PHARMACOGENETIC ANALYSIS OF GLUTAMATE SYSTEM GENE VARIANTS AND CLINICAL RESPONSE TO CLOZAPINE

by

Danielle L. Taylor

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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and Clinical Response to Clozapine

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ABSTRACT

Altered glutamatergic neurotransmission is implicated in schizophrenia etiology and response to the atypical antipsychotic clozapine. Response to clozapine is highly variable and partly depends on genetic factors as indicated by twin studies. The association of 17 glutamate system gene variants and clinical response to clozapine was investigated in a sample of European schizophrenia/schizoaffective patients deemed resistant/intolerant to previous pharmacotherapy. Categorical and continuous response measures were assessed following six months of clozapine treatment using change in Brief Psychiatric Rating Scale Scores, and were analyzed using Pearson’s chi-squared test or analysis of covariance, respectively. No significant associations were observed for all analyses after correction for multiple testing. Prior to correction, several nominally significant associations were observed, the most notable being between the SLC6A9 rs16831558 variant and change in positive symptoms ($p_{uncorrected}=0.008$, $p_{corrected}=0.08$). This finding warrants further investigation in larger well-characterized samples to clarify the role of SLC6A9 in clinical response to clozapine.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis supervisor Dr. James Kennedy for providing me with the opportunity to undertake a Master’s degree in his lab. His continued support, guidance and witty sense of humor have made these last few years in graduate school an altogether enriching experience. I would also like to thank the members of my Program Advisory Committee, Dr. Joanne Knight, Dr. Daniel Müller and Dr. Gary Remington for their patience and constructive feedback. I extend many thanks to my fellow colleagues in the Neurogenetics Department for providing guidance on protocols and statistical analyses, and for helping to troubleshoot obstacles that popped up along the way. To the Pharmacogenetics crew, thank you all for the many laughs, good food and good times. Much recognition goes out to my friends on the volleyball team for providing a carefree environment where I could forget about the worries of the work week. Thank you to my family for continuing to support me on my long scholarly journey. Finally, I would like to acknowledge the lab technicians, trainees, collaborators, faculty mentors and funding sources for their contribution to this project.
CONTRIBUTIONS

Dr. James Kennedy, Dr. Daniel Müller, Dr. Joanne Knight and Dr. Gary Remington helped design this thesis project. Patient blood samples were provided by Dr. Herbert Meltzer, Dr. Jeffrey Lieberman and Dr. Steven Potkin. Dr. Arun Tiwari provided guidance regarding statistical analyses of genotype data and Dr. Vanessa Gonçalves provided guidance regarding use of the Applied Biosystems OpenArray® genotyping platform. A very special thank you is extended to the patients for their willingness to participate.
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<td>Δ</td>
<td>change</td>
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<td>α</td>
<td>alpha; probability associated with a type I error, statistical significance criteria</td>
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<td>antipsychotic-induced weight gain</td>
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<td>BDNF</td>
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<td>Bill S-201</td>
<td><em>Genetic Non-discrimination Act</em> (Canada)</td>
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<td>BNEG</td>
<td>Brief Psychiatric Rating Scale negative symptom subscale</td>
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<td>bipolar disorder</td>
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<td>Ca²⁺</td>
<td>calcium ion</td>
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<td>Centre for Addiction and Mental Health</td>
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<td>CaMKII</td>
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<td>cAMP</td>
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<td>CATIE</td>
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<td>CDN</td>
<td>Canadian dollars</td>
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<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
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<td>CGI</td>
<td>Clinical Global Impressions Scale</td>
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<tr>
<td>ChIP-seq</td>
<td>chromatin immunoprecipitation sequencing</td>
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<td>CHOR</td>
<td>Clozapine, Haloperidol, Olanzapine, Risperidone Study</td>
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CHRM1  cholinergic receptor, muscarinic 1
chrN   chromosome number
CI     confidence interval
CIA    clozapine-induced agranulocytosis
CI-OCD clozapine-induced obsessive compulsive disorder
CLZ    clozapine
CNS    central nervous system
Cons   evolutionarily conserved variant
COS    childhood onset schizophrenia
CPZ    chlorpromazine
CREB   cyclic AMP response element-binding protein
CRESTAR clozapine pharmacogenetics study
CSF    cerebrospinal fluid
CVD    cardiovascular disease
CYP1A2  cytochrome-P450 isoenzyme family 1, subfamily A, polypeptide 2 gene
CYP2D6  cytochrome-P450 isoenzyme family 2, subfamily D, polypeptide 6 gene
D’     Normalized linkage disequilibrium constant between two loci
DAAO   d-amino acid oxidase gene
DAAO   d-amino acid oxidase enzyme; DAO
DAOA   d-amino acid oxidase activator gene
DAOA   d-amino acid oxidase activator protein; G72
dbSNP  National Centre for Biotechnology Information short genetic information database
DISC1  disrupted in schizophrenia 1 gene
DLPFC  dorsolateral prefrontal cortex
DNA  deoxyribonucleic acid
DNAse-seq  DNAse I hypersensitivity site sequencing
DRD1  dopamine D1 receptor gene
DRD2/D2  dopamine D2 receptor
DRD2  dopamine D2 receptor gene
DRD3  dopamine D3 receptor gene
D-ser  D-serine
DSM-III-R  Diagnostic and Statistical Manual, 3rd Edition Revised
DSM-IV  Diagnostic and Statistical Manual, 4th Edition
DSM-5  Diagnostic and Statistical Manual, 5th Edition
DTNBPI  dystrobrevin binding protein 1 (dysbindin) gene
DTNBPI  dystrobrevin binding protein 1
DZ  dizygotic
EAAT1  excitatory amino acid transporter 1
EAAT2/GLT1  excitatory amino acid transporter 2; glutamate transporter 1
EHM  enhancer associated histone mark
emPCR  emulsion polymerase chain reaction
ENCODE  Encyclopedia of DNA Elements Project
EPS  extrapyramidal symptoms
EPSCs  excitatory postsynaptic currents
EPSPs  excitatory postsynaptic potentials
eQTL  expression quantitative trait loci
ESE/ESS  exonic splicing enhancer/exonic splicing silencer
EUR  European sample
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<th>Term</th>
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<td>FuncPred</td>
<td>National Institute for Environmental Health Sciences SNP Functional Prediction database</td>
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<td>FWER</td>
<td>family-wise error rate</td>
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<td>G</td>
<td>guanine nucleotide base</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GAD1</td>
<td>glutamate decarboxylase 1 gene</td>
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<td>GAD1</td>
<td>glutamate decarboxylase 1 protein</td>
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<td>GAIN</td>
<td>Genetics Association Information Network</td>
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<td>GATA1</td>
<td>GATA-binding factor 1; globin transcription factor 1; erythroid transcription factor 1</td>
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<td>GATA-binding factor 4; transcription factor GATA-4</td>
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<td>GCS</td>
<td>glycine cleavage system</td>
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<td>GDH</td>
<td>glutamate dehydrogenase</td>
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<td>GERP</td>
<td>genomic evolutionary rate profiling</td>
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<td>GINA</td>
<td><em>Genetic Information Non-discrimination Act</em> (US)</td>
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<td>Gln</td>
<td>glutamine</td>
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<td>GLS</td>
<td>glutaminase; L-glutamine aminohydrolase</td>
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<td>Glu</td>
<td>glutamate</td>
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<td>GLUR1</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor subunit 1 protein; GluA1</td>
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<td>Gly</td>
<td>glycine</td>
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<td>GlyT1</td>
<td>glycine transporter 1</td>
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<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor subunit 1 gene</td>
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<td>GRIN1</td>
<td>N-methyl-D-aspartate receptor subunit 1 gene</td>
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<tr>
<td>GRIN2A</td>
<td>N-methyl-D-aspartate receptor subunit 2A gene</td>
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GRIN2B  N-methyl-D-aspartate receptor subunit 2B gene
GRM2   metabotropic glutamate receptor 2 gene
GRM3   metabotropic glutamate receptor 3 gene
GS     glutamine synthetase
GWAS   genome wide association study
H₂O₂   hydrogen peroxide
Haploreg Broad Institute Genomic Functional Annotation database
HRH1   histamine receptor H1 protein
HRH2   histamine receptor H2 gene
HTR1A  5-hydroxytryptamine (serotonin) receptor type 1A gene
HTR2A  5-hydroxytryptamine (serotonin) receptor type 2A gene
HTR2C  5-hydroxytryptamine (serotonin) receptor type 2C gene
HWE    Hardy-Weinberg equilibrium
IP₃    inositol 1,4,5-triphosphate
k      independent number of tests conducted in a statistical analyses
K⁺     potassium ion
kb     nucleotide kilobases
kDa    kilodalton
kg     kilogram
LD     linkage disequilibrium
LGC    Laboratory of the Government Chemist
LOD    log of the likelihood odds ratio; confidence in D’
L-ser  L-serine
LTP    long term potentiation
LY2140023 pomaglumetad methionil; metabotropic glutamate receptor 2/3 agonist
MAF minor allele frequency
MAPK mitogen activated protein kinase
MC motif change
MC4R melanocortin receptor 4 gene
MDD major depressive disorder
MDR multifactor dimensionality reduction
METH-IP methamphetamine-induced psychosis
mg milligrams
Mg+ magnesium ion
mg/d milligrams per day
mGluR2 metabotropic glutamate receptor 2
mGluR3 metabotropic glutamate receptor 3
mGluR5 metabotropic glutamate receptor 5
MIM Mendelian inheritance in man gene code
min(s) minute(s)
miRNA micro ribonucleic acid
MK-801 (+)-D-aspartate 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-iminedizocilpine (dizocilpine)
mL millilitres
Mn/Mj major/minor allele
mPFC medial prefrontal cortex
mRNA messenger ribonucleic acid
MZ monozygotic
n number; study sample size
NA  not available
Na⁺ sodium ion
Nacc nucleus accumbens
NB neuroblastoma cell line
NCBI US National Centre for Biotechnology Information
ng nanograms
ng/mL nanograms per millilitre
NGS next generation sequencing
NH₃ ammonia
NHGRI US National Human Genome Research Institute
NIEHS National Institute of Environmental Health Sciences
NMDA N-methyl-D-aspartate
NMDAR N-methyl-D-aspartate receptor
NR1 N-methyl-D-aspartate receptor subunit 1
NR2A N-methyl-D-aspartate receptor subunit 2A
NR2B N-methyl-D-aspartate receptor subunit 2B
NRG1 neuregulin 1 gene
NRG1 neuregulin 1 protein
NXRN1 neurexin 1 gene
NXRN1 neurexin 1 protein
OCD obsessive compulsive disorder
OLZ olanzapine
OR odds ratio
PANSS Positive and Negative Syndrome Scale
PB protein bound
<table>
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<td>principle components analysis</td>
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<td>polymerase chain reaction</td>
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<td>PFC</td>
<td>prefrontal cortex</td>
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<td>PGC</td>
<td>Psychiatric Genomics Consortium</td>
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<td>personal genome machine</td>
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<td>promoter-associated histone mark</td>
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<td>protein kinase C</td>
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<td>postsynaptic density protein 95</td>
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<td>q-value</td>
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<td>r²</td>
<td>correlation coefficient between loci</td>
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<td>Stanford University Centre for Genomics and Personalized Medicine SNP annotation database</td>
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<td>R/NR</td>
<td>responder/non-responder</td>
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<tr>
<td>RR</td>
<td>relative risk; risk ratio</td>
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<td>reference single nucleotide polymorphism identification number</td>
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<td>schizophrenia</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>SMR</td>
<td>standardized mortality ratio</td>
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<td>SNARE</td>
<td>soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptor</td>
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<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
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<td>serine racemase</td>
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<td>succinate</td>
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<td>thymine nucleotide base</td>
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<td>T-box transcription factor 18 protein</td>
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<td>TCA</td>
<td>tricarboxylic acid cycle; Krebs cycle; citric acid cycle</td>
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<td>tardive dyskinesia</td>
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<td>transcription factor</td>
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<td>transcription factor binding site</td>
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<td>treatment-resistant schizophrenia</td>
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<td>vesicular glutamate transporter</td>
</tr>
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Chapter 1

