genomic brain database today. The Adult Human Brain Atlas data set was used in this study to analyze genetic expression, and neuronal density of tissue extracted from patients with schizophrenia and in matched controls.

**Microarray.** DNA microarrays provide information about thousands of genes simultaneously, and are highly useful for gene expression profiling. In order to increase accuracy of the data, AIBS uses at least two probes per gene, and organizes these genes into specific categories, including the Schizophrenia Associated Category, which will be used for the current study (AIBS, 2012). The microarray data is presented in the form of a heat map indicating gene expression levels and organized by gene symbol. The genes are categorized by donor and brain structure, with raw data scores available for each gene, along with a full ontology of the gene (AIBS, 2012).
**Colorimetric In Situ Hybridization (CISH).** The Allen Atlas provides CISH data that can be used to localize messenger ribonucleic acid (mRNA) within a sample of tissue, and is helpful in finding gene expression patterns. CISH is an automated method using nucleic acid probes that can localize target mRNA. CISH has low occurrences of background labeling, yet is highly sensitive, and can provide a high cellular resolution (Webber & Cohen, 2010). The detection of target mRNA is important due to its protein encoding properties that affect the expression of a gene (Webber & Cohen, 2010). mRNA provides coding information that is needed for the biosynthesis of a protein, which is an important factor in the activity of a gene. It is necessary to target the activity and location of mRNA using CISH in order to observe the gene expression patterns in cells.

**Nissl Staining.** Localization of the cell body and dendrites of neurons is made possible via Nissl stained images provided by AIBS. This is a method that stains extranuclear cytosol that contains RNA proteins, and reveals the condition of a cell, which allows for labeling of neurons (Pilati, Barker, Panteleimonitis, & Donga, 2008). Tissue slice images are also taken throughout the brain that allows for the visualization of neuronal density and location of a cell. This method is extremely important for looking at neuronal organization and for detecting any neuropathological abnormalities that are associated with a disease.
**Limitations.** Despite the high quality and accuracy of these data sets provided by the Allen Brain Institute, there are several limitations. Since the Allen Institute is still in process of data collection, many genes are not available from donors affected by schizophrenia, and access to different brain regions is limited. Biomarkers such as transcription factors, enzymatic activity, and neurotransmitter secretion are also currently unavailable. Completely matching between affected and unaffected individuals is difficult, which is a limitation similarly found with all postmortem tissue experiments. It is necessary to always take potential confounding factors into account, such as, medication, substance abuse, comorbidity, pH levels of the tissue, post mortem interval (PMI), and tissue preservation methods (Halim et al., 2008; Lipska et al., 2006).

With the limitations in mind, there is still much to be offered from the use of human postmortem tissue. Human postmortem brain studies are critical for understanding pathophysiology of schizophrenia, and genetic advances have brought about an even more useful approach that focuses on risk-associated gene expression (Kleinman et al, 2011). Past literature has shown that transcripts associated with genetic risk are highly identifiable and significantly noticeable in affected individuals (Kleinman et al, 2011). The information that postmortem tissue provides, further supports that using human brain tissue can still be used as a resource for understanding the genetic complexities found in mental illnesses. Postmortem tissue studies have brought about many significant and successful findings that have already improved treatment methods for neurodegenerative disorders such as Parkinson’s Disease (Ehringer & Hornykiewicz, 1960). Investigating
genetic neuropathology will provide a better understanding of neuropsychiatric disorders, and may eventually aide in the identification of new therapeutic targets for mental illnesses (Karam et al., 2010; Kleinman et al., 2011; Seillier & Guiffrida, 2009).

Nissl Staining, and CISH allow for the identification of important biomarkers. High-resolution Nissl images from the Allen Atlas were useful for anatomic comparisons between donors, and CISH data allowed for expression of RGS4 and COMT to be analyzed. All of these methods provide large datasets that can be used for detailed analysis on a specific gene or pathway, and can provide evidence for pathophysiological differences between individuals with and without schizophrenia.

Post Mortem Studies. Postmortem studies commonly find inconsistent results, and it has been difficult to determine concrete differences between schizophrenia patients and controls (Harrison, 1999). In order to minimize the affect of post mortem confounding variables, the Allen Brain Institute follows a specific protocol when obtaining brain tissue (AIBS, 2011). The pH levels, PMI, and methodology for fixation all fit within narrow criteria for minimal tissue or cell degradation. The Atlas also provides detailed information about the diagnostics of the patients so that analysis can be done as accurately as possible. Even though limitations arise when using post mortem tissue, it is still a leading method for observing biomarkers in the developed human brain. It has been widely agreed upon that postmortem tissue is an acceptable method to observe genetic expression between disease states (Kleinman et al., 2011; Lewis & Moghaddam, 2006).
Dorsolateral Prefrontal Cortex

Abnormalities in the frontal lobe are a major finding associated with schizophrenia, and dopamine inputs specific to the PFC are known to be associated with cognitive impairments in patients with schizophrenia (Durstewitz & Seamans, 2008; Howes & Kapur, 2009; Volpe, Mucci, Quarantelli, Galderisi, & Maj, 2012). Neuroimaging studies report abnormal function of the frontal lobe and altered dopamine function in the DLPFC of schizophrenic patients (Durstewitz & Seamans, 2008; Kegeles et al. 2010; Walton et al., 2012). Several neuroimaging studies have also revealed dysfunctional connections and abnormal functioning in the DLPFC (Kegeles et al., 2010; Zhou et al., 2007).

Many studies have aimed to find observable differences in frontal lobe activity between unaffected and affected patients. Magnetic resonance Imaging (MRI) scans have revealed decreased volume, and altered activity in the frontal cortex of patients from any stage of the disorder (Pantelis et al., 2005; Spence et al., 2000; Walton et al., 2012). It was first thought by researchers that the anatomical differences in patients was due to long term medication effects: However, studies have found that decreased brain volume and abnormal frontal lobe function are symptoms of the disorder before treatment (Walton et al., 2012).

Walton and colleagues (2012), investigated frontal lobe functioning in patients at first episode before medication, in order to determine if pharmaceuticals play a role in the atrophic features found in schizophrenia. Patients and controls performed a working memory task during functional magnetic resonance image (fMRI) recordings, which
revealed a significant reduction in memory function and decreased DLPFC volume in schizophrenic individuals (Walton et al., 2012). These findings show that atrophy in the DLPFC are not due to the use of antipsychotics, but may be a pathological marker that results from the disorder. It has been hypothesized that more severe symptoms are associated with an increased amount of atrophy (Hazlett et al., 2008).

Previous studies have also found reduced amount of gray matter within the prefrontal and the temporal cortex, and more specifically within the DLPFC and hippocampus (Callicott et al., 2000). Callicott and colleagues (2000), also used fMRI scans to observe anatomical abnormalities while testing executive function in a control and schizophrenia affected group. They specifically investigated PFC activity with working memory performance. Noticeable differences were found in affected individuals, including impaired working memory with increased DLPFC activation. The researchers concluded that there are significant and observable alterations of cortical activity, especially in the DLPFC, which can be considered characteristics of schizophrenia (Callicott et al., 2000).

Zhou and colleagues (2007) focused on the bilateral DLPFC, which also showed a reduction in connectivity between the striatum, parietal lobe, thalamus, and the posterior cingulate gyrus in schizophrenic patients. The schizophrenic individuals were first episode patients, and increased activity was found between the DLPFC and the limbic system. This further elucidates that there is a change in DLPFC activity and functional disconnectivity in first episode patients with schizophrenia (Zhou et al., 2007). Studies
consistently report observable connectivity and anatomical abnormalities in schizophrenic individuals, however the aberrations remain inconsistent, therefore warranting deeper analysis on brain structure changes in schizophrenic individuals (Olabi et al., 2011).
Figure 1. A schematic overview of the Prefrontal cortex. The left side illustrates the Dorsolateral, Medial, and Orbital, regions of the prefrontal cortex, along with the Brodmann areas and gyri. Adapted from “The neuroanatomy of schizophrenia: circuitry and neurotransmitter systems,” by J.H. Fallon, I.O. Opole, & S.G. Potkin, 2003, Clinical Neuroscience Research, 77, p. 79. Copyright 2012 Elsevier B.V.
Schizophrenia and Genetics

The direction of postmortem studies has been directed towards candidate gene identification and genetic linkage association studies. It is well established that schizophrenia has a strong genetic background, with twin studies and meta-analyses that estimate the heritability of schizophrenia to be 80% (Allen et al., 2008; Barch, 2005; Bray et al., 2003; Harrison & Weinberger, 2005). Many of these twin studies have emphasized that genes are highly involved in the pathogenesis of schizophrenia, though shared with environmental risk factors (Cardno & Gottesman, 2001; Kleinman et al., 2011). Increased risk for psychiatric illness has also been found to be high within families, further supporting the hypothesis that psychosis has a strong genetic etiology, and may one day be useful for clinical diagnostics (Arajarvi et al., 2006). The interaction between genetic and environmental factors is considered a critical component for risk of schizophrenia (Allen et al., 2008). Due to the overall agreement that schizophrenia is highly heritable, it is appropriate for studies concerned with this disorder to focus on the genetic background of schizophrenic individuals.

GWAS studies have become a large source for the advancement of genetic investigation for mental illness. Ripke and colleagues (2011) recently performed a GWAS for bipolar disorder and schizophrenia, which identified susceptibility genes specific to these disorders. This was one of the largest genetic studies on schizophrenia to date, which included 21,856 individuals of European ancestry, along with a replication study including a sample size of 29,839 subjects (Ripke et al., 2011). Similarly, Yue and
researchers (2011), performed a GWAS study in a Hans Chinese population on over 10,000 subjects, and found strong susceptibility loci for schizophrenia, including many similar to the genes identified by Ripke and colleagues (2011). Many of these identified schizophrenia susceptibility genes are now known to affect specific downstream factors, alter other genes, and correlate with changes in specific brain regions (Kleinman et al., 2011; Yue et al., 2011). Much of the research is still based on candidate gene studies, and if interpreted accurately these studies are important for finding characteristics specific to schizophrenia (Cannon & Keller, 2005).

Changes in working memory, attention, and executive function have also been found to correlate with genetic variations found in patients with schizophrenia. Understanding the relationship between these factors will be helpful in gaining insight to the symptoms of schizophrenia (Barch, 2005; Beck & Rector, 2005; Durstewitz & Seamans, 2008). Important questions remain, and scientists have still not identified a specific DNA variant perfectly associated with schizophrenia. Therefore, it is crucial to look at all candidate genes and changes in genetic expression in order to demonstrate associations among risk genes, and to establish biomarkers. Finding associations between genes and the pathology of schizophrenia may aide in finding biomarkers that could help identify at risk individuals (Cannon & Keller, 2005).
Schizophrenia Associated Signaling Pathways

Past literature reveals that there are many different pathways involved in the pathogenesis of schizophrenia, and understanding the role of each pathway is extremely difficult. Many analyses have set out to examine thousands of associations and linkages between genes, which have helped in understanding the disorder (Allen et al., 2008). Much of the current research focuses on penetrance of alleles and common allele characteristics that may act together to increases risk (Karam et al., 2010). Many studies are identifying alleles within specific signaling pathways, and are examining the relationship of genetic alterations that may be responsible for symptoms of schizophrenia.

Glutamatergic Signaling Pathway. In the past Glutamatergic targets were a main focus of mental illness research. Phencyclidine (PCP) is an N-Methyl-D-aspartate (NMDA) antagonist, which induces psychotic states similar to schizophrenic symptoms. PCP has been used in many mouse behavioral and pharmacological studies to model aspects of schizophrenia (Seillier & Guiffrida, 2009). Moghaddam and Adams (1998), treated mice with PCP while blocking metabotropic glutamate receptors (mGluR). The blockage of mGLUR suppressed the effects of PCP on locomotor activity, and behavioral movements associated with PCP were suppressed (Moghaddam & Adams, 1998). One specific metabaotropic glutamate receptor mGluR5, is also thought to play a role in regulation of the NMDA receptor in the forebrain, and may play a critical role in schizophrenia. One meta-analysis reviews pathways involved in schizophrenia includes glutamatergic, GABAergic, and cholinergic pathways, along with their reaction to
pharmacotherapy (Karam et al., 2010).

**γ-Aminobutyric acid (GABA) Signaling Pathway.** The 67-isoform of glutamic acid decarboxylase (GABA67), a synthetic enzyme for GABA, is also of interest due to its regulatory properties on GABA production, specifically in the DLPFC (Hashimoto et al., 2003). Decreased GABA has been found to alter activity and structure of pyramidal neurons in the PFC, and aberrations in pyramidal cells are strongly related to deficits in working memory and cognitive function (Lewis & Moghaddam, 2006). Rao, Williams, and Goldman-Rakic (2000) also found that abnormalities in GABA neurotransmission in the DLPFC correlated with a decrease in cognitive performance, and future research on the role of GABA is warranted.

**Cholinergic Signaling Pathway.** The cholinergic system is also a candidate pathway that may be important for treating negative and cognitive symptoms found in schizophrenia. Cholinergic pathways connect to all areas in the cerebral cortex, and abnormalities in cholinergic neurotransmission affect attention, working memory, and motivation, all common symptoms of schizophrenia (Berman, Talmage, & Roe, 2007). Therefore, many studies using animal models and postmortem tissue focus on the alpha7 nicotinic (a7) receptor. Researchers have found decreased levels of the a7 nicotinic receptor in the PFC and hippocampus of schizophrenic individuals, which further supports the hypothesis that the cholinergic system is important for understanding schizophrenia (Freedman et al., 2008).
All of these signaling pathways need to be further investigated in detail before the advancement of new drug treatment and diagnostic tests can take place. Many of these studies address major signaling pathways associated with schizophrenia, and these pathways may represent targets for the development of new pharmacological treatment (Freedman et al., 2008; Karam et al., 2010).

**Dopaminergic Signaling Pathway.** The general dopamine hypothesis predicts that the positive symptoms of schizophrenia are due to hyperactivity of dopaminergic transmission from mostly D2 receptors (Kegeles et al., 2010; Kessler et al., 2009). Past and present evidence suggests that dopaminergic dysfunction is a major contributor to the positives symptoms of schizophrenia, and many studies report changes in dopaminergic activity in the striatum, substantia nigra, thalamus, hippocampus, and cortex (Kessler et al., 2009; Pantelis et al., 2007; Seamans & Yang, 2004). The dopaminergic pathway is not likely to be the only component, however, understanding this major pathway in the brain, specifically within the DLPFC, can lead to important knowledge of the disorder. Dysregulation of dopaminergic pathways is not only relevant to schizophrenia, but dysfunction of dopamine is also strongly associated with Parkinson’s Disease, other mood disorders, and attention hyperactivity disorder (ADHD) (Carlsson, 1988).

The dopaminergic signaling pathway is important for working memory and executive performance, and alterations in the activity of this pathway may reduce cognition and influence the severity of psychosis (Carlsson, 1988; Walton et al., 2012). The development and success of antipsychotic medication created a new way to treat
positive symptoms in schizophrenia, and eventually led to the dopamine hypothesis (Kegeles et al., 2011). Antipsychotics are dopamine antagonists that directly block dopamine receptors (Figure 2), the anti-dopaminergic properties of these drugs, suggest that positive symptoms of schizophrenia are due to an excess of dopamine signaling (Carlsson, 1988). The most commonly used antipsychotic drugs are responsible for receptor regulation, and alter properties at the dopamine synapses. Drug based evidence is prevalent; however a drawback is that current treatments act at receptors and downstream mechanisms. Recent evidence suggests that drugs that act on upstream factors may control these dopaminergic pathways, and could be more effective (Howes & Kapur, 2009). Therefore, genes that regulate dopaminergic neurotransmission are of major interest when examining schizophrenia.

Most antipsychotics improve positive symptoms, specifically hallucinations and delusions. However, they are unable to improve cognitive deficits, which are a major symptom of schizophrenia (Howes & Kapur, 2009; Karam et al., 2010). Mixed hypotheses have also arisen due to findings that lowered dopamine activity is found in affected patients with negative symptoms or more chronic diagnoses (Van Kammen & Kelley, 1990). To localize dopaminergic hyperactivity, Kegeles and colleagues (2010) used positron emission tomography (PET) scans to observe differences in D2 receptors in the striatum between groups. Patients with schizophrenia were found to have higher synaptic dopamine concentration. Modern methods are supporting the original dopamine hypothesis by also finding increased activity of dopamine transmission in the striatum,
prefrontal cortex, and hippocampus (Kegeles et al., 2010; Pantelis et al., 2007). Due to the strong amount of evidence that dopamine plays a critical role in schizophrenia, it is necessary to investigate genes that may cause altered activity in the dopaminergic pathway.
Figure 2. *Role of Dopamine Antagonists.* All antipsychotic drugs are antagonists of Dopamine D2 receptor. The antipsychotics block D2R activation and suppress dopaminergic signaling. The binding of dopamine and D2R causes G-Protein signaling, which inhibits adenylate cyclase and regulates K+ and Ca2+ channels. Adapted from “Subunit-selective modulation of GABA type A receptor neurotransmission and cognition in schizophrenia,” by Lewis et al., 2008, American Journal of Psychiatry, 165, p. 1587. Copyright 2006 American Medical Association.
Dopaminergic Signaling Pathway Associated Genes