INTRODUCTION

1.1 Schizophrenia Overview

The disease concept of schizophrenia (SCZ) first emerged in the middle of the 19\textsuperscript{th} century as psychiatrists within Europe began noting disorders of mental dysfunction that arose in early adulthood and often progressed to chronic deterioration. Several early terms were used to refer to patients presenting with these symptoms, including “démence précoce” meaning precocious onset of cognitive decline in France (Morel, 1860), and “adolescent insanity” in Scotland (Clouston, 1904). Borrowing from Morel’s term, German neurologist Emil Kraepelin (1856-1926) termed this condition ‘dementia praecox’ in 1887 and characterized the disorder as nine different “clinical forms” (Kraepelin, 1899). Approximately two decades later, the Swiss psychiatrist Eugen Bleuer (1857-1939) coined the term ‘schizophrenia’ on the 24\textsuperscript{th} of April, 1908 (Bleuer, 1911). The term was derived from two Greek words, ‘schizo’ meaning to tear or to split, and ‘phrenia’ meaning the mind.

In the present day, SCZ is characterized as a chronic, lifelong neuropsychiatric disorder accompanied by positive, negative and cognitive symptoms that are diagnosed according to the Diagnostic and Statistical Manual of Mental Health Disorders 5\textsuperscript{th} Edition (DSM-5) (American Psychiatric Association, 2013). According to this recent version of the DSM, a patient presenting with positive symptoms, or psychosis, exhibits psychotic behaviours that are not present in healthy individuals. The hallmark positive symptoms of SCZ include auditory hallucinations (hearing voices), delusions (fixed false beliefs), disorganized speech, and grossly disorganized or catatonic behaviour. In contrast, the negative and cognitive
symptoms of SCZ manifest as deficits in normal functioning and include characteristics such as affective flattening, poverty of speech (alogia), lack of motivation (avolition) and the inability to experience pleasure (anhedonia). Cognitive symptoms include deficits in attention, working memory, reasoning and processing speed. Affective symptoms and social interaction deficits are also considered appropriate symptom categories of SCZ. Together, these symptoms collectively contribute to the debilitating social and occupational dysfunction commonly observed in patients suffering from this disorder.

1.1.1 Epidemiology and Time Course

SCZ affects approximately 15 out of every 100,000 people each year (median incidence, central 80% range: 7.7-43) and almost 1% of the world’s population will suffer from SCZ sometime in their lifetime (median lifetime morbid risk: 7.2 per 1000 persons) (Jablensky et al., 1992; McGrath et al., 2008). The onset of SCZ usually occurs during adolescence between the ages of 18 and 35 years old (Hafner et al., 1994). Interestingly, the disease manifests two to three years later in females than in males (Reicher et al., 1990) and may be caused, in part, by the anti-dopaminergic effects of estrogen (Seeman & Lang, 1990).

The time course of SCZ typically follows three distinct phases: the prodrome, the acute or active phase and the residual phase. SCZ prodrome is the period leading up to psychosis and is marked by abnormal behaviours and psychological states relating to cognition, emotion, perception, communication, motivation and sleep (Klosterkotter et al., 2001). Nonspecific clinical symptoms such as depression, anxiety, social isolation and school and/or occupational dysfunction may also present during this time (Larson et al., 2010). The acute phase of SCZ, otherwise known as the ‘first episode’ of psychosis, occurs once the classic positive symptoms of SCZ manifest. This period is often accompanied by cognitive and
social decline (Hafner et al., 1999). Following the first episode, patients are commonly hospitalized, given a psychological assessment and diagnosed according to their symptoms. Patients often begin a trial with antipsychotic (AP) medication, which is the mainstay in SCZ treatment. Once positive symptoms are brought under control through the use of APs, the residual phase begins. Unfortunately, this phase is often associated with a number of adverse drug side effects and the presence of persistent negative and cognitive symptoms.

1.1.2 Deficits in Quality of Life

Individuals suffering from SCZ live shorter, lower quality lives in comparison to members of the general population (standardized mortality ratio (SMR): 2.6, central 80% range: 1.2-5.8) (McGrath et al., 2008). In addition, SCZ patients are often at higher risk of suicide (SMR: 12.9) (Saha et al., 2007; Zai et al., 2012). Indeed, approximately 5% of patients commit suicide in their lifetime, usually near disease onset (Palmer et al., 2005). Cardiovascular disease (CVD) is also more common in patients with SCZ and is primarily attributable to unhealthy diet, lack of exercise, excessive smoking and co-morbid substance abuse (Laursen, Munk-Olsen & Vestergaard, 2012). Compared to the general population, SCZ patients are 35% more likely to smoke cigarettes and 20% more likely to abuse alcohol and other substances. A subset of patients who are diagnosed with treatment-resistant schizophrenia (TRS) exhibit even higher rates of these health risk behaviours. In addition, patients often suffer from motor side effects collectively called extrapyramidal symptoms (EPS), which include tardive dyskinesia (TD) (30%), akathisia (36%) and parkinsonism (37%) (reviewed by Kennedy et al., 2014).

Health utility scales are useful for assessing the effect of disease status on a patient’s quality of life. On a scale from 0 to 1, zero representing death and one representing perfect health,
severe, moderate and mild forms of SCZ have been assigned health utility scores of 0.56 (standard deviation (SD): 0.11), 0.62 (SD: 0.22) and 0.79 (SD: 0.09), respectively (Centre for the Evaluation of Value and Risk in Health, 2011). TRS is also associated with poorer quality of life that is approximately 20% lower than patients with SCZ who achieve remission (0.61 vs. 0.75) (Kennedy et al., 2014). Alleviation of SCZ symptomatology through successful therapeutic intervention usually increases a patient’s quality of life and stresses the need for optimizing treatment outcomes (Bobes et al., 2007).

1.1.3 Burden to Society, Patients and Caregivers

The total financial burden of SCZ in Canada in 2004 was estimated to be $6.85 billion CDN, accounting for direct healthcare and non-healthcare costs, as well as productivity lost due to unemployment (Goeree et al., 2005; Kennedy et al., 2014). Furthermore, TRS patients report annual healthcare costs ranging 3-to-11 times higher than schizophrenia patients who respond to treatment ($66,360-$163,795 vs. $15,500-$22,300 per year). In addition, SCZ is ranked as one of the top 10 causes of disability among people in developed countries (Murray & Lopez, 1996; Barbato, 1998). Areas that are most prone to disability include social functioning in relation to self-care, occupational performance, family relationships and relationships in society as a whole (Janca et al., 1996). Stigma against individuals with mental health disorders is lower than in the past, however, individuals with SCZ still experience high degrees of rejection, discrimination and social isolation that ultimately affects their sense of self and well-being (Goffman, 1963). Schizophrenia also represents a substantial burden to caregivers (Schene et al., 1998; Gutierrez-Maldonado et al., 2005; Vasudeva et al., 2013), with approximately 25% of patients living with a relative after diagnosis (Torrey, 2013).
1.2 Etiology and Pathophysiology

The cause of SCZ is not known, however, various hypotheses have been posited to explain why this disorder manifests in particular individuals. These hypotheses propose SCZ emerges due to a combination of risk factors that include environmental exposures and genetic predisposition that ultimately lead to abnormalities in brain structure and function. A brief overview of the etiology and pathophysiology of SCZ is presented below.

1.2.1 Environmental Risk Factors

Several environmental risk factors are thought to increase the likelihood of developing SCZ. These factors include: male sex (relative risk (RR): 1.42, 95% Confidence Interval (CI): 1.30-1.56) (Aleman et al., 2003), first- and second-generation migrant status (RR: 2.7, 95% CI: 2.3-3.2 and 4.5, 95% CI: 1.5-13.1, respectively) (McGrath et al., 2004; Cantor-Graae & Selten, 2005), urban residence (RR: 2.4, 95% CI: 2.13-2.70) (Marcelis et al., 1998; Mortensen et al., 1999), advanced paternal age (Malaspina et al., 2001; El-Saadi et al., 2004; Sipos et al., 2004) and cannabis use (Boydell et al., 2007; De Sousa et al., 2013). Pre- and postnatal exposure to stress and infection (Buka et al., 2001; Brown et al., 2004; Mortensen et al., 2007), as well as prenatal nutritional deprivation (Susser & Lin, 1992; St Clair et al., 2005) may also increase one’s risk for developing SCZ.

1.2.2 Genetic Predisposition

SCZ is also caused in part by genetic factors as evidenced by twin, family and adoption studies. For instance, studies analyzing the incidence of SCZ within families indicate that children are at an increased risk for developing SCZ if other family members are affected. This risk increases as the degree of relation to the affected family member increases. As was
mentioned earlier in Section 1.1, the risk of developing SCZ in the general population is approximately 1%, however this risk jumps to 6% for a child born to an afflicted parent and even higher to 9% for a person who has an afflicted sibling (Gottesman, 1991).

Twin studies have been particularly useful in understanding the contribution of genes to SCZ etiology because twins share varying degrees of genetic similarity (reviewed by Cardno & Gottesman, 2000). More specifically, dizygotic twins share exactly half their genes while monozygotic twins, or identical twins, have genotypes that are exact duplicates. Twin studies in SCZ research have yielded proband-wise concordance rates of 41-65% in monozygotic twins and 0-28% in dizygotic pairs (reviewed by Gottesman, 1991). The amount of concordance observed between twins as well as observed and expected resemblance between relatives is useful for estimating trait heritability (Visscher et al., 2008). Researchers involved in twin and family studies estimate the heritability of SCZ to be approximately 80-85% (reviewed by Kendler, 1983). Thus, genetic factors are the largest known risk factor for SCZ.