Disrupted in Schizophrenia 1 (DISC1). Many different genes play a role in the regulation of the dopaminergic pathway, including DISC1, which codes for the scaffolding protein that is important for binding other proteins involved in the dopaminergic pathway. Kamiya and colleagues (2005) observed impairments in neurite growth and abnormal neuronal migration in mouse cell cultures after suppression of DISC1. Neuronal orientation and function were significantly impaired and overall there was abnormal development of the cerebral cortex (Kamiya et al., 2005). Using PET scans Kessler and colleagues (2009) also found increased levels of dopamine receptor D2 in the substantia nigra, which strongly correlated with disorganized thinking and delusions found in schizophrenic individuals. Unlike positive symptoms, negative symptoms are thought to be related to deficits associated with the D1 receptor, and alterations in dopamine signaling have been found in the prefrontal cortex of those suffering from negative symptoms (Buchanan et al., 2007). Overall, findings indicate that positive and negative symptoms are due to a dysfunction in dopamine signaling. Many researchers suggest that understanding the factors that cause the imbalance between D1:D2 activity, will provide insight to the cognitive, positive, and negative symptoms observed in schizophrenia (Buchanan et al., 2007). The exact upstream and downstream targets are still undefined; therefore future research investigating the mechanisms that regulate dopamine signaling is warranted.
**Catechol-O-methyltransferase (COMT).** COMT is an enzyme encoded by the COMT gene, and is responsible for the regulation of catecholamines, such as dopamine (Figure 3) (Karayiorgou & Gogos, 2004). Past literature has shown that alterations in COMT activity affect neurotransmission, however its particular role in schizophrenia is unknown (Kates et al., 2006). It is now widely agreed upon that cognitive deficits and other symptoms of psychosis are related to dysfunction in the dopaminergic system of the PFC (Karayiorgou & Gogos, 2004). The PFC is strongly regulated by the enzyme COMT, which is also a direct regulator of dopaminergic metabolism (Durstewitz & Seamans, 2008). It has not been determined if it is high or low activity of COMT that contributes to schizophrenia, but researchers have begun to focus on the A valine158 methionine (Val158Met) allele which affects the stability of the protein, thus altering dopamine metabolism (Bray et al., 2003; Karayiorgou, Simons, & Gogos, 2010).
Figure 3. *Role of COMT and RGS4 in Dopamine Signaling.* COMT is an enzyme encoded by COMT gene and is important for the degradation of catecholamines. G-coupled protein receptors are encoded by the gene RGS4 which is important for cell signaling and neurotransmitter release.
Many studies have been directed towards understanding the function and role of the val158met polymorphism found in COMT. The polymorphism involves a valine (val) to methionine (met) substitution. The met/met individuals have higher dopamine levels than val/val individuals, due to lower enzymatic activity (Durstewitz & Seamans, 2008). Eisenberg and colleagues (2009) observed changes associated with the COMT val158met polymorphism by using regional cerebral blood flow (rCBF) PET scans on 25 medication free patients and 47 control subjects. In patients with schizophrenia, the presence of the val (risk) allele predicted a decrease in regional cerebral blood flow in the right DLPFC and the right superior temporal gyrus. Interestingly, the presence of the val allele also predicted increased rCBF in the amygdala and parahippocampal gyrus. It is understood that the val risk allele is found in patients with schizophrenia, and the presence of the val/val allele predicts decreased function in the PFC and lowered limbic activation (Eisenberg et al., 2009). Mier, Kirsch, and Meyer-Lindenberg (2010) also observed the pharmacological effects on val/val subjects. The val/val individuals showed an increase in executive function tasks when given a COMT inhibitor, while met/met individuals showed the reverse.

Other evidence that supports the theory that COMT is associated with schizophrenia is based on 22q11 deletion syndrome (22q11DS). 22q11DS is caused by 22q11.2 microdeletions, and 25-30% of children with 22q11.2 will develop schizophrenia (Karayiogou et al., 2010). In addition, 1-2% of sporadic non-familial schizophrenia has
been linked to 22q11 DS (Karayiorgou et al., 2010; Liu et al., 2002; Xu et al., 2008). The COMT gene is located in the 22q11 chromosome, which emphasizes the importance in understanding COMT (Figure 4) (Shifman et al., 2002). Knockout mouse models have been made with a deletion on the 22q11.2 region. These mice had visible developmental abnormalities such as, impairments in dendritic growth and lowered dendritic spine density (Mukai et al., 2008; Paterlini et al., 2005). Researchers have also found that there is a significant decrease in glutamatergic synapses and impaired connectivity between the hippocampus and prefrontal cortex with microdeletions on the 22q11 locus (Mukai et al., 2008; Sigurdsson, Stark, Karayiorgou, Gogos & Gordon, 2010). These findings suggest that polymorphisms associated with COMT may contribute to the developmental abnormalities in the PFC found in schizophrenic populations (Durstewitz & Seamans, 2008).

Contrary to other studies, researchers have found there to be no association between COMT polymorphisms and schizophrenia (Strous, Lapidus, Viglin, Kolter, & Lachman, 2005). Many studies have also been conducted to understand the inconsistencies that arise from COMT activity, and many have found COMT to not be associated with schizophrenia (Okochi et al., 2009). Okochi and colleagues performed a mutation scan and meta-analysis to evaluate COMT risk factors in schizophrenia, where they concluded that COMT did not play a major role in schizophrenia. Many findings do not support the hypotheses regarding associations between COMT polymorphisms and schizophrenia, and some have concluded that COMT is not a risk factor for schizophrenia (Strous et al.,...
2005; Nieratschker et al., 2010). Alternatively, researchers found a significant association between COMT polymorphisms and manic symptoms found in schizophrenia (DeRosse et al., 2006). All of these findings lead to a complex hypothesis about the role of COMT, and it is necessary to investigate any associations of COMT activity and schizophrenia.
Figure 4. The 22q11 locus. The illustration shows the presence of catechol-O-methyltransferase (COMT) on the 22q11 chromosomal region. All of these genes have been identified as susceptibility genes for schizophrenia, and a microdeletion on 22q11 has a high comorbidity with schizophrenia. Adapted from “Signaling pathways in schizophrenia: emerging targets and therapeutic strategies” by Karam et al., 2010, Trends in Pharmacological Science, 31, p. 385. Copyright 2012 Elsevier.
Regulator of G-protein coupled protein receptors (RGS4). RGS4 is a guanine triphosphate (GTP) ase-activating protein responsible for regulating G-protein coupled receptors (GPCR), and has also been consistently linked with dopamine receptor signaling (Figure 5) (Min et al., 2011). GPCR’s are a major mediator of neurotransmitters such as dopamine, and researchers specifically found that RGS4 plays a major role in inhibitory regulation of D2R and D3R signaling (Min et al., 2011). The common agreement remains that RGS4 has a direct interaction with dopamine signaling, however the complex interactions are still unexplained (Taymans et al., 2004). Ultimately, RGS4 has been identified as a schizophrenia susceptibility candidate gene and further investigation on the role of this gene should be implemented.
Evidence is suggested from functional biology, microarray data, postmortem studies along with linkage association studies, that RGS4 is strongly linked to the pathophysiology of schizophrenia (Talkowski et al., 2006; Williams et al., 2004). Mirnics, Middleton, Marquez, Lewis, & Levitt (2001) performed a postmortem DNA microarray study looking at expression of RGS4 in the DLPFC. They found reduced expression of RGS4 in the DLPFC of patients with schizophrenia, which may contribute to the phenotype of schizophrenia.

Talkowski and colleagues (2006) performed a genotype based meta-analysis in order to resolve controversies found with the role of RGS4 in schizophrenia. The findings indicated that there was a consistent reduction of RGS4 in affected individuals as well as changes in RGS4 mRNA levels (Talkowski et al., 2006). Recently, another study found that polymorphisms of RGS4 are associated with a reduction in DLPFC gray matter volume (Prasad et al., 2005). Using MRI scans and genetic assays, researchers examined RGS4 polymorphisms and features of different brain structures in antipsychotic naive first episode patients. There was a significant decrease in brain volume when there was a decreased presence of RGS4, which suggested that RGS4 may be a major contributor to the anatomical characteristics found in schizophrenia (Prasad et al., 2005). Associations have also been observed between RGS4 gene polymorphisms and schizophrenia in Indian, UK, and European populations (Chowdari et al., 2002; Morris et al., 2003; Williams et al., 2004). Together, the evidence addressed above supports the hypothesis that there is a relationship between the activity of RGS4 and schizophrenia.
Much of the current research has found a strong association of RGS4 and schizophrenia, however there are also contradictory findings that cause disagreement among researchers. A study looking within the Chinese Han population yielded no significant evidence supporting RGS4 as a susceptibility gene for the disorder (Guo et al., 2006). Furthermore, Chowdari and colleagues (2002) found a decreased expression of RGS4 in patients with schizophrenia within an Indian population, however did not find differences in RGS4 expression between affected and non affected groups in their US sample. Due to the contradiction in results between populations, ethnicity should also be considered when looking at genetic polymorphisms associated with a disease. The alterations found between ethnicities are unlikely to be due to chance, and understanding why this occurs requires further investigation.

Elucidation of the role of RGS4 in schizophrenia is warranted, and gene expression profiling should be combined with other analyses in order to determine if RGS4 contributes to the anatomical aberrations found in schizophrenia. Schizophrenia is a genetically complex disorder, and the inconsistencies that arise across studies emphasize the need for more detailed studies focusing on each individual gene to be replicated.

**Genotypic Differences**

**Gender.** Anatomical differences between genders has been of interest to researchers, and many experimental, as well as imaging studies, have been done in order to find morphometric, anatomical, and behavioral differences between men and women. Overall, results have indicated anatomical connectivity patterns, brain size, cognition,
structural and synaptic density are different between males and females (Bocklandt & Vilain 2007; Yan et al. 2011; Rabinowicz, Dean, McDonald-Cumber Petot, & Courten- myers; 1999; Alonso-Nanclares, Gonzales-Soriano, Rodriguez, & De-Felipe; 2008). Rabinowicz et al., (1999) found significantly higher neuronal density and neuronal number in males than females, however the researchers also concluded that females had an increase in neuropril and processes between neurons. Alonso-Nancleres et al. (2008), also found a significant difference in synaptic density with men having a 33% increase in synaptic density in the temporal neocortex. Conversely, other researchers have found an increase in neuronal density in women, which contradicts the general findings (Witelson, Glezer & Kigar; 1995). It is also understood that men have higher grey matter density, but in many regions women have higher grey matter volume, thus could be the reason for the recorded lower density found inn women (Luders, Gaser, Narr, & Toga; 2009). Despite controversy in findings, there is an overall agreement that there are neurobiological differences between men and women, and this should be taken into consideration when trying to understand the pathology of any neurodevelopmental disorder.