Adoption studies are another important tool for distinguishing the influence of heredity and environment on human traits. Generally, traits shared between biological parents and a child that is given up for adoption is explained by genetic factors, while traits shared between adoptive or ‘environmental’ parents and an adopted child is attributed to environmental influence (Plomen et al., 1997). The first adoption study in SCZ was proposed in 1959 (Kety, 1959) and investigated whether adopted away children of parents (usually mothers) with SCZ were at increased risk for developing the disorder. Adoption studies that followed found that children who are raised in foster homes or institutions and whose biological parent had SCZ developed SCZ more often than children of controls (Heston,
Similar studies have replicated these findings and provide sufficient evidence to support a role of genetics in SCZ (Higgins, 1966; Rosenthal et al., 1968; Higgins, 1976; Tienari & Wynne, 1994).

Molecular genetic studies aim to identify genes involved in disease etiology. Genetic linkage studies follow the segregation of marker alleles from an affected lineage to offspring in families (Riley & McGuffin, 2000), while genetic association studies assess the contribution of loci to disease presentation in populations. Results from linkage studies in SCZ have been mixed, with limited replication of findings. Positive results are generally accepted as significant if they replicate in several additional studies. To date, SCZ has been linked to numerous chromosomal regions including chromosome 1, 2, 4, 5-10, 13, 15, 18, 22 and the X chromosome. The breadth of these findings exceeds the scope of this paper and have been reviewed elsewhere (Riley & McGuffin, 2000; Riley & Kendler, 2006). Select candidate genes and chromosomal regions are summarized in Table 1.1.

1.2.2.1 Chromosome 6: 6p24-p22

Linkage findings for chromosome 6 first emerged from studies involving Irish families with a high density of broad-spectrum psychiatric disorders (Straub et al., 2002). One particular gene within this region of chromosome 6, the dystrobrevin binding protein 1/dysbindin gene (DTNBP1) has been, in general, positively associated with SCZ (Funke et al., 2004; Numakawa et al., 2004; Williams et al., 2004). Altered expression of DTNBP1 in certain brain regions has also been reported in patients with SCZ (Weickert et al., 2004). Although the exact function of DTNBP1 in the brain is unknown, this protein appears to have a role in glutamatergic neurotransmission (Talbot et al., 2004; Shao et al., 2011).
1.2.2.2 **Chromosome 8: 8p22-p21**

Findings from the Maryland family sample, as well as data from numerous pedigrees of individuals with differing ethnic background have provided linkage support for chromosome 8 (Pulver et al., 1995). Association findings have confirmed that a candidate gene located within this region called neuregulin 1 (NRG1) is associated with increased risk for SCZ (Stefansson et al., 2003; Williams et al., 2003; Corvin et al., 2004). The neuregulin 1 protein (NRG1) is a CNS-expressed molecule involved in the expression and activation of neurotransmitter receptors (reviewed by Harrison & Law, 2006). Of interest to the glutamatergic neurotransmitter system, NRG1 regulates N-methyl-D-aspartate glutamate receptor (NMDAR) function via a postsynaptic density protein (PSD-95) (Gu et al., 2005).

1.2.2.3 **Chromosome 11: 11q22-q21**

The chromosome 11 region was first implicated in SCZ etiology in a linkage study involving two Japanese pedigrees (Nanko et al., 1992), and was later confirmed in a separate study involving a large Canadian pedigree (Maziade et al., 1995; Gurling et al., 2001). One particular gene of interest lying within chromosome region 11q22 is the dopamine receptor D2 gene (DRD2). This gene is of particular interest because all antipsychotic medications on the market for the treatment of SCZ symptoms have blockade of the dopamine D2 receptor (DRD2) as a common mechanism of action (Seeman & Lee, 1975). A large meta-analysis of variants within DRD2 indicates this gene is associated with SCZ (Glatt et al., 2003).

1.2.2.4 **Chromosome 13: 13q14-q32**

Data from a mixed sample of UK and Japanese families initially suggested linkage to chromosome 13 (Lin et al., 1995). This region is of particular interest because approximately 120 known genes of interest are located here including the serotonin receptor type 2A gene
(HTR2A), D-amino acid oxidase activator gene (DAOA) and the d-amino acid oxidase gene (DAAO). Association studies involving HTR2A variants in SCZ risk have produced mixed results (reviewed by Serretti et al., 2007), while studies assessing association with response to atypical APs have yielded more consistently positive findings (Arranz et al., 1995; Burnet & Harrison, 1995; Masellis et al., 1995; Nothen et al., 1995). Based on meta-analysis, DAOA has been implicated in SCZ risk (Detera-Wadleigh & McMahon, 2006), as well as DAAO (Chumakov et al., 2002; Schumacher et al., 2004; Yang et al., 2013). Functionally, the d-amino acid oxidase activator protein (DAOA) activates d-amino acid oxidase enzyme (DAAO/DAO) that is responsible for the oxidation of D-amino acids such as D-serine in the brain. Because D-serine acts as a co-agonist at the NMDAR, variable activity of DAOA and/or DAAO may therefore contribute to SCZ pathophysiology by affecting glutamatergic neurotransmission.

1.2.2.5 Polygenic Inheritance

Recently, a very large genome wide association study (GWAS) consisting of up to 36,989 cases and 113,075 controls from the psychiatric genomics consortium (PGC) identified 128 independent associations spanning 108 conservatively defined loci with SCZ (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The top 128 loci included DRD2 and many other genes related to glutamatergic function such as the metabotropic glutamate receptor 3 gene (GRM3), the NMDAR subunit 2A gene (GRIN2A) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR) subunit 1 gene (GRIA1). As implicated by this recent GWAS, SCZ is considered a complex trait that almost certainly follows a polygenic mode of inheritance (Gottesman & Shields, 1967; McClellan et al., 2007; International Schizophrenia Consortium et al., 2009). As opposed to a Mendelian
mode of inheritance where a single gene is responsible for disease presentation, multiple genes and the interaction among these genes likely contribute to the development of SCZ.

**Table 1.1 Select Candidate Genes & Chromosomal Regions Associated with Schizophrenia**

<table>
<thead>
<tr>
<th>Gene Region</th>
<th>Linkage &amp; Candidate Gene Association Findings</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>1q32-42</td>
<td>Balanced 1:11 translocation identified in a large Scottish pedigree with a chromosomal breakpoint at 1q42.1</td>
<td>(St Clair et al., 1990; Shaw et al., 1998; Hovatta et al., 1999; Brzustowicz et al., 2000; Miyoshi et al., 2003; Ozeki et al., 2003; Sumiyoshi et al., 2004)</td>
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<tr>
<td></td>
<td><strong>Disrupted in schizophrenia 1 gene (DISC1):</strong> Plays a role in cytoskeletal regulation and may affect neuronal migration, neurite outgrowth and intracellular transport</td>
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<td>6p24-p22</td>
<td>Linkage findings identified in Irish families with a high density of broad spectrum psychiatric disorders</td>
<td>(Straub et al., 2002; Funke et al., 2004; Numakawa et al., 2004; Talbot et al., 2004; Williams et al., 2004; Shao et al., 2011)</td>
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<tr>
<td></td>
<td><strong>Dystrobrevin binding protein 1/dysbindin gene (DTNBP1):</strong> Altered expression in SCZ brains; appears to have a role in glutamatergic neurotransmission</td>
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<tr>
<td>8p22-p21</td>
<td>Genetic data from the Maryland sample and several other pedigrees of different ethnic origin provide evidence to support linkage of this region to SCZ</td>
<td>(Pulver et al., 1995; Stefansson et al., 2003; Williams et al., 2003; Corvin et al., 2004; Gu et al., 2005)</td>
</tr>
<tr>
<td></td>
<td><strong>Neuregulin 1 gene (NRG1):</strong> Associated with increased risk for SCZ; regulates NMDAR function through postsynaptic density protein</td>
<td></td>
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<tr>
<td>11q22-q21</td>
<td>This region was first implicated in a linkage study involving two Japanese pedigrees and was later confirmed in a large Canadian pedigree</td>
<td>(Nanko et al., 1992; Maziade et al., 1995; Gurling et al., 2001; Glatt et al., 2003; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014)</td>
</tr>
<tr>
<td></td>
<td><strong>Dopamine Receptor D2 gene (DRD2):</strong> Primary target of AP medications; meta-analyses indicate this gene is positively associated with SCZ</td>
<td></td>
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<tr>
<td>13q14-q32</td>
<td>Data from a mixed sample of UK and Japanese families initially suggested linkage to this region</td>
<td>(Arranz et al., 1995; Burnet &amp; Harrison, 1995; Lin et al., 1995; Masellis et al., 1995; Nothen et al., 1995; Chumakov et al., 2002; Schumacher et al., 2004; Detera-Wadleigh &amp; McMahon, 2006; Serretti et al., 2007; Yang et al., 2013)</td>
</tr>
<tr>
<td></td>
<td><strong>Serotonin receptor 2A gene (HTR2A):</strong> Mixed results in SCZ etiology; positive results for association with AP response</td>
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</tr>
<tr>
<td></td>
<td><strong>D-amino acid oxidase activator gene (DAOA):</strong> Activates D-amino acid oxidase protein; positively associated with SCZ</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>D-amino acid oxidase gene (DAOA/DAAO):</strong> Oxidizes D-amino acids such as NMDAR co-agonist D-serine; positively associated with SCZ</td>
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</tr>
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</table>
1.2.3 *Brain Structural Abnormalities*

Several brain structural abnormalities have been observed in patients with SCZ (reviewed by Buckley, 2005). In brief, these structural abnormalities include: reduced cortical thickness (Voineskos et al., 2013), gradual lateral and third ventricle enlargement (Johnstone et al., 1976; Mathalon et al., 2001; Kasai et al., 2003), subtle decreases in thalamic volume (Andreasen et al., 1994; Kemether et al., 2003), an enlarged caudate nucleus (Jernigan et al., 1991), smaller temporal lobes (Wright et al., 2000; Csernansky et al., 2002) and reduced left-right asymmetry (anisotropy) (Nakamura et al., 2012; Lee et al., 2013). Brain structural abnormalities in patients with SCZ have been difficult to reproduce across studies because they are relatively subtle and are also influenced by patient characteristics such as age, gender, chronicity of illness, medication use and co-morbid substance abuse (McCarley et al., 1999; Arango et al., 2003).

1.2.4 *Neurochemical Abnormalities*

In addition to brain structural aberrations, several neurochemical abnormalities have been observed in patients with SCZ. The majority of these abnormalities relate primarily to the dopamine, glutamate, serotonin and the inhibitory neurotransmitter γ-aminobutyric acid (GABA) neurotransmitter systems. The roles of serotonin and GABA in SCZ etiology are beyond the scope of this text and have been reviewed elsewhere (Meltzer, 1995a; Aghajanian & Marek, 2000; Wassaf & Kochan, 2003; Nakazawa et al., 2012).

1.2.4.1 *The Dopamine Hypothesis*

Much of SCZ research has focused on perturbations in the dopamine neurotransmitter system. The groundwork for the ‘dopamine hypothesis of SCZ’ was conducted by Arvid Carlsson (1923-) in the late 1950s, who won the Nobel prize in 2000 for his contribution, and
by Jacques van Rosum in the late 1960s (reviewed by Seeman, 1987). Furthermore, Seeman & colleagues showed in 1975 that the efficacy of AP drugs was tightly correlated with affinity of the drugs for DRD2 receptors (Seeman & Lee, 1975). Hence, the dopamine hypothesis was based largely on observations that dopamine-enhancing drugs, such as amphetamines, were capable of reproducing positive symptoms observed in patients with SCZ (Lieberman et al., 1987), and that AP drugs which treat psychosis do so primarily by blocking DRD2 receptors (Carlsson & Lindqvist, 1963; Seeman & Lee, 1975).

Biological data has also provided evidence to support the dopamine hypothesis of SCZ. A large proportion of the dopaminergic neurons in the human brain originate in the ventral tegmental area (VTA), and project to the prefrontal cortex (mesocortical pathway) and to subcortical limbic brain regions (mesolimbic pathway). Dopamine activity in prefrontal regions appears to play an inhibitory role on dopamine release in subcortical areas (Pycock, Kerwin & Carter, 1980; Kolachana, Saunders & Weinberger, 1995; Karreman & Moghaddam, 1996). From these findings, SCZ has been characterized as a disorder caused by deficits in dopamine transmission (hypodopaminergia) in the prefrontal cortex, consequently leading to over-activity of dopamine circuits (hyperdopaminergia) in the striatum (reviewed by Davis et al., 1991). Hypofrontality in SCZ patients is evidenced by reduced cerebral blood flow in the frontal cortex and may be related to low dopaminergic activity in that region (Davis et al., 1991; Rubin et al., 1991; Andreasen et al., 1992). Positive symptoms are thought to arise due to striatal hyperdopaminergia, while dopamine hypoactivity in the prefrontal cortex is proposed to account for negative and cognitive symptoms.
1.2.4.2  The Glutamate Hypothesis

Despite the usefulness of the dopamine hypothesis, cortical abnormalities observed in SCZ appear to be more complex than the originally proposed hypofrontality. Some posit that the dopamine system of SCZ patients may in fact be normal, yet suffer from abnormal regulation (Grace, 2012), and hints towards the involvement of several neurotransmitter systems in SCZ’s etiology. For instance, a large amount of evidence has characterized SCZ as a neurodevelopmental disorder accompanied by perturbations in glutamatergic neurotransmission (Weinberger, 1987; Olney & Farber, 1995; Mohn et al., 1999; Olney et al., 1999). Much of the rationale supporting this ‘glutamate hypothesis of SCZ’ stems from observations that administration of NMDAR antagonists, ketamine and phencyclidine (PCP), induce SCZ-like symptoms in healthy controls and exacerbate psychotic symptoms in SCZ patients (Luby et al., 1959; Luisada, 1978; Lahti et al., 2001). Interestingly, glutamate action, specifically at the NMDAR, has been shown to regulate dopaminergic neurotransmission (Zweifel et al., 2008; Parker et al., 2010).

Several theories regarding glutamate’s involvement in SCZ etiology have been proposed. One overarching theory suggests that NMDAR glutamatergic hypofunction is responsible for creating the positive and negative symptoms of SCZ through dysregulation of mesolimbic and mesocortical dopamine neurons, respectively. More specifically, hypofunction of the inhibitory glutamatergic pathway that projects from cortical pyramidal neurons to dopamine neurons in the VTA may cause tonically uninhibited dopamine release from the mesolimbic pathways and result in positive symptoms, while hypofunction of the stimulatory glutamatergic pathway that acts on mesocortical dopamine neurons could cause the
hypodopaminergia that is responsible for negative, cognitive and affective symptoms (Stahl, 2007). The glutamate hypothesis of SCZ is discussed in more detail in the following section.

1.3 Glutamatergic Dysfunction in Schizophrenia

A great deal of evidence has characterized SCZ as a disorder related to aberrant glutamate signalling. The following section provides a general overview of the glutamate system with particular emphasis placed on receptors, transporters and enzymes involved in glutamatergic neurotransmission. This section concludes with a thorough summary of literature on glutamate abnormalities in SCZ.

1.3.1 The Glutamate System

Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (CNS) (Curtis et al., 1960). Glutamate neurotransmission plays an essential role in physiological processes such as synaptic plasticity and induction of long term potentiation (LTP), processes that are both implicated in memory and learning (Harris et al., 1984; Morris et al., 1986). Various receptors, transporter and enzymes are involved in the proper functioning of glutamatergic neurotransmission. There are two main groups of excitatory glutamate receptors, the ligand gated ion channels which include the NMDAR, AMPAR and kainate types of receptors, and the metabotropic G-protein coupled receptors (mGluRs) which are subdivided into group I, group II and group III (Foster & Roberts, 1981; Watkins & Evans, 1981; Watkins & Collingridge, 1994; Pin & Duvoisin, 1995). Extracellular levels of glutamate and other relevant neurotransmitters such as glycine are carefully regulated by the action of transporters. Once taken up into neurons and/or surrounding microglia, glutamate and glycine are either converted to other chemical compounds or metabolized.
through the action of several intracellular enzymes. A schematic representation of a neuronal synapse is shown in Figure 1.1 and highlights the role of a number of key receptors, transporters and enzymes relevant to the glutamate system. This figure also highlights GABAergic neurons (top right corner), which are highly important in brain function due to their general inhibitory role. It should also be mentioned that GABA and glutamate are in a complex interplay, however this thesis largely focused on investigating the role of glutamate system abnormalities. Therefore, please note that this diagram is an oversimplified representation of a synapse, for example, in that the role of GABAergic neurons is not fully depicted.
Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian central nervous system with roles in cognition, memory and learning. Precise regulation of glutamate signaling must be maintained as excitotoxicity can result from excessive glutamatergic stimulation. (1) Glutamate is derived from glutamine (Gln) through the action of the enzyme glutaminase (GLS) or from the tricarboxylic acid (TCA) cycle, and is packaged into presynaptic vesicles by vesicular glutamate transporters (vGluTs). (2) Vesicles release their contents into the synaptic cleft through vesicle-membrane fusion, which is facilitated by SNARE proteins. (3) Presynaptic and postsynaptic glutamate binding activates metabotropic (mGlu) and ionotropic (NMDA, AMPA, Kainate) glutamate receptors, downstream signal transduction cascades and synaptic plasticity. (4) Glutamate is recycled from the synapse by glutamate transporters (EAAT1/2), which are expressed predominantly on astroglia (glial cells). Once inside astroglia, glutamate is converted to glutamine through glutamine synthetase (GS) and is then exported for re-uptake by glutamatergic neurons. (5) Glutamine may also be taken up by GABAergic interneurons, converted first into glutamate by GLS, and then to GABA by glutamate decarboxylase 1 (GAD1). Glycine (Gly) acts independently as an inhibitory neurotransmitter, or in concert with glutamate neurotransmission as a co-agonist at the NMDAR. (6) Glycine is recycled from the synapse by glycine transporters (GlyT1) which are located primarily on astrocytes. Once taken up by astrocytes, glycine can be metabolized to α-ketoglutaric acid (α-keto acid), ammonia (NH₃) and hydrogen peroxide (H₂O₂) through a series of intermediate steps: glycine to L-serine (L-ser) catalyzed by the glycine cleavage system (GCS), L-serine to D-serine (D-ser) catalyzed by serine racemase (SRR), D-serine to the final products via the D-amino acid oxidase enzyme, that is activated by D-amino acid oxidase activator protein (G72). Adapted from Pharmacology Biochemistry and Behavior, 100(4). Mark J. Niciu, Benjamin Kelmendi & Gerard Sanacora. Overview of Glutamatergic Neurotransmission in the Nervous System, 656-64. Copyright (2012) with permission from Elsevier.
1.3.1.1  

**Ionotropic Glutamate Receptors**

NMDAR and AMPAR are principally expressed on dendritic spines of postsynaptic neurons (Kharazia et al., 1996) where they function in signaling during induction of synaptic plasticity and long-term modulation of synaptic strength (Asztely & Gustafsson, 1996; Shapiro, 2001). Kainate receptors mostly localize to postsynaptic membranes where they function to modulate synaptic response to glutamate (Van den Pol, 1994; Haak, 1999). Kainate receptors also localize to surrounding glia and presynaptic neurons where they modulate a variety of cellular functions including glutamate reuptake and release, and presynaptic excitability (Cartmell & Schoepp, 2000; Huettner, 2003; Grueter & Winder, 2005; Rodriguez-Moreno, 2006).

The NMDAR is a hetero-tetramer composed of two obligatory GluN1 (NR1) subunits and either two GluN2 (NR2A-D) or two GluN3 (NR3A-B) subunits (Monyer et al., 1992) (Figure 1.2). Subunit composition varies during development and results in various receptor isoforms with different physiological and pharmacological properties (McBain & Mayer, 1994). The NMDAR is implicated in additional processes such as neuronal migration (Komuro & Rakic, 1993) and differentiation (Pearce et al., 1987), response to trophic factors (Black, 1999), dendritic spine development (Tian et al., 2007), LTP, and memory and learning (Massey et al., 2004). Given the importance of the NMDAR in neuronal processes, abnormal function and/or development of this receptor system could lead to the deficits in learning and memory that are observed in patients with SCZ.

NMDAR channel activation utilizes a coincidence detection type mechanism that requires simultaneous binding of glutamate to the NR2 subunit (Laube et al., 1997; Anson et al., 1998), binding of an endogenous co-agonist, either glycine or D-serine, to the NR1 subunit.
(Kuryatov et al., 1994; Hirai et al., 1996) and removal of a voltage-dependent block by external magnesium (Mg$^{2+}$) (Mayer et al., 1984; Nowak et al., 1984). Channel activation produces excitatory postsynaptic potentials (EPSPs) and increases calcium (Ca$^{2+}$) and sodium (Na$^+$) concentration inside the cell (Schiller et al., 1998). Intracellularly, calcium acts as a secondary messenger with downstream effects on protein kinases such as mitogen activated protein kinase (MAPK) (Xia et al., 1996) and transcription factors such as cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (Sala et al., 2000). NMDAR action is also associated with effector kinases such as protein kinase C (PKC) (Zheng et al., 1997), protein kinase A (PKA) (Tingley et al., 1997) and calcium/calmodulin-dependent kinase II (CaMKII) (Omkumar et al., 1996).