COMT has been specifically identified as closely interacting with estrogen, and gene transcription of COMT has been found to be inhibited by estrogen (Xie, Ho, & Ramsden, 1999). There have been other studies further implicating a strong influence of estrogen on COMT ranging from fMRI and volumetric studies, and overall researchers
hypothesize that there are alterations associated with the COMT gene between males and females (Kates et al., 2006; Kempton et al., 2009). Interestingly, RGS4 has not been found to be alternatively expressed between genders, which indicates that some genes may be more hormones dependent than others. Hormone specific affects on gene expression or anatomical features should be considered in postmortem analysis, and these gender differences may shed light on epigenetic features of the disease.

**Ethnicity.** Studies observing differences between ethnicities are few as well as inconsistent, therefore it is difficult to come to any conclusions concerning neuroanatomical or genotypic differences between ethnicities. When McLeod, Fang, Luo, Scott & Evans (1994), compared COMT activity in 195 African Americans to 202 white individuals, they found that the black population had COMT activity that was significantly higher than the white population (P < .001). However, Malhotra et al., (2002) compared met/val and val/val groups in their cognitive performance, and did not find significant differences within the ethnicity subgroup (F=0.03, df=2, 70, p=0.97), or differences between sex ($\chi^2=4.67$, df=2, p=0.10). Also contradictions in literature looking at RGS4 polymorphisms has been accounted to ethnicity, and ethnic background should be considered to be a confounding factor in genetic analysis (Chowdari et al., 2002; Guo et al., 2006). Further research concerning other anatomical differences such as gray matter volume, neuronal density, synaptic density and patterns, have not been studied in depth, therefore a large amount of questions remain to be answered, and future
postmortem research should include ethnicity as a post-hoc sub group for testing.

**Smoking.** Smoking can cause a number of alterations in the physical health of an individual, and it is known that patients with schizophrenia tend to smoke at much higher rates than the general population (Freedman et al., 2008). It has also been found that 80% of individuals with schizophrenia smoke, and is currently determined as a form of self-medication that affects the cholinergic pathway (Freedman et al., 2008). Due to the large amount of schizophrenia patients who smoke, it is extremely difficult to exclude smokers from cohorts. Therefore, including individuals who smoke may be contributing to the contradictory results, as well as causing the decreased gene expression or lower neuronal densities that have been associated with schizophrenia in past research.

Lipska and colleagues (2006), performed an in depth analysis looking at pre and postmortem factors that may cause differing results among studies for neuropsychiatric disease. The researchers found that RNA quality, specifically in the white matter was negatively affected by smoking (Lipska et al., 2006). Smoking can cause many pathological changes such as RNA damage, changes in transcription factors, as well as negatively affecting general physical health (Martinet et al., 2004). Smoking as well as substance abuse can cause changes in the neurochemical baseline, which also can impact gene expression (Martinet et al., 2004).

Smoking has been found to cause damage to white matter, cell damage, and may expedite deterioration of RNA and mRNA. This damage is likely caused by low blood
oxygen levels, cerebrovascular disease, and heart disease, all of which are physical symptoms of long term smoking (Lipska et al., 2006). Lipska and colleagues (2006) also found that even when testing non-diseased individuals, the smoking groups still had significantly lower pH levels, indicating lower RNA quality. The researchers concluded that it is likely that smoking may be the reason for an overall decrease in RNA quality and mRNA expression (Lipska et al., 2006). The huge influence that smoking has on brain tissue, and the prevalence of schizophrenic smokers, may be a reason why some researchers generally find lower pH, lower neuronal density, decreased gene expression, and increased tissue acidity in schizophrenia (Sipos et al., 2005).

Different genotypes and external variables such as smoking and drug abuse, should be included when studying the etiology of a disease state. Gene expression, including upstream factors that moderate transduction, may be different from one person to the next, which may ultimately be the cause of inconsistent findings in the literature. There are many different possible gene x gene and gene x environment interactions that could be the cause of schizophrenia, and Figure 6 summarizes just a few of these interdependent mechanisms that could cause the schizophrenia phenotype. This emphasizes the importance of subgroup testing in postmortem studies. Therefore the current study specifically investigated schizophrenia between and within genotypic sub groups such as Gender, Ethnicity, and history of smoking.
Figure 6. *Gene x environment influences on phenotype*. An overview of the possible pathophysiological pathways that cause the phenotype of schizophrenia. 

Data Analysis

Microarray assays and ISH can be used to examine the extent of gene expression and aide in identification of genes involved in schizophrenia using different methods of statistical analysis (Vawter et al., 2002). In a study performed by Mirnics and colleagues (2000), microarray and Fluorescence in situ hybridization (FISH) analysis were necessary in order to characterize the molecular mechanisms of schizophrenia. The study compared affected and non-affected subject tissue, while controlling for demographics such as age, race, sex and PMI (Mirnics et al., 2000). They retrieved data by performing their own microarray assay and FISH, and compared results to the mean and distribution of other genes. The researchers were able to quantify the extent of gene expression from microarray analysis by performing a Chi-square and t-test. The analysis for between groups of ISH was statistically analyzed using analysis of variance (ANCOVA), with pH and tissue storage time as covariates, and the diagnosis as the dependent variable (Mirnics et al., 2000). The researchers also used descriptives, Pearson’s correlation, and factor analysis to find quantifiable differences between groups. The researchers concluded that the schizophrenic subjects had decreased activity in specific proteins responsible for presynaptic activity. This methodology was used as a framework for the current investigation to determine neuroanatomical differences in schizophrenia by examining protein activity between a control and schizophrenia tissue group.
Researchers also performed Loewess normalization in order to normalize signal intensities from an entire array (Ivanov, Dvoriantchikovaa, Nathansone, McKinnond, & Shestopalova, 2006). To compare gene expression levels they divided ratios of genes expressed between two groups. Furthermore, Vawtera and colleagues (2002) performed a microarray assay looking for relative gene expression in the DLPFC from three groups of patients with schizophrenia (N=15) and three matched control groups (N=15). The researchers found a consistent decrease in expression of different genes by comparing z-ratio differences between groups (Vawtera et al., 2002).

Histological analysis is also extremely important for understanding anatomical characteristics specific to a disorder. Images of postmortem tissue slices can be quantified by cell counting methods, and by observing anatomical differences. Ivanov and colleagues (2006) quantified oligodendrogial density in the PFC of patients with schizophrenia via cell counting and histological processing techniques. Cell counting was performed using image analysis software (Image Expret, v.100, FEI), and density was calculated using the formula: \( Nv = Q/v \text{ (dis)} \). The researchers used STATISTICA software package, and post hoc Duncan’s test (\( a<0.05 \)) to estimate number of cells per slice. They also used ANCOVA and multiple regression analysis to see the effect of age, PMI, and fixation time (Ivanov et al., 2006). Ivanov and colleagues (2006) observed genetic activity as a marker for specific neuronal cells. The researchers found an increased activity of genes associated with the retinal ganglion cell pathway. Observing gene expression can be used to reveal neuronal changes and can even reveal a
neuropathy, therefore the current study uses similar techniques to identify differences
between schizophrenia and control groups.

**Summary**

Schizophrenia is a debilitating brain disorder that affects 1% of the world population, it is highly heritable however little is understood about the disease (McEvoy, 2007). The gene x environment interactions are highly complex, and due to heterogeneity, the specific cause of this disorder is still unknown (Bray et al., 2003). Many projects and institutes such as the Allen Institute for Brain Science have made it their goal to facilitate researchers with high quality data in order to accelerate the understanding of complex brain disorders. The Allen Brain Atlas provides data collected from techniques such as microarray, CISH, and Nissl staining from postmortem tissue, to provide detailed genetic and histological information necessary for gaining insight on schizophrenia.

Large GWAS studies have also become a large contributor to understanding schizophrenia by identifying susceptibility genes that may indicate risk for developing the disorder. However, schizophrenia is a result of multiple susceptibility genes in combination with other epigenetic and environmental factors, which leaves many questions unanswered (Karayiorgou et al., 2010). Regardless of complexity, it is known that genetic variations are responsible for symptoms such as cognitive, anatomical, and histological abnormalities in schizophrenia (Barch, 2005; Beck & Rector, 2005; Durstewitz & Seamans, 2008).
The dopaminergic pathway is a major area of interest for researchers and has been a leading hypothesis that dysfunction in dopamine neurotransmission is a key contributor to schizophrenia (Carlsson, 1988; Howes & Kapur, 2009; Kessler et al., 2009). The genes COMT and RGS4 play a key role in the modulation of activity of the dopaminergic signaling pathways, and have also been repeatedly identified as candidate risk genes for schizophrenia (Mirmics, Middleton, Stanwood, Lewis, & Levitt, 2001; Okochi et al., 2009; Talkowski et al., 2006; Taymans et al., 2004; Ripke et al., 2011; Yue et al., 2011). Alterations or presence of polymorphisms in COMT and RGS4 have also been found to be associated with dysfunction in neuronal organization, decreased neuronal density, altered connectivity, and impaired cognitive performance (Bray et al., 2003; Mukai et al., 2008; Sigurdsson et al., 2010). Past research indicates that there is an association between genes that regulate the dopaminergic signaling pathway and the pathology of schizophrenia, and research focusing on these mechanisms is vital.