The remaining two ionotropic glutamate receptors, AMPA and kainate, both form tetrameric complexes consisting of GluR1-4 subunits (AMPA) or GluR5-7 and KA1-2 subunits (kainate). Both receptors are widely expressed in the mammalian CNS and mediate fast excitatory neurotransmission in response to the binding of glutamate (Khakh & Henderson, 2000). The function of AMPA and kainate ionotropic glutamate receptors is controlled in part by RNA editing of subunits (Higuchi et al., 1993), as well as alternative splicing of messenger RNA (mRNA) transcripts (Sommer et al., 1990). Ionotropic glutamate receptor function, the AMPAR in particular, is also regulated by receptor number, localization and facilitation at synapses ultimately controlling such processes as LTP-like strengthening of neocortical synapses after sensory stimulation (Takahashi et al., 2003), learning in the hippocampus (Whitlock et al., 2006) and response to stress hormones (Groc et al., 2008; Martin et al., 2009; Yuen et al., 2011).
Figure 1.2 The Ionotropic N-methyl-D-aspartate Glutamate Receptor

Functional NMDARs are constructed from assemblies of four subunits (two NR1 and either two NR2A-D or NR3A-B). Subunit composition varies and results in various receptor isoforms that have different physiological and pharmacological properties. NR1 subunit is encoded by eight alternatively spliced variants of GRIN1, and contains the binding site for endogenous co-agonists glycine and D-serine. NR2 subunits contain the glutamate recognition site and binding sites for various other antagonists. The NMDAR channel is normally blocked at resting membrane potential by magnesium (Mg$^{2+}$) in a voltage-dependent manner and is activated following depolarization of the postsynaptic membrane. Receptor activation occurs through a coincidence detection type mechanism and requires both glutamate and glycine binding. Receptor activation leads to calcium (Ca$^{2+}$) and sodium (Na$^{+}$) influx, and potassium (K$^{+}$) efflux, facilitating long-term potentiation and synaptic plasticity. Antagonists of the NMDAR include: Memantine (treats Alzheimer’s disease), Selfotel (discontinued anxiolytic/anticonvulsant), MRZ 2/576 (research use) and Ifenprodil (discriminates NMDAR subpopulations). Adapted from Pharmacological Reviews 50. Wojciech Danysz and Chris G. Parsons. Glycine and N-Methyl-D-Aspartate Receptors: Physiological Significance and Possible Therapeutic Applications, 597-664. Copyright (1998) with permission from American Society for Pharmacology and Experimental Therapeutics.
1.3.1.2 Metabotropic Glutamate Receptors

Like the kainate receptors, mGluRs modulate synaptic responses to glutamate at postsynaptic membranes (Van den Pol, 1994; Haak, 1999) and modulate a variety of cellular functions at presynaptic neurons (Cartmell & Schoepp, 2000; Huettner, 2003; Grueter & Winder, 2005; Rodriguez-Moreno, 2006). The metabotropic glutamate receptors mediate signaling through 7-transmembrane region G-protein coupled receptors (Pin et al., 2003). The three subclasses are groups I, II and III and they signal through different mechanisms. Group I (mGluR1, 5) signals through G-protein activation that is coupled to protein kinase activation (ie PLC), and subsequently leads to the formation of inositol 1,4,5-triphosphate (IP₃) and intracellular release of calcium (Hermans & Challiss, 2001). In contrast, group II (mGluR2, 3) and group III (mGluR4, 6, 7, 8) receptors couple to inhibitory G-protein signaling whereby inhibition of adenylyl cyclase activity subsequently leads to reduction in cAMP production (Conn & Pin, 1997). The mGluR receptors are tightly regulated by formation of various alternatively spliced isoforms which control several aspects of receptor function, including surface receptor expression, G-protein coupling, and interaction with scaffolding proteins in the postsynaptic density (PSD) (Stowell & Craig, 1999; Tu et al., 1999; Pin et al., 2003).

Drugs targeting metabotropic glutamate receptors have been investigated for the treatment of SCZ (reviewed by Li et al., 2015). LY404039, a specific and potent mGluR2/3 agonist developed by Eli Lilly & Co, is one example of a drug from this class (Monn et al., 2005). Experiments with mGluR2/3 knockout mice suggest that the antipsychotic-like behavioural effects of LY404039 are caused by mGluR2 activation and further implicate the glutamate system in SCZ etiology (Rorick-Kehn et al., 2007; Fell et al., 2008). Clinical studies investigating this compound for the treatment of SCZ indicate it was well tolerated and
lacked the adverse side effects typically associated with dopaminergic agents such as weight gain, EPS and hyperprolactinemia (Adams et al., 2013). Unfortunately, efficacy results for this compound were inconsistent. More specifically, the initial proof of concept study showed LY404039 and the active control olanzapine (OLZ) significantly decreased patient symptoms compared to placebo (Patil et al., 2007), however a Phase 2 (inpatient, dose-ranging study) reported ‘inconclusive results’ as both LY2140023 and OLZ failed to separate from placebo (Kinon et al., 2011). Finally, a Phase 2 (acute, fix-dosed study) showed that LY404039 did not separate from placebo in the primary efficacy endpoint unlike the active control risperidone (Kinon et al., 2013). Development of this drug was discontinued following the last study. Further research into LY2140023 by Eli Lilly has indicated that response to this compound may be more favourable in a subgroup of patients that carry a select set of 23 single nucleotide polymorphism (SNP) gene variants (Liu et al., 2012). Interestingly, 16 of these 23 SNPs are located in the HTR2A gene. Studying the relationship between genetic variation and drug response is called pharmacogenetics and will be discussed in more detail in Section 1.5.

1.3.1.3  

**Amino Acid Transporters**

Amino acid transporters control the reuptake of amino acids, such as glutamate and glycine, from the synapse into neurons and/or surrounding neuroglia. Two amino acid transporters are involved in glutamatergic neurotransmission: the excitatory amino acid transporters (EAATs) 1-5 and the glycine transporters (GlyTs) 1 and 2. The EAATs are responsible for the rapid reuptake of synaptic glutamate into astrocytes and control extracellular concentrations of glutamate (reviewed by O'Shea, 2002). Strict control of extracellular glutamate concentrations is necessary to prevent excitotoxicity and cell death (Coyle &
Puttfarcken, 1993; Szatkowski & Attwell, 1994). Of the five EAATs, EAAT2 (otherwise known as GLT1) is the chief glutamate transporter in the forebrain and is responsible for more than 90% of glutamate reuptake into surrounding astrocytes (Bar-Peled et al., 1997; Furuta et al., 1997).

The second type of transporter involved in glutamatergic neurotransmission is responsible for controlling levels of the amino acid glycine. Within the nervous system, glycine carries out a dual role as both an inhibitory neurotransmitter at glycine receptors and as a co-agonist at the NR1 subunit of the NMDAR to mediate excitatory neurotransmission (Johnson & Ascher, 1987). The primary neuronal glycine transporter, GlyT1, is expressed predominantly on astrocytes and is responsible for tightly regulating glycine concentrations in the vicinity of excitatory synapses through reuptake of glycine into neighbouring astroglia (Smith et al., 1992; Zafra et al., 1995; Cubelos et al., 2005). Interestingly, changes in GlyT1 activity have been shown to alter NMDAR-mediated neurotransmission as evidenced by increased NMDAR excitatory postsynaptic currents (EPSCs) following GlyT1 antagonism (Bergeron et al., 1998; Chen et al., 2003; Kinney et al., 2003) and by increased NMDAR function in GlyT1 knockout mice (Gabernet et al., 2005).

Drugs targeting the glycine system have also been investigated for SCZ. One such drug inhibits GlyT1 function in an attempt to ameliorate the NMDAR hypofunction associated with SCZ etiology (reviewed by Hashimoto, 2010). One such glycine transporter inhibitor bitopertin (RG1678), developed Roche, has been investigated as an adjunct for persistent negative symptoms in SCZ. Five clinical trials have been conducted so far, however the results have been non-significant (ClinicalTrials.gov identifiers: NCT01192867, NCT01192906, NCT01192880, NCT01235520, and NCT01235559). Various clinical studies
have also investigated the efficacy of adjunct glycine, D-serine or D-cycloserine (NMDAR co-agonists) for the treatment of SCZ (reviewed by Javitt, 2004). On a more positive note, the majority of these studies have reported large effect-size improvements in negative and cognitive symptoms (Potkin et al., 1992; Heresco-Levy et al., 1996; Tsai et al., 1998; Goff et al., 1999; Javitt et al., 2001).

1.3.1.4 Glutamate System Enzymes

There are several key enzymes involved in regulating glutamatergic neurotransmission. These enzymes include glutaminase (GLS) and glutamine synthetase (GS), which are responsible for interconverting glutamate and glutamine, glutamate decarboxylase 1 (GAD1), which converts glutamate to GABA, and various other enzymes involved in glycine metabolism.

Glutamine synthetase is an astrocytic enzyme responsible for converting glutamate to glutamine (Meister, 1974; Martinez-Hernandez et al., 1977). Glutamine is released extracellularly as a neuroprotective form of glutamate where it can be taken up by neurons and deaminated back into glutamate by the enzyme glutaminase (Goldstein, 1967; Curthoys & Watford, 1995). This pattern of compartmentalization and interconversion constitutes what is known as the ‘glutamate-glutamine cycle’ (Shank & Aprison, 1981). This cycle ensures three events: 1) glutamate is rapidly removed from the synapse to prevent neurotoxicity; 2) glutamate is converted to the ‘neuroprotective carrier’ glutamine; and 3) glutamine is made available in neuronal compartments and allows for the regeneration of glutamate for excitatory signaling (Daikhin & Yudkoff, 2000).
Besides reuptake into glutamatergic neurons, glutamine can also be taken up by GABAergic interneurons and metabolized to GABA by GAD1 (Erlander et al., 1991). GABA is an inhibitory neurotransmitter that controls fundamental processes such as neurogenesis (Fagiolini et al., 2004; Ge et al., 2006), movement, and tissue development (Nakatsu et al., 1993). A mutation in the glutamate decarboxylase 1 gene (GAD1) in humans results in a disorder known as spastic cerebral palsy (Lynex et al., 2004), while GAD1 knockout mice have dramatically reduced GABA levels and die at birth due to a severe cleft palate (Asada et al., 1997).

Lastly, a series of enzymes are responsible for the metabolism of glycine to α-ketoglutaric acid (α-keto acid), ammonia (NH₃) and hydrogen peroxide (H₂O₂). Upon entry into glial cells through the action of GlyT1, glycine is first converted to L-serine (L-ser) by the glycine cleavage system (GCS) (Kikuchi, 1973). L-serine is then converted to D-serine (D-ser) by the enzyme serine racemase (SRR) (Wolosker et al., 1999), and then converted to the final products α-keto acid, NH₃ and H₂O₂ via D-amino acid oxidase (Dixon & Kleppe, 1965). Like glycine, D-serine is an endogenous agonist at the NR1 subunit of the NMDAR and plays a role in regulating excitatory neurotransmission (Matsui et al., 1995).

1.3.2 Glutamate System Abnormalities in Schizophrenia

Numerous glutamate system abnormalities have been reported in patients with SCZ giving further credence to the glutamate hypofunction hypothesis. For instance, postmortem studies show abnormalities in NMDAR subunit expression in patients with SCZ, including increased NR2B transcript in the thalamus (Clinton & Meador-Woodruff, 2004)) and temporal lobes (Grimwood et al., 1999), as well as higher NR1 expression in the dorsolateral prefrontal cortex (DLPFC) (Dracheva et al., 2001), substantia nigra (Mueller et al., 2004) and superior
temporal cortex (Nudmamud-Thanoi & Reynolds, 2004). In addition, more recent data has indicated that unmedicated patients with SCZ have increased hippocampal glutamate in comparison to healthy controls (Kraguljac et al., 2013).

Preclinical models also point to glutamate system abnormalities in modeling SCZ-related behaviours. Rats administered PCP and ketamine exhibit hyperlocomotion in an open field taken to emulate positive symptoms (Sturgeon et al., 1979; Nabeshima et al., 1987), deficits in social behaviour (Sams-Dodd, 1995, 1998) and immobility in the forced swim test (Noda et al., 1995, 2000) as behavioural models for negative symptoms, and impairments in various learning paradigms to imitate the cognitive deficits seen in patients with SCZ (reviewed by Mouri et al., 2007). Another behavioural model for SCZ involves a phenomenon called pre-pulse inhibition (PPI) wherein, in healthy individuals, the application of a small stimulus (typically auditory) before a strong stimulus reduces or inhibits the response to the stronger stimulus. This PPI function is impaired in most patients with SCZ and is linked to dysfunctions in sensorimotor gating and protection from sensory overload that can be modeled in animals (Braff et al., 1992; Lipska et al., 1995). Administration of PCP or (+)-D-aspartate 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-iminedizocilpine (MK-801) to postnatal rats during key neurodevelopmental periods induces PPI deficits in adulthood, linking glutamate to this important model of SCZ (Harris et al., 2003; Takahashi et al., 2006).
1.4 Schizophrenia Pharmacotherapy

Antipsychotic medications are the mainstay in the pharmacological treatment of SCZ (reviewed by Tandon, 2011). This section begins with an overview on the history and mechanism of action of antipsychotic drugs in psychiatry, with a particular focus on clozapine. Next, a brief review on the use of symptom rating scales in measuring response will be presented. This section concludes with a discussion on CLZ’s unique efficacy for the treatment of TRS and factors that contribute to variability in CLZ response.

1.4.1 Antipsychotic Medication Overview

Prior to the advent of antipsychotic therapy, patients suffering from SCZ were chronically ill, confined to mental health hospitals and subjected to alternative treatment options such as electroconvulsive shock therapy (ECT) and lobotomies, in which a portion of the patient’s brain was removed (Moniz, 1937). The pioneer typical (first-generation) antipsychotic drug, chlorpromazine (CLZ), was serendipitously discovered in the early 1950s following attempts to develop a synthetic anti-malaria drug during WWII (Gilman et al., 1944). Rather than use as an anti-malaria agent, CPZ was found to have anaesthetic-like properties with added benefits: patients administered CPZ required lower doses of anesthetic and were able to better withstand the stress of surgical trauma (Laborit & Huguenard, 1951). CPZ was initially supplied to two hospitals in Paris – the Central Military Hospital called Val-de-Grâce and the psychiatric clinic of Sainte-Anne Hospital, to treat psychiatric disorders (Delay et al., 1952). The first patient administered CPZ was a 57-year-old laborer admitted to hospital due to erratic and uncontrolable behaviour. Following 3-weeks of CPZ treatment, the patient appeared normal and was discharged (Hamon et al., 1952). In the decades
following, other typical APs were developed including: haloperidol, fluphenazine, thioridazine, loxapine, perphenazine, trifluoperazine and thiothixene.

Very shortly after the introduction of CPZ and its derivatives, extrapyramidal symptoms including parkinsonism and akathisia became recognized as side effects associated with the use of APs (Steck, 1954; Sigwald et al., 1959). From efforts to develop an AP that was free of EPS came the birth of the second generation (atypical) APs, the first of which was clozapine (CLZ). First manufactured by G. Stille at Wander Pharmaceuticals in Bern, Switzerland in the early 1960s, CLZ was associated with little to no EPS (Hippius, 1996). After two open studies in 1966 (Gross & Langner, 1966; Bente et al., 1966) and one double-blind study in 1971 (Angst et al., 1971), CLZ was briefly marketed yet quickly withdrawn due to reports of a life-threatening side effect in Finland populations (reviewed by Idanpaan-Heikkila et al., 1997). This side effect is now recognized as clozapine-induced agranulocytosis (CIA), which occurs in ~1% of CLZ-treated patients. CLZ was reintroduced into the US market in 1990 following a landmark clinical trial that proved CLZ’s superior efficacy for TRS patients (Kane et al., 1988). CLZ is currently a 3rd line treatment for patients with treatment-resistant or intolerant forms of SCZ and is prescribed in combination with mandatory blood monitoring (Moore et al., 2007). CLZ also has the propensity to cause weight gain and metabolic syndrome. CLZ’s success quickly led to the development of additional drugs in the atypical class including: risperidone, olanzapine, quetiapine, ziprasidone, aripiprazole, paliperidone, iloperidone, asenapine and lurasidone.

1.4.2 The Mechanism of Action of Antipsychotic Drugs

All AP medications share a common primary mechanism of action, which involves blockade of DRD2 (Carlsson & Lindqvist, 1963; Seeman & Lee, 1975; Creese et al., 1976). For this
reason, DRD2 has been an important access point to the circuitry underlying the symptoms of SCZ, however, may not be the primary mechanism through which CLZ achieves therapeutic effect. All AP medications have variable binding profiles and affinities for different combinations of receptors. CLZ’s multi-receptor profile likely accounts for its ability to improve some domains of cognition and social function for patients with TRS (Hagger et al., 1993; McGurk, 1999). Besides having a weaker affinity for DRD2, CLZ has a high affinity for 5-HT2A, histamine H1 (HRH1/H1), muscarinic M1 (CHRM1/M1) and adrenergic α-1A receptor (ADRA1A) (Meltzer et al., 1989a), and a moderate affinity for the serotonin type 1A receptor (5-HT1A) and the adrenergic receptor α-2A receptor (ADRA2A) (Schotte et al., 1996). This binding profile, more specifically the weak affinity for 5-HT2A compared to DRD2, is thought to account for CLZ’s lower propensity to cause EPS (Meltzer et al., 1989b).

1.4.3 Treatment Response Measures

Response to AP medication is commonly assessed using symptom rating scales (reviewed by Mortimer, 2007). Several scales assessing symptom severity in SCZ have been used for this purpose, the most common of which are the Brief Psychiatric Rating Scale (BPRS) (Overall & Gorham, 1962), the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987) and the Clinical Global Impression Scale (CGI) (Guy, 1976). Symptom severity is assessed at baseline prior to treatment and then again following a trial of AP medication. Change in symptom scores from baseline can be used as either a categorical measure of response in which ‘responders’ are those who exhibit a decrease in scores beyond a certain threshold, or a continuous response measure whereby percent score reduction is calculated using the following formula: % score change = [(6 month score - baseline score)/(baseline score)] x
100%. The threshold used to define categorical response in this thesis was a 20-point or more decrease in BPRS scores. This measure of response is commonly used, however, is somewhat arbitrary and fails to reflect a patient’s personal, social and cognitive functioning – factors that hold great weight as to how a patient will function in society (Mortimer, 2007). Despite this limitation, the use of rating scales to assess response has proven useful in research studies.

1.4.4 Clozapine for Treatment-Resistant Schizophrenia

As mentioned in Section 1.1.3, TRS is particularly burdensome to the healthcare system and drastically decreases the likelihood of optimal disease outcome. The most commonly used definition of TRS involves: 1) failure to respond to at least two periods of treatment in the preceding five years with neuroleptic agents from at least two different chemical classes (doses equivalent ≥1000 mg/day of CPZ for six weeks) without significant symptomatic relief; and 2) no period of good functioning within the preceding five years (Kane et al., 1988). This definition of TRS first emerged from the landmark clinical trial, which proved CLZ was superior to CPZ for treatment resistant forms of SCZ (Figure 1.3). More specifically, 268 patients who met criteria for TRS were entered into a double-blind comparison study that lasted 6 weeks. Patients were randomly assigned to either one of two groups: CLZ (up to 900mg/day) or CPZ (up to 1,800mg/day). Response was defined as: 1) a 20% decrease or more in BPRS total score; 2) a CGI score less than or equal to 3; and 3) a BPRS total score equal to or less than 35, using 0-6 ratings. Results showed that a significantly greater number of treatment-resistant patients responded to CLZ than to the prototypical AP CPZ (30% vs 4%, p=0.001). The results of this trial were responsible for
restoring CLZ to a position of utility in psychiatry for the treatment of patients who have failed at least two previous drug trials or for those who develop intolerable side effects.

![Graph showing comparison between clozapine and chlorpromazine in treating treatment-resistant schizophrenia](image)

**Figure 1.3 Clozapine is Superior to Chlorpromazine for Treatment-Resistant Schizophrenia**

Results from a landmark clinical trial illustrated clozapine’s unique efficacy over the prototypical antipsychotic chlorpromazine (CPZ) for the treatment of persistent positive symptoms in patients deemed resistant to conventional pharmacotherapy. Mean change in total Brief Psychiatric Rating Scale (BPRS) scores were plotted each week of the study for patients treated with clozapine (solid line, n=126) or chlorpromazine with adjunct benztropine mesylate therapy (broken line, n=139) (p<0.001). Reprinted from *Archives of General Psychiatry* 45(9). John Kane, Gilbert Honigfield, Jack Singer, Herbert Meltzer and Clozaril Collaborative Study Group. Clozapine for the Treatment-Resistant Schizophrenic: A Double-blind Comparison with Chlorpromazine, 789-96. Copyright (1988) with permission from Elsevier.
Several additional trials have been conducted assessing CLZ’s unique efficacy for TRS. For instance, one such study comparing clozapine, haloperidol, olanzapine and risperidone (CHOR) in TRS found that CLZ and OLZ had similar antipsychotic efficacy, however, CLZ was the most effective for treating negative symptoms (Volavka et al., 2002). Additional studies indicate CLZ shows greater benefits for TRS as compared to OLZ (Conley et al., 1999; Meltzer et al., 2008). Results from the Clinical Antipsychotic Trials of Intervention Effectiveness Project (CATIE) also suggest that CLZ is superior to other AP drugs for treatment resistance (McEvoy et al., 2006). Briefly, CATIE was a double-blind randomized clinical trial funded by the National Institute of Mental Health (NIMH) that compared the effectiveness of first- and second-generation APs used to treat SCZ. Patients who discontinued treatment in the first phase with one of four newer atypical APs (olanzapine, quetiapine, risperidone or ziprasidone) because of inadequate efficacy (n=99) were randomly assigned to either open-label CLZ (n=49) or blinded treatment to an alternative atypical in the second phase. Discontinuation due to lack of efficacy was significantly less in the CLZ group than in the other four groups (11% vs ~40%). It should be noted that this study disallows for firm conclusions regarding the advantages of CLZ because of the unblinded nature with which CLZ was used. However, given the results from other studies, CLZ does appear to be superior for TRS.