Many of the abnormalities that are believed to occur due to genetic aberrations are within the PFC. The PFC is responsible for executive functioning, which is significantly impaired in patients with schizophrenia. Many studies using rCBF, fMRI and PET scans have shown that there is an observable dysfunction in activity and connectivity within the PFC, along with a lowered performance in executive thinking (Calicott et al., 2000; Hashimoto et al., 2003; Hazlett et al., 2008; Zhou et al., 2007). Due to consistent findings that the PFC is affected in patients with schizophrenia, the current study performed a detailed analysis on genetic contributions and histological abnormalities within the
DLPFC of schizophrenia postmortem tissue. The current study also performed analysis within and between subgroups of gender, ethnicity, and smoking in order to account for confounding factors that may be a contributor to the contradictory findings in the postmortem research.

Past research identifying genes that indicate risk for schizophrenia has been ambiguous, and there are many contributors that may cause psychiatric disease. Major focus has been on the dopaminergic pathway and the PFC, therefore the current study investigated specific candidate genes associated with the dopaminergic signaling pathway within the DLPFC in order to locate and validate areas of importance. The current study also performed analysis within and between subgroups of gender, ethnicity, and smoking in order to account for confounding factors that may be a contributor to the contradictory findings in postmortem research.
CHAPTER III

STATEMENT OF PROBLEM

Candidate genes that indicate a high risk for schizophrenia have been identified and found to be correlated with different anatomical aberrations (Cannon & Keller, 2005; Kleinman et al., 2011). Microarrays, ISH, and Nissl staining techniques have revealed that genetic polymorphisms are associated with abnormal neural development and strongly influence the occurrence of mental illness (Arajarvi et al., 2006; Durstewitz & Seamans, 2008; Yue et al., 2011).

Despite the large amount of evidence pointing to specific candidate genes that may indicate an increased risk for schizophrenia, much of the research has been ambiguous. Many studies have been unable to replicate past findings, and sometimes suggest a different candidate gene. Emphasis is being placed on replication studies, and a tighter control on variables has been implemented in order to minimize confounding factors (Allen et al., 2008; Guo et al., 2006). RGS4 and COMT have continually been found to be risk factor genes for schizophrenia, and both strongly influence the dopaminergic pathway (Min et al., 2011; Strous et al., 2005). Therefore, due to the role of COMT and RGS4, the current study investigated changes in expression of these genes in the DLPFC.

MRI, fMRI, rCBF, and PET scans observing anatomical abnormalities and functional changes have also provided significant evidence that there are identifiable neuroanatomical characteristics in schizophrenic patients (Barch, 2005; Beck & Rector,
2005; Kegeles et al., 2010; Walton et al., 2012; Volpe, Mucci, Quarantelli, Galderisi, & Maj, 2012). Due to the prevalence of past literature indicating an overall association with abnormalities in the PFC and schizophrenia, the current study focused on neuronal density changes within the DLPFC in order to further validate past research (Durstewitz & Seamans, 2008; Howes & Kapur, 2009; Volpe et al., 2012).

Overall, replications of postmortem studies focusing on schizophrenia have been inconsistent, however, techniques and procedures are continually being refined in order to minimize the affects of confounding variables. With a more conservative regulation of pH, PMI, and fixation methods, research findings may become more consistent. In addition, gene interaction and the histopathology of schizophrenia are extremely complex and need to be examined at the most basic level for the advancement of understanding this disorder. Therefore, the current study used the Allen Brain Atlas to analyze the histological and genetic characteristics of schizophrenia, and specifically focused on genes associated with dopaminergic pathways within the DLPFC, between schizophrenic and healthy control groups and within different subgroups of schizophrenics.
Hypotheses

This study tests the hypotheses that neuronal density is decreased in schizophrenia, and that expression of two genes related to dopaminergic neurotransmission, RGS4 and COMT, will be decreased in schizophrenia. Primary hypotheses below state expected differences between the entire group of schizophrenics and control brains. Secondary hypotheses state expected differences among subgroups within the schizophrenic group.

Primary Hypotheses

Hypothesis 1a. When Nissl stained images are compared between the control group and schizophrenia group, there will be an overall decrease of neuronal density within the DLPFC of the schizophrenia cohort.

Rationale for Hypothesis 1a: Neuronal organization and lowered neuronal density in the PFC have been continually linked to schizophrenia in previous studies (Harrison & Weinberger, 2005). Therefore, it is expected that there will be a significant difference in neuronal density within the DLPFC of the schizophrenic group in the current study.

Hypothesis 1b. When compared to the control group, there will be an overall decrease in expression of COMT in the DLPFC of the schizophrenia group.

Hypothesis 1c. When compared to the control group, there will be an overall decrease in expression of RGS4 in the DLPFC of the schizophrenia group.
Rationale for hypotheses 1b-1c. The dopaminergic system has repeatedly been shown to contribute to the pathology of schizophrenia, and is a leading signaling pathway for investigation. RGS4 and COMT are genes that play a regulatory role in the dopaminergic system, and genome-wide association studies have repeatedly identified these as risk factor genes for schizophrenia (Morris et al., 2003; Nieratschker et al., 2010; Okochi et al., 2009; Williams et al., 2004). Therefore, it is expected that there will be a significant decrease in expression of RGS4 and COMT in the DLPFC of affected tissue when compared to the healthy control group.

**Secondary Hypotheses**

**Hypothesis 2a.** There will be differences in neuronal density between schizophrenic and control brains when analyzed within sex.

**Hypothesis 2b.** There will be differences in COMT expression between schizophrenic and control brains when analyzed within sex.

**Hypothesis 2c.** There will be differences in RGS4 expression between schizophrenic and control brains when examined within sex.

**Hypothesis 2d.** Brains from male and female schizophrenics will differ on neuronal cell density in the DLPFC.

**Hypothesis 2e.** Brains from male and female schizophrenics will differ on COMT expression.

**Hypothesis 2f.** Brains from male and female schizophrenics will differ on RGS4 expression.
Rationale for hypotheses 2a – 2f. Many studies have found higher neuronal density and cell counts in males, as well as finding higher synaptic and gray matter density in specific regions in males (Alonso-Nancleres et al., 2008; Rabinowicz et al., 1999). However, contradictory results have arisen and some researchers have found increase in neuronal density in women, along with higher gray matter volume (Witelson, Glezer & Kigar; 1995). Due to the large amount of inconsistencies in gender studies, this study will examine differences in gene expression and histological differences between sexes.

**Hypothesis 2g.** There will be differences in neuronal density between schizophrenic and control brains when analyzed within each ethnic group.

**Hypothesis 2h.** There will be differences in COMT expression between schizophrenic and control brains when analyzed within each ethnic group.

**Hypothesis 2i.** Brains from African American and White schizophrenics will differ on neuronal cell density in the DLPFC.

**Hypothesis 2j.** Brains from African American and White schizophrenics will differ on COMT expression in the DLPFC.

**Hypothesis 2k.** Brains from African American and White schizophrenics will differ on RGS4 expression in the DLPFC.

Rational for hypotheses 2g – 2k. Ethnicity has also been a concern for confounding or contradictory results found in neuropathological studies, however ethnicity has not been as extensively studied as gender differences. Responsiveness to
antipsychotics has been found to be different between African Americans and white populations, supporting the idea that gene regulation may differ between populations (Fijal et al., 2009). Researchers have also found differences in COMT activity between African American individuals and White individuals, however other anatomical differences have not been studied (Mcleod, Fang, Luo, Scott, & Evans, 1994). Therefore, the current study will look at neuronal density and COMT gene expression between a schizophrenic African American group and schizophrenic White group. The current study will also compare control to schizophrenia groups within the ethnic groups in order to see if controlling for these potential confounds changes the results of the overall comparison between schizophrenic and control brains.

**Hypothesis 2l.** Brains from cigarette smokers and non-smokers will differ on neuronal cell density in the DLPFC.

**Hypothesis 2m.** Brains from cigarette smokers and non-smokers will differ in COMT expression within the schizophrenic group.

**Hypothesis 2n.** Brains from cigarette smokers and non-smoker will differ in RGS4 expression within the schizophrenic group.

Rationale for hypotheses 2l-2n. Cigarette smoking is a variable that has been shown to alter RNA quality, expression of transcription factors and other genes, and changes in neurochemical baseline (Lipska et al., 2006; Martinet et al., 2004). Cigarette smoking has also been associated with cell damage and shown to negatively affect white matter (Lipska et al., 2006). Therefore, the current study will examine neuronal density
and gene activity differences in schizophrenic smoking versus schizophrenic non-smoking individuals.