1.4.5 Variability in Clozapine Response

Response to CLZ is highly variable and likely influenced by a combination of clinical, demographic, environmental and genetic factors (reviewed by Arranz & Munro, 2011). Non-genetic factors that alter clinical response include: treatment adherence (Dunbar-Jacob & Mortimer-Stephens, 2001), previous drug exposure and duration of illness (Perkins et al.,
diet, smoking and concomitant medications (Meyer, 2001; de Leon, 2004), as well as
gender, ethnicity and age (Palego et al., 2002). Response to APs is also overshadowed by the
occurrence of adverse side effects that result in less than optimal treatment outcomes.
Regarding genetic factors, twin and family studies of CLZ response indicate concordance for
response (Vojvoda et al., 1996). Like SCZ, response to CLZ is thought to be a complex trait
likely depending on the multiplicative effect of several genes that are involved in drug action
(International Schizophrenia Consortium et al., 2009; Sadee, 2013). However, drug response
phenotypes likely depend on fewer genes than those involved in disease etiology due to the
relatively narrow pathway a drug follows through the body to mediate therapeutic effect
(Johnson et al., 2011; Ma & Lu, 2011). Identifying genetic variation with the ability to
improve treatment outcome remains an active area of research and is discussed in the
following section.
1.5 Pharmacogenetics of Clozapine Response

Pharmacogenetics (PGx) is the study of gene variation as it relates to drug response and the development of adverse side effects. This section will touch on the history of PGx, summarize findings from PGx studies on CLZ response with a particular focus on glutamate system related genes, and will conclude by exhibiting how ENCODE data can be used to select functional candidate SNPs for PGx studies.

1.5.1 Pharmacogenetics Overview

The roots of pharmacogenetics travel as far back as 1866 with the work of Gregor Johann Mendel (1822-1884) on the garden pea (Pisum sativum L.) and the establishment of the rules of heredity (Mendel, 1866). Since Mendel’s time, there have been several noteworthy individuals who have contributed to our present day understanding of the relationship between genetics and drug response. Noteworthy contributions include: 1) twin studies indicating the influence of multiple genes on the pharmacokinetics of various drugs between the years of 1957-1970 (Vesell, 1978); 2) development of the term ‘pharmacogenetics’ by Friedrich Vogel in 1959 (Vogel, 1959); and 3) revolutionary works by a man named Werner Kalow (1917-2008), deemed the ‘grandfather of pharmacogenetics’, who published the first monograph on PGx entitled ‘Phamacogenetics – Heredity and the Response to Drugs’ in 1962 (Kalow, 1962). The advent of polymerase chain reaction (PCR) in 1985 was also a significant contribution to PGx as it paved the way for gene cloning experiments and allowed for further characterization of genes involved in drug response (Mullis et al., 1986). Shortly thereafter in 1987, a very important family of liver enzymes called the cytochrome P450 enzymes (CYPs), such as CYP2D6 and CYP1A2, were cloned and polymorphisms within these genes were linked to drug response (Distlerath et al., 1985). CYP enzymes are
particularly relevant to the field of psychiatry because they are responsible for metabolizing a large proportion of the currently prescribed antipsychotics and antidepressants (reviewed by Guengerich, 2008).

Present day pharmacogenetics studies in psychiatry hope to elucidate gene variants that alter response to psychotropic medications. The current prescribing method for AP medications utilizes a ‘one size fits all’ approach and a suitable drug is identified through trial and error. During this trial and error period, a subset of patients is plagued by lack of efficacy and side effects due to toxicity. Generalized treatment plans are designed to satisfy the needs of the majority of patients and do not account for patients that lie at the extremes of the distribution. Treatment plans also fail to consider the biological patient-specific differences that cause certain individuals sharing similar symptoms to react differently to the same medication. Pharmacogenetics studies seek to identify the portion of patient-specific differences that lie within the genome. Once predictive genetic markers are identified, they can be used to guide therapeutic dosing and to warn against increase risk for developing adverse drug reactions (Phillips et al., 2001). To illustrate current advancements in the field of psychiatry thus far, positive results involving the use of genetic testing in depression have been reported (Hall-Flavin et al., 2013; Winner et al., 2013; Altar et al., 2015). In addition, our Neurogenetics section at the Centre for Addiction and Mental Health (CAMH) in Toronto is currently leading a randomized control trial of an enhanced version of the GeneSight Psychotropic Test for treatment response in SCZ.

1.5.2 Clozapine and Glutamatergic Neurotransmission

An important aspect of PGx studies is variant selection. Variants are often selected from genes involved in known pharmacodynamic and pharmacokinetic pathways that relate to
drug action, and often include variants that are located within drug metabolizing enzymes, membrane transporters, receptors and target proteins (Sadee, 2013). A thorough understanding of drug action can help guide selection of genetic variants that may play a role in response. As mentioned above, CLZ has a unique binding profile and may achieve therapeutic effect in part by modulating glutamatergic neurotransmission. A great deal of evidence supports this theory and is summarized in Table 1.2. The information presented in this table was used to guide gene selection for the work presented herein.

**Table 1.2 Evidence Supporting Clozapine Interacts with the Glutamate System**

<table>
<thead>
<tr>
<th>Main Findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CLZ Attenuates NMDAR Antagonist-induced Behaviours in Preclinical Models</strong></td>
<td></td>
</tr>
<tr>
<td>PPI Impairments</td>
<td></td>
</tr>
<tr>
<td>Clozapine (5.0mg/kg) antagonized the ability of PCP (1.0mg/kg) as well as MK-801 (0.5mg/kg) to repeatedly and robustly decrease PPI in a rat model, without interfering with PPI itself</td>
<td>(Bakshi et al., 1994)</td>
</tr>
<tr>
<td>Clozapine (7.5kg/mg), but not haloperidol (0.1mg/kg), significantly restored PPI in ketamine (1, 3, or 6mg/kg) treated rats</td>
<td>(Swerdlow et al., 1998)</td>
</tr>
<tr>
<td>Clozapine (2.5mg/kg) but not haloperidol (0.035mg/kg) successfully reversed PCP-induced PPI deficits in a primate model (Cebus apella)</td>
<td>(Linn et al., 2003)</td>
</tr>
<tr>
<td>Clozapine (5 or 10 mg/kg), but not risperidone (0.1 and 1mg/kg) restored deficits in PPI induced by MK-801 (0.3mg/kg) in a rat model</td>
<td>(Bubenikova et al., 2005)</td>
</tr>
<tr>
<td>Clozapine (1.25 or 2.5mg/kg) significantly attenuated PPI-impairments induced by MK-801 (0.05mg/kg) in a rat model</td>
<td>(Levin et al., 2007)</td>
</tr>
<tr>
<td><strong>Hyperlocomotion</strong></td>
<td></td>
</tr>
<tr>
<td>Clozapine and haloperidol (variable doses) were moderately effective in blocking PCP-induced (5.0mg/kg) locomotor stimulation in rats</td>
<td>(Freed et al., 1984)</td>
</tr>
<tr>
<td>Clozapine (2.5 or 5mg/day) and olanzapine (0.25 or 0.5mg/kg) potently antagonized MK-801-induced (0.05 or 1.1mg/kg) locomotion and falling assay (MK-801-LF) in rats</td>
<td>(Corbett et al., 1995)</td>
</tr>
<tr>
<td>Clozapine (5-20mg/kg) and haloperidol (0.01-0.1mg/kg) progressively potentiated hyperlocomotion induced by PCP administration (3.2mg/kg) in rats</td>
<td>(Sun, Hu &amp; Li, 2009)</td>
</tr>
</tbody>
</table>
## Cognitive and Social Deficits

| Acute administration of clozapine (5mg/kg) but not haloperidol (0.05mg/kg) or chlorpromazine (2mg/kg) to rats significantly reversed PCP-induced cognitive impairments using an operant reversal-learning paradigm following sub-chronic PCP administration (2mg/kg, twice daily for 7 days) | (Abdul-Monim et al., 2006) |
| Clozapine (2.5 or 5mg/day) and olanzapine (0.25 or 0.5mg/kg) significantly reversed social withdrawal induced by PCP (2mg/kg) in rats | (Corbett et al., 1995) |

## CLZ Alters Glutamate Receptor Expression, Density and Binding

### NMDAR

*In situ* hybridization of riboprobes for NMDAR subunits NR2A, NR2B and NR1 splice variants indicated increased NR1 and NR2B subunit expression in rat brains following subchronic administration of clozapine (30mg/kg) | (Meshul et al., 1996) |

Chronic clozapine administration (30mg/kg/day for 21 days) resulted in a 20% increase of NMDA receptors localized to the dentate gyrus of the hippocampus in rat brains | (Giardino et al., 1997) |

Chronic administration of clozapine (30mg/kg/day for 30 days) increased NMDAR binding in insular and parietal cortices in rat brain using radio-ligand binding and quantitative autoradiography with [*H]CGP 39653 | (Ossowska et al., 1999) |

Rats chronically administered clozapine (45mg/kg/day for 6 months) showed up-regulated NMDAR binding in the nucleus accumbens, and down-regulated expression of NR2A in the hippocampus and PFC and NR1 in the dorsolateral PFC as indicated by quantitative receptor autoradiography with [*H]MK-801 | (Schmitt et al., 2003) |

*In vivo* single photon emission tomography (SPET) using the NMDAR tracer [*123*I]CNS-1261 indicated significant reductions in relative NMDAR binding in the left hippocampus of medication-free, but not clozapine-treated SCZ patients, as compared to healthy subjects | (Pilowsky et al., 2006) |

### AMPAR

Immunoblotting procedures in rat brains showed clozapine administration (35-40mg/kg) significantly increased AMPAR subunit GluR1 expression in the medial prefrontal cortex (mPFC) | (Fitzgerald et al., 1995) |

Clozapine administration significantly increased AMPAR density in the frontal and anterior cingulated cortices compared with normal controls in a rat model | (Spurney et al., 1999) |

### CLZ Increases Glutamate Concentrations

Acute administration of clozapine (25mg/kg) but not haloperidol (0.5 or 1.0mg/kg) significantly increased extracellular glutamate levels over time in the mPFC of free-moving rats as indicated by *in vivo* intracerebral microdialysis | (Daly & Moghaddam, 1993) |

Chronic clozapine administration (20mg/kg/day for 21 days) stimulated glutamate release from the nucleus accumbens in rats as indicated by *in vivo* microdialysis | (Yamamoto & Cooperman, 1994) |

Clozapine treatment significantly increased serum glutamate concentrations in 7 patients with SCZ after switching from conventional neuroleptics | (Evins et al., 1997) |
CLZ Activates Excitatory Glutamatergic Neurotransmission

| Clozapine administration (20mg/kg) potentiated NMDAR-mediated synaptic responses in the dentate gyrus of chronically prepared rabbits | (Kubota et al., 1996, 2000) |
| Clozapine administration produced a marked facilitation (300-400%) of NMDAR-evoked responses and produced bursts of EPSPs caused by increased release of excitatory amino acids in pyramidal cells of the mPFC in rat brain slices as indicated by intracellular recording and single-electrode voltage clamp | (Arvanov et al., 1997; Chen et al., 2003; Ninan et al., 2003) |
| Administration of CLZ reversed PCP effects on pyramidal cell firing and cortical synchronization as recorded extracellularly with glass micropipettes, and prevented PCP-induced increases in c-fos expression in PFC pyramidal neurons as indicated by in situ hybridization | (Kargieman et al., 2007) |

To summarize, CLZ is thought to interact with the glutamate system due to its ability to block NMDAR antagonist-induced behaviours in preclinical models including: PPI impairments induced by PCP (Bakshi et al., 1994; Linn et al., 2003), MK-801 (Bubenikova et al., 2005; Levin et al., 2007) and ketamine (Swerdlow et al., 1998), as well as hyperlocomotion, social deficits and impaired cognitive function induced by PCP and MK-801 (Freed et al., 1984; Corbett et al., 1995; Abdul-Monim et al., 2006; Sun et al., 2009).