Summary

Signaling pathways, genotype, and anatomical and physiology differences are all major contributors to schizophrenia, and understanding these mechanisms can lead to new pharmacological treatment (Javitt, 2008; Karam et al., 2010; Kleinman et al., 2011). Much of the past research observing histological and etiological factors associated with mental illness have been inconsistent. Replication and further data analysis on specific genes within a more detailed and narrow range is necessary in order to minimize the conflicting findings from past research. Smoking, gender, and ethnicity may have a huge influence on the RNA quality, neuronal physiology and morphology, and gene expression within the brain. Therefore, subgroups will be made out of the different genotypic groups including ethnicity, gender, and history of smoking, in order to account for any other neurobiological differences that may not be a result of the disorder.
CHAPTER IV

METHODS

**Tissue Samples.** Tissue specimens for the Allen Atlas were provided by the National Institute for Mental Health (NIMH) after obtaining informed consent from legal next-of-kin. Postmortem clinical diagnoses and a psychiatric history were given by board-certified psychiatrists, diagnostics consisted of family interviews, and were based on the Structured Clinical Interview for DSM-IV (SCID-CV). Cause of death, toxicology results, and history of cigarette smoking were obtained by a neuropathologist upon examination of autopsy results. Postmortem interval (PMI) was measured as hours passed between recorded time of death and time of tissue freezing. PMI’s over 36 hrs were excluded unless the pH values indicated that tissue quality was sufficient for planned analyses, and all tissues were required to have a pH>6. Ages under 20, and suicide as cause of death were all excluded from the database. However, positive toxicology results, indicating recent drug or alcohol use, were not excluded from the schizophrenia cohort.
Subjects. Information about control specimen was obtained similarly to the affected tissue, and the subjects met the criteria listed above from a neuropathological examination. Individuals who contributed control brains were confirmed to have no history of mental illness, drug abuse, drug overdose, poisoning, or to have committed suicide. A table summarizing the subject demographics can be seen below (Table 1), and further information on the procedures and criteria for tissue qualifications, tissue fixation, and dissection methods are available at http://www.alleninstitute.org/.
Table 1

*Subject Demographics*

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**Instrumentation.** The images were downloaded directly from the Allen Brain Atlas (Figure 7 & 8) and processed in ImageJ, a publicly available application that allowed for detailed image analysis (rsbweb.nih.gov/ij/). ImageJ is a flexible image processing program, which was used to quantify the brain slice images for statistical comparisons. Specifically, ImageJ was used to count cells, estimate cell densities, and quantify the intensity of fluorescence signals associated with the molecular probes of interest. Output of ImageJ was then manually pasted into an excel spreadsheet for statistical analysis. Two image slices from each brain were analyzed and averaged, with 3 different analyses for each slice: Nissl staining, CISH for the RGS4, and CISH for COMT (Figure 7 & 8). The information extracted from CISH images was the average brightness intensity for each image, and the information extracted from Nissl stained sections was cell density. Background fluorescence intensity was controlled for by ensuring that extracellular fluorescence differed no more than 10% between images. To determine cell density, a threshold of 200 pixels was applied to the image in order to allow ImageJ to identify cells automatically without experimenter bias, and a threshold of 150 was set to analyze average brightness of CISH data. Thresholding assigned all pixels to an intensity value of 0 or 1, based on a preset criterion. Thresholding was necessary to allow ImageJ to detect structural features (e.g., individual cells) within the image. Nissl staining revealed neuronal cell nuclei, and cell density was expressed as the average number of cells per sample.
Figure 7. *In situ hybridization image for RGS4*. The image on the left is a detailed ROI of ISH tissue for RGS4. The image on the right is oriented such that dorsal is on the top and ventral is on the bottom. Adapted from The Allen Human Brain Atlas. Copyright 2012 Allen Institute for Brain Science.
Figure 8. *Nissl stained ROI*. The image on the left is a Nissl stained ROI of from the image shown on the right. The image on the right is oriented such that dorsal is on the top and ventral is on the bottom. Adapted from The Allen Human Brain Atlas. Copyright 2012 Allen Institute for Brain Science.
Data Analysis

Twenty tissue samples were examined, pH levels, RNA integrity and PMI were matched with a pH>6.0, and two brain slices from each tissue specimen were averaged, for a total of 19 samples per group. The expression levels (fluorescence intensity) of RGS4 and COMT, and neuronal densities, were compared between the schizophrenia group (N=19) and control group (N=19) in the DLPFC regions using independent means sample \( t \)-test. Cell density, RGS4, and COMT expression means were compared between the control and schizophrenia group, and all statistical analyses were done using PASW, with significance set at 0.05% for all planned comparisons.

Three subgroups were created for sex, ethnicity, and smoking, and comparisons were made between schizophrenic and controls brains within these groups (see Chapter III). A summarized table of number of subjects within each comparison group can be seen in Table 2, with each subject consisting of two tissue slices that were measured independently, and then averaged to give a single value for each brain.
Table 2

*Comparison Groups*

<table>
<thead>
<tr>
<th>Tissue Group</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizophrenia Total</td>
<td>Male (N=4)</td>
<td>White (N=9)</td>
<td>Smoker (N=3)</td>
</tr>
<tr>
<td>(N=19)</td>
<td>Female (N=4)</td>
<td>African American (N=9)</td>
<td>Non-smoker (N=3)</td>
</tr>
<tr>
<td>Control Total</td>
<td>Male (N=4)</td>
<td>White (N=9)</td>
<td>N/A</td>
</tr>
<tr>
<td>(N=19)</td>
<td>Female (N=4)</td>
<td>African American (N=9)</td>
<td>N/A</td>
</tr>
</tbody>
</table>
CHAPTER V

RESULTS

Neuronal density, COMT and RGS4 expression levels were compared between schizophrenia and controls subjects, and within subgroups composed by sex, ethnicity, and smoking status.

**Neuronal Density.** Analysis of Nissl staining revealed that neuronal density for schizophrenia (M=0.143, SD=0.203) and control tissue (M=0.134, SD=0.178); t(36)=0.153, *p*=0.879 was not significantly different (Table 3).

**CISH.** COMT expression in the schizophrenia group (M=120.2, SD=8.77) was not significantly different than COMT expression in the control group (M=119.2, SD=5.99); t(36)=0.425, *p*=0.673. RGS4 expression also did not differ between the schizophrenia group (M=95.0, SD=8.38) and control group (M=93.4, SD=7.13); t(36)=0.656, *p*=0.516.
Table 3

*Neuronal Density and CISH Intensity*

<table>
<thead>
<tr>
<th></th>
<th>Schizophrenia M(SD)</th>
<th>Control M(SD)</th>
<th>t</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuronal Density</td>
<td>.143 (.203)</td>
<td>.134 (.178)</td>
<td>.15</td>
<td>36</td>
</tr>
<tr>
<td>CISH COMT</td>
<td>120.2 (8.77)</td>
<td>119.2 (5.99)</td>
<td>.43</td>
<td>36</td>
</tr>
<tr>
<td>CISH RGS4</td>
<td>95.0 (8.38)</td>
<td>93.4 (7.13)</td>
<td>.656</td>
<td>36</td>
</tr>
</tbody>
</table>
Post Sub-Group Tests

**Gender.** Neuronal density, COMT and RGS4 expression levels were compared between schizophrenia and controls within female subjects. COMT expression for females within the schizophrenia group (M=118.3, SD=9.14) was not significantly different from females in the control group (M=118.4, SD=2.09); t(6)=0.029, $p=0.979$ (Figure 9). RGS4 expression for the female schizophrenia group (M=93.8, SD=11.52) was not significantly different than RGS4 expression for the female control group (M=93.4, SD=7.41); t(6)=0.055, $p=0.958$. Furthermore, analysis of Nissl images revealed that neuronal density for females in the schizophrenia group (M=0.099, SD=0.088) was not significantly different than in the female control group (M=0.097, SD=0.006); t(6)=0.452, $p=0.670$.

In addition, Neuronal density, COMT and RGS4 expression levels were also analyzed between schizophrenia and controls within male subjects. Expression levels of COMT for males in the schizophrenia group (M=113.9, SD=8.91) was not significantly different from males in the control group (M=118.9, SD=7.45); t(6)=0.852, $p=0.427$ (Figure 10). Additionally, RGS4 expression in the male schizophrenia group (M=96.4, SD=6.30) did not significantly differ from the male control group (M=91.4, SD=6.55); t(6)=1.11, $p=0.308$. Nissl Analysis revealed that there was no significant difference in neuronal density between the male schizophrenia group (M=0.101, SD=0.002) and the male control group (M=0.094, SD=0.005); t(6)=1.89, $p=0.130$. 
Neuronal density, RGS4 and COMT expression levels were then compared between male and female schizophrenia subjects. COMT expression was not significantly different between the schizophrenia male and female group $t(6)=0.721, p=0.498$ (Table 4). RGS4 expression also did not differ between the female schizophrenia group and male schizophrenia group $t(6)=0.403, p=0.703$. In addition, neuronal density in the female schizophrenia group was not significantly different than the male schizophrenia group $t(6)=0.088, p=0.935$. 


Table 4

*COMT and RGS4 Expression*

<table>
<thead>
<tr>
<th></th>
<th>Schizophrenia M(SD)</th>
<th>Control M(SD)</th>
<th>t</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female COMT</td>
<td>118.3 (9.14)</td>
<td>118.4 (2.09)</td>
<td>.03</td>
<td>6</td>
</tr>
<tr>
<td>Female RGS4</td>
<td>93.8 (11.52)</td>
<td>93.4 (7.41)</td>
<td>.06</td>
<td>6</td>
</tr>
<tr>
<td>Male COMT</td>
<td>113.01 (8.91)</td>
<td>118.9 (7.45)</td>
<td>.85</td>
<td>6</td>
</tr>
<tr>
<td>Male RGS4</td>
<td>96.4 (6.31)</td>
<td>91.4 (6.55)</td>
<td>1.11</td>
<td>6</td>
</tr>
<tr>
<td>Female Neuronal Dens</td>
<td>.099 (.088)</td>
<td>.097 (.006)</td>
<td>.45</td>
<td>6</td>
</tr>
<tr>
<td>Male Neuronal Dens</td>
<td>.101 (.002)</td>
<td>.094 (.005)</td>
<td>1.89</td>
<td>6</td>
</tr>
</tbody>
</table>
RGS4 and COMT expression was determined based off of CISH intensity using parameters set for automatic analysis by ImageJ.
**Ethnicity.** To see if there were any differences specific to an ethnic group, neuronal density and COMT expression levels between schizophrenia and controls were compared within African American subjects. There was no significant difference in COMT expression between the schizophrenia African American group (M=122.5, SD=9.67) and the control African American group (M=117.5, SD=6.04); t(16)=1.26, \(p=0.233\) (Table 5). There was also not a significant difference in neuronal density between the schizophrenia African American group (M=0.201, SD=0.302) and the control African American group (M=0.094, SD=0.009); t(16)=1.07, \(p=0.316\).
<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Schizophrenia M(SD)</th>
<th>Control M(SD)</th>
<th>t</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA COMT</td>
<td>122.5 (9.67)</td>
<td>117.5 (6.04)</td>
<td>1.26</td>
<td>16</td>
</tr>
<tr>
<td>White COMT</td>
<td>118.9 (8.58)</td>
<td>122.5 (5.64)</td>
<td>.77</td>
<td>16</td>
</tr>
<tr>
<td>AA Neuronal Density</td>
<td>0.201 (.302)</td>
<td>0.094 (.009)</td>
<td>1.07</td>
<td>16</td>
</tr>
<tr>
<td>White Neuronal Density</td>
<td>0.097 (.007)</td>
<td>0.092 (.006)</td>
<td>1.58</td>
<td>16</td>
</tr>
</tbody>
</table>
Neuronal density and COMT expression levels were then compared between schizophrenia and control subjects within the group of White subjects. COMT expression within White schizophrenia subjects (M=118.9, SD=8.58) did not differ from the White control subjects (M=122.5, SD=5.64); \( t(16)=0.769, p=0.455 \). Neuronal density within the White schizophrenia group (M=0.097, SD=0.007) was also not different than the White control group (M=0.092, SD=0.006); \( t(16)=1.58, p=0.134 \).

There were no significant differences in COMT expression between the African American schizophrenia group and the White schizophrenia groups \( t(16)= 0.769, p=0.455 \). In addition, the neuronal density of the schizophrenia African American group did not significantly differ from the neuronal density of the schizophrenia White group \( t(16)=1.04, p=0.331 \).
Figure 10. Neuronal Density. Neuronal Density was calculated by total number of cells over the entire area. The neuronal densities were then averaged between the two slices for each brain sample.
Smokers versus Non-Smokers. COMT expression among schizophrenic smokers (M=118.9, SD=5.86) and schizophrenia non-smokers (M=119.9, SD=5.44) was not significantly different $t(4)=0.230, p=0.829$ (Table 6). It was also found that RGS4 expression within the schizophrenia smoking group (M=100.4, SD=5.71) did not significantly differ from RGS4 expression in the non-smoking group (M=99.7, SD=6.82); $t(4)=0.134, p=0.900$. Furthermore, neuronal density among schizophrenic smokers (M=0.095, SD=0.007) was not significantly different than the schizophrenic nonsmoking group (M=0.100, SD=0.008); $t(4)=0.812, p=0.463$.

Overall there were no significant differences between schizophrenia and control subjects, even after comparing subgroups. Differences were also not found within schizophrenia subjects within variables such as sex, ethnicity and smoking. In addition, in order to confirm that Image J parameters were not set too low, analyses were re-run using an increased threshold. This was done in order to make sure the parameter limitations did not prevent observation of differences in gene expression or cell counts. Again there were no significant differences found, therefore the original determined threshold was maintained for analysis.
Table 6

*Smokers versus Non-Smokers*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Smoking M(SD)</th>
<th>Non Smoking M(SD)</th>
<th>$t$</th>
<th>$df$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schizophrenia COMT</td>
<td>118.9 (5.86)</td>
<td>119.9 (5.44)</td>
<td>.23</td>
<td>4</td>
</tr>
<tr>
<td>Schizophrenia RGS4</td>
<td>110.4 (5.71)</td>
<td>99.7 (6.82)</td>
<td>.13</td>
<td>4</td>
</tr>
<tr>
<td>Schizophrenia Neuronal Density</td>
<td>0.095 (.007)</td>
<td>0.102 (.008)</td>
<td>.81</td>
<td>4</td>
</tr>
</tbody>
</table>
CHAPTER VI

DISCUSSION

The current findings do not support the hypotheses regarding associations between alterations in COMT and RGS4 expression in the DLPFC of patients with schizophrenia. The findings of the current study also suggest that lowered neuronal density in the DLPFC is not particular to schizophrenia. In order to test for confounds such as race, ethnicity, or smoking; subgroups were individually compared, but differences in gene expression and neuronal density remained unchanged in these subsample comparison groups. Explanations for the lack of significance in this study can be due to a myriad of different variables, including antemortem factors, postmortem factors, an interaction between different etiological factors, along with a copious amount of uncontrolled confounds that alter phenotype in an individuals’ lifetime. Quantification of images was straightforward, and the protocol was the same between all tissue slices, therefore skewed or flawed results due to analysis error are minimal. This section will discuss many variables that may be the reason why the current study failed to find significance.
Postmortem Factors

Results from postmortem research for psychiatric disease have been ambiguous, and despite concerted efforts to maintain specific protocols or methodology, findings continue to come up contradictory. Many factors can contribute to these inconsistencies, however due to the highly controlled and well maintained scientific method implemented by the AIBS, postmortem factors, such as extraction method, RNA integrity, and PMI, are not considered confounds that influenced the results of the current study.

Epigenetic Factors

It is now understood that there are many gene x gene and gene x environment interactions that could lead to the complex etiology of schizophrenia. Many factors introduced during a human life can alter DNA methylation and protein regulation, which ultimately determine gene expression and phenotype (Figure 11) (Eccleston, DeWitt, Gunter, & Nath, 2007; Oh, & Petronis., 2008). Postmortem research alone does not allow for the control or visualization of gene x environment interactions, therefore, postmortem studies have been limited in their ability to make patterned observations. Epigenetic factors may ultimately be the cause for the high variability and heterogeneity between individuals, and are the reason researchers are unable to identify a single biomarker for schizophrenia.
**Medication.** The current study did not find a strong uniform pattern in gene expression due to confounding factors such as medication, substance misuse, length of disease state, or smoking (Dixon, Haas, Weiden, Sweeney, & Frances, 1991; Harrison & Weinberger 2005; Keshavan, Tandon, Boutros, & Nasrallah, 2008; McCullumsmith & Meador-Woodruff, 2011). Medication effects are particularly considered to be a confound in detecting physiological markers in schizophrenia. It is likely that long term medication affects molecular activity within the brain, influencing many neurophysiological functions and even causing epigenetic alterations (Hakak et al., 2001; Kleinman et al., 2011). Some studies have shown they’re to be alterations in genetic expression after psychotropic medication, and long term drug treatment has been shown to alter neuroplasticity of the brain (Haka et al., 2001; Mccullimsmith & Meador-Woodruff, 2011; Talkowski et al., 2006; Tkachev et al., 2003).

Medication effects are a huge hurdle for postmortem studies that focus on schizophrenia, and due to a high percentage of patients having been exposed to drugs and medication throughout much of their lifetime, drug effects may have caused identification of false biomarkers for the disease. The medication status of the tissue provided by AIBS is left unknown, and researchers are unable to make any correlations between gene expression and medication due to unavailable resources. When RGS4 expression was compared between schizophrenia patients treated with antipsychotics and not treated with antipsychotics at least three months prior to death, a study reported a significant decrease
in RGS4 mRNA in the caudate nucleus of the medicated individuals (Erdely, Tamminga, Roberts, & Vogel., 2006). Researchers have also gone further and found that after exposing rats to chronic antipsychotic treatment, COMT mRNA levels were significantly increased (Chen & Chen., 2007). It is likely that patient tissue used in our analysis was exposed to antipsychotics, and alterations in mRNA levels and gene expression may be differentially regulated between treated and non-treated individuals. Therefore, a conclusive decision cannot be reached due to the possibility that medication may have highly confounded the data, and It is warranted that AIBS allow visualization of medical history in order to account for drug effects.