Functional studies in preclinical models also suggest that CLZ alters glutamate receptor subunit expression, binding and density. More specifically, CLZ administration has been shown to: increase AMPAR density in the frontal and anterior cingulate cortices; increase GluR1 expression in the medial prefrontal cortex (mPFC) (Fitzgerald et al., 1995; Spurney et al., 1999); increase NR1 and NR2B expression in select brain regions (Meshul et al., 1996); decrease NR2A expression in the PFC and hippocampus (Schmitt et al., 2003); increase NMDAR expression in the hippocampus (Giardino et al., 1997); increase NMDAR binding in insular and parietal cortices, as well as nucleus accumbens (NAcc) (Ossowska et al., 1999); increase mGluR2 signaling (Fribourg et al., 2011); decrease EAAT2 expression in cerebral cortex (Melone et al., 2001); increase synaptic glycine concentrations (Javitt et al., 2011).
down-regulate promoter methylation of **GAD1** (Dong et al., 2008). Lastly, an *in vivo* single photon emission tomography (SPET) study using the NMDAR tracer $[^{123}]$CNS-1261 showed that CLZ significantly reduced relative NMDAR binding in the left hippocampus of medication-free, but not clozapine treated SCZ patients, as compared to healthy subjects (Pilowsky et al., 2006).

CLZ also alters glutamate concentrations and activates excitatory glutamate neurotransmission. More specifically, CLZ administration has been shown to: increase extracellular glutamate levels in the mPFC and NAcc as evidenced by *in vivo* micro-dialysis in free-moving rats (Daly & Moghaddam, 1993; Yamamoto & Cooperman, 1994); increase amplitude of excitatory postsynaptic potentials (EPSPs) elicited by single electrical stimulation in the dentate gyrus of chronically prepared rabbits (Kubota et al., 1996, 2000); increase EPSPs in pyramidal cells of the mPFC in rat brain slices (Arvanov et al., 1997; Chen & Yang, 2002; Ninan et al., 2003); and increase serum glutamate concentrations in SCZ subjects switched from conventional neuroleptics to CLZ (Evins et al., 1997). Altogether, these findings suggest that CLZ’s therapeutic benefits may in part be mediated by re-establishing a balance in glutamatergic neurotransmission. Therefore, genetic variation within glutamate system-related receptors, transporters and enzymes may be ideal candidates for PGx studies in CLZ response.

### 1.5.3 Polymorphisms in Glutamate System and Related Genes

A comprehensive review on the pharmacogenetics of CLZ response has recently been conducted (Kohlrausch, 2013). Studies investigating the association among glutamate system genes and CLZ response are summarized in Table 1.3 below.
<table>
<thead>
<tr>
<th>Gene/polymorphism</th>
<th>Sample</th>
<th>Main Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRIN1 rs11146020</td>
<td>N=183 European-Caucasian &amp; N=49 African-American SCZ/SA patients deemed resistant/intolerant to AP therapy; Response assessed using BPRS following 6 months of CLZ therapy</td>
<td>All genotype, allele and haplotype association analyses were negative</td>
<td>(Hwang et al., 2011)</td>
</tr>
<tr>
<td>GRIN2A GT-repeat</td>
<td>N=183 European-Caucasian &amp; N=49 African-American SCZ/SA patients deemed resistant/intolerant to AP therapy; Response assessed using BPRS following 6 months of CLZ therapy</td>
<td>All genotype, allele and haplotype association analyses were negative</td>
<td>(Hwang et al., 2011)</td>
</tr>
<tr>
<td>GRIN2B rs1806201</td>
<td>N=100 Chinese SCZ/SA patients deemed resistant/intolerant to AP therapy; Response assessed using BPRS after a minimum of 8 weeks</td>
<td>No significant associations were reported, however, CC carriers had a marginally higher mean clozapine dosage (p=0.013)</td>
<td>(Hong et al., 2001)</td>
</tr>
<tr>
<td>GRIN2B rs1806201</td>
<td>N=100 Chinese SCZ/SA patients deemed resistant/intolerant to AP therapy; Response assessed using BPRS after a minimum of 8 weeks</td>
<td>No significant associations were reported, however, mean clozapine dosage was higher for CC carriers (p=0.18)</td>
<td>(Chiu et al., 2003)</td>
</tr>
<tr>
<td>GRIN2B rs10193895</td>
<td>N=183 European-Caucasian &amp; N=49 African-American SCZ/SA patients deemed resistant/intolerant to AP therapy; Response assessed using BPRS following 6 months of CLZ therapy</td>
<td>All genotype, allele and haplotype association analyses were negative</td>
<td>(Hwang et al., 2011)</td>
</tr>
<tr>
<td>NRXN1 rs1045881</td>
<td>N=169 European-Caucasian SCZ/SA patients deemed resistant or intolerant to AP therapy; Response assessed using BPRS following 6 months of CLZ therapy</td>
<td>Post hoc analysis indicated a genotype of rs1045881 was significantly associated with BPRS negative symptom score</td>
<td>(Lett et al., 2011)</td>
</tr>
</tbody>
</table>
Briefly, the neurexin-1 gene (*NXRN1*) was previously associated with CLZ response in our group (Lett et al., 2011). The rs1045881 variant was significantly associated with change in BPRS negative symptom score following six months of CLZ monotherapy (*p*=0.033). The neurexin-1 protein (NRXN1) is a neuron-specific cell-surface molecule necessary for normal activity of the NMDAR (Kattenstroth et al., 2004). The remaining glutamate system gene variants are polymorphisms located in the NMDAR subunit genes *GRIN1*, *GRIN2A* and *GRIN2B*. Unfortunately, all variants within these genes were reported to be non-significant with CLZ response (Hong et al., 2001; Chiu et al., 2003; Hwang et al., 2011). Two studies did, however, report that patients carrying the CC-genotype of *GRIN2B* variant rs1806201 required marginally higher mean CLZ dosages than the other genotype groups (Hong et al., 2001; Chiu et al., 2003). Given the fact relatively few pharmacogenetics studies investigating the contribution of glutamate system gene variation to CLZ response have been conducted thus far, additional studies are of high priority.

1.5.4 *Identifying Functional Gene Variants using ENCODE*

Gene variant selection can also be approached through the use of functional annotation databases such as the University of California Santa Cruz (UCSC) Genome Browser (Kent et al., 2002), Haploreg (Ward & Kellis, 2012), RegulomeDB (Boyle et al., 2012) and the National Institute for Environmental Health Sciences (NIEHS) Functional SNP Prediction Database (Xu & Taylor, 2009). These databases provide access to data generated by the Encyclopedia of DNA Elements (ENCODE) project. In more detail, the ENCODE project is a public consortium research effort launched by the US National Human Genome Research Institute (NHGRI) with the aim to decipher all of the functional elements within the human genome (ENCODE Project Consortium, 2012). Of interest to candidate SNP selection is
mapping of functional elements such as transcription factor binding sites (TFBS), DNase I hypersensitivity sites and regions of histone modification from data pooled from 1,640 data sets and 147 different cell types. Originally, a large portion of non-coding DNA identified during the Human Genome project was believed to have little importance (International HapMap Consortium, 2003), however, ENCODE reports the majority of the human genome (~80%) is found to be functional in some way. In fact, many non-coding variants and SNPs identified to be associated with disease through GWAS have been found to lie within ENCODE-annotated functional regions that lie outside of protein-coding genes. To illustrate the use of ENCODE data in SNP selection, Figure 1.4 shows search results displaying ENCODE functional data for the glycine transporter 1 gene (SLC6A9) variant rs16831558 genotyped within this study, as presented by the UCSC Genome Browser.

Figure 1.4 Identifying Functional Promoter Variants in SLC6A9 Using ENCODE Data

University of California Santa Cruz (UCSC) Genome Browser track results for the glycine transporter 1 gene (SLC6A9) approximately 5kb upstream from the promoter region. Search results highlight the location of the reference SNP rs16831558 under ‘Single Nucleotide Polymorphisms’ (dbSNP 142) (highlighted in the red box) relative to intron 1 of SLC6A9 (indicated by the green arrow). Search settings were customized using drop-down options located in the page below. More specifically, ‘Integrated Regulation from ENCODE’ under
‘Regulation’ was set to ‘show’ and ‘common SNPs (142)’ located under ‘Variation’ was set to ‘pack’. Functionally, the rs16831558 was associated with: Layered H3K27Ac histone mark (lysine 27 of the H3 histone protein acetylated), with colours indicating cell type in which H3K27Ac mark was identified [Blue: K562 (myelogenous leukocyte), Purple: NHEK (epidermal keratinocytes), Pink: NHLF (normal lung fibroblast)]; DNAse I Hypersensitivity Cluster represents areas of open chromatin where regulatory regions likely reside, with darkness of the boxes indicating extent of hypersensitivity; Txn Factor ChIP indicates regions of transcription factor binding as determined by chromatin immunoprecipitation (ChIP-seq) DNA binding motif assays; and Vista HMR-Conserved Non-Coding Human Enhancers from LBNL that identify distant-acting transcriptional enhancers in the human genome. This genome browser screen shot was constructed using human genome assembly version 19 and can be accessed by visiting: http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&position=chr1:44494763-44508289 (Kent et al., 2002)

Several functional elements reported in Figure 1.4 deserve further explanation. For instance, K27Ac is a histone mark (modified histone protein) in which lysine 27 of the H3 histone protein has been acetylated as determined by a ChIP-seq assay. This histone mark is thought to increase transcription possibly by blocking the spread of the repressive histone mark H3K27Me3 (tri-methylation of lysine 27 on H3) (Bernstein et al., 2005). Another functional element is the DNAse I Hypersensitivity sites that locate regions of open chromatin where regulatory elements such as promoters, enhancers and silencers likely reside (Song & Crawford, 