Length of disease state, increased cortisol, hormone alterations, or smoking can also alter DNA methylation, transcriptional regulation, and enzymatic activity, all of which can affect many downstream cellular mechanisms (Eccleston et al., 2007; Keshavan et al., 2008; Oh & Petronis, 2008). The Allen Institute for Brain Sciences did not document or list the length or history of medication, and was unable to control for many of these other potentially confounding variables. Therefore, any of these factors might have influenced gene expression of the tissue under investigation, yielding inconclusive evidence. The lack of accounting for medication history or any of these other factors may be the major cause of variability between findings, and after review, future research concerning schizophrenia should consider medication and clinical history as a confounding factor. Furthermore, concerted efforts between researchers should be
made in order to observe combinations of many different epigenetic factors to find conclusive identify biomarkers of schizophrenia.
Figure 11. *A schematic overview of Epigenetic Mechanisms.* Many factors are now being considered to impact gene expression and the occurrence of disease phenotypes, including pharmaceutical drugs. Adapted from “Study Probes Environment-Triggered Genetic Changes in Schizophrenia,” by NIMH, 2008, Science Update. Copyright 2012 National Institute of Mental Health.
Gender. Generally, postmortem studies are very limited due to low tissue availability, therefore many studies are unable to match between groups or exclude comorbidity factors. This study attempted to compare schizophrenia to controls within and between male and female groups. The ISH analysis revealed there were no significant differences for schizophrenia and controls, even when broken down by sex. Even though neurophysiological differences between sexes have been reported in schizophrenia, this study did not find any sex differences in the biological measures analyzed (Table 3) (Gur et al., 1994). One reason for a lack of finding a physiological patterns with gender may be due to a low sample size of only four brain specimen for each affected gender group (Table 1).

COMT has been specifically identified as closely interacting with estrogen, and gene transcription of COMT has been found to be inhibited by estrogen. Estrogen is expected to be different between males and females, which lead to the expectation of a detectable sex differences in the current analysis on COMT expression, however no such differences were found (Table 2) (Xie, Ho, & Ramsden, 1999). There has been evidence supporting differing COMT expression between genders, however not with RGS4, which also implies that some genes may be more hormone dependent than others.

Due to the contradictions between past literature and the current study, future research should particularly examine sex differences with larger sample sizes. The
interaction between genes and hormones may be stronger than expected, and research investigating the direct interaction between schizophrenia-related genes and hormones is warranted.

Ethnicity. The current findings did not yield any significant differences when comparing brains of African American and White schizophrenic individuals, or comparing brains of African American and White individuals across disease state (Table 4). Differences in RGS4 haplotypes, a combination of alleles, have been found between different populations. A few studies have shown there to be differences in RGS4 genotype in an African American population, which may be what alters their responsiveness to antipsychotic drugs (Mirnics, 2008). Results vary in the literature, and postmortem research on RGS4 expression that includes gender and ethnicity differences are minimal (Chowdari et al., 2002). A reason for the lack of significant findings in the current study, might be that the genotypic or physiological differences expected to be found between these two ethnicities was not specific to RGS4 and COMT mRNA levels. Also the sample size, with each ethnic group consisting of nine tissue samples, might also have been too low to notice a pattern. The findings of the current study in combination with past literature, argues for future research to examine differences schizophrenia
**Subtypes of Schizophrenia.** The differences between one schizophrenic individual to another has also been widely documented, presenting many difficulties for researchers, and the DSM also has had difficulty determining a uniform diagnosis for the disease (Keshavan et al., 2008). Interactions between different gene or environmental factors may also lead to the observable sub-phenotypes of schizophrenia (Figure 12). Some studies find differences in anatomical features related to the severity of symptoms and subtype of schizophrenia, suggesting that when looking for disease markers it may be best to analyze patients within etiological or pathophysiological subgroups (Gur et al., 1994). The schizophrenia disease phenotype is so variable that is has been difficult to specify a pattern in biomarkers (Harrison, 1999; Keshavan et al., 2008). Phenotypic, biological, and etiological factors all may cause the differences found between subtypes of schizophrenia, therefore it is possible that this study did not find any patterns because of the inability to identify and study subgroups of schizophrenia (Keshavan et al., 2008).
Figure 12. Complex etiology and pathophysiology contributing to schizophrenia subtypes. Adapted from “Schizophrenia, “Just the Facts” What we know in 2008. 2. Epidemiology and etiology” by Adapted from “Schizophrenia, Just the Facts: what we know in 2008 part 1,” by R. Tandon, M.S. Keshavan, and H.A. Nasrallah, 2008, Schizophrenia Research, 1, p. 5. Copyright 2012 Elsevier B.V.
Pathophysiology

**Brain Region.** In addition up to 50% of alterations in schizophrenia are non-specific and within white matter, which is mostly myelinated axons and glial cells, therefore this study would have been unable to pick up on differences from only observing the general neuronal density (Keshevan et al., 2008). One reason for not finding differences in neuronal density in the current study, may be that schizophrenia is not a disease of apoptosis. Many studies have found there to be observable anatomical traits associated with the disease such as decreased white matter in the DLPFC, which is made up of glial cells and myelinated axons, both of which are not visible the Nissl staining (Glatnz & Lewis, 2000; Kalus, Muller, Zuschratter, & Senitz, 2000; Sweet, Henteleff, Zhang, Sampson, & Lewis, 2009).

**Histology.** Much of the literature suggests that schizophrenia is a disease of the axons or dendrites, and cognitive phenotypes may be due to altered connectivity or decreased neuritic growth, and not a result of apoptosis. Axon labeling, neurite organization, dendritic spine density or length, are all important pathophysiological markers believed to be altered in schizophrenia. Analysis of Nissl stained images limited the current study to only looking at cell bodies and general neuronal density in the DLPFC, and we were unable to look at more specific biomarker types or other brain areas, which may be associated with the disease. Furthermore, decreased or altered organization of pyramidal neurons, along with altered synapse morphology, has been
associated with schizophrenia (Lewis, Fish, Arion, Gonzalez-Burgos, 2011). It is possible that only certain types of neuronal cells may be altered in schizophrenia, and that these neurons were undetected in the current analysis. Also, reasoning behind our the lack of significant findings, may be that differences in RGS4 or COMT might not be specifically within the DLPFC. Other studies have found differences in RGS4 expression in the cingulated gyrus, superior frontal gyrus and insular cortex, thus indicating that gene expression might be altered in more specific subcortical areas of the brain, rather than just specific to the DLPFC (Erdely et al., 2008). Finally, it is known that tissue atrophy can cause cell density analyses to be inaccurate, specifically, density may increase due shrinkage of surface area. It is possible that this effect may alter neuronal density calculations in schizophrenia postmortem research (Harrison & Weinberger, 2005).

**Conclusion**

Oleh Hornykiewicz’s (1960) was the first to use postmortem methodology for clinical research where he found decreased dopamine in patients with Parkinson’s disease. This method of using postmortem tissue changed the way neurological disease is studied, and after 50 years, research still follows the traditional study design of comparing diseased state versus a non-affected group (Ehringer & Hornykiewiz, 1960; Kleinman et al., 2011). Despite the information postmortem research provided for Parkinson’s disease, the use of postmortem tissue for psychiatric disease has been convoluted and contradictory. Due to the difficulty in matching between groups, low
sample sizes, the inability to control for epigenetic and environmental confounding factors, postmortem research provides little information on psychiatric disease (Kleinman et al., 2011). In addition, increasing the number might allow the visualization of patterns, however effect size may decrease in this process, a problem that is possible in the large GWAS studies. Therefore, before we can make any conclusions about neuropsychiatric disease, a method of investigation that either eliminates or controls for confounding factors should be implemented.

The current study did not find any differences between schizophrenia and control tissue, which can be due to confounding variables that blur the ability to see any patterned alterations in neuronal density, RGS4 and COMT expression. It is also possible that the differences in RGS4 and COMT expression along with neuronal density do not exist, however this cannot be determined until confounds are better accounted for. The current study was limited by the type of data made available by the Allen Institute, and enzymatic activity, transcription, and downstream effectors, all of which are important to look at when studying pathology of a disease state, were unavailable for analysis. It is also highly likely that many of the pathological aberrations found in schizophrenia are from long-term medication effects, substance abuse, and length of disease, all of which were not available for the current study. Due to the lack of medical history, we cannot make any conclusive statements on our findings.
Past literature emphasizes the strong possibility that genetic factors alone do not account for the psychiatric disease, and interactions between genes and environmental factors are what predisposes an individual to schizophrenia (Eccleston et al., 2007; Keshavan et al., 2008). Characterization of biomarkers associated with schizophrenia is greatly needed in order to help in diagnosis and treatment, and concerted efforts between researchers should investigate all possible genetic and epigenetic interactions in order to better understand schizophrenia. Postmortem factors were controlled at the highest quality by Allen Institute for Brain Sciences, however, there remain other factors that can strongly influence the pathophysiology of the disease that were not documented or controlled. RGS4 and COMT might be insufficient or nonspecific markers, and modifier genes or environmental influences combined with other factors may cause the complex heterogeneous phenotype of schizophrenia. The results of this study must be determined inconclusive due to limitations. Furthermore, the Allen Institute for Brain Sciences database is not sufficiently developed to conduct rigorous tests of pathophysiological theories of schizophrenia. Therefore, future studies of this type should be performed using this promising public research tool once the Institute’s database is expanded with greater numbers of brain samples and more information about each sample is provided.
CHAPTER VII

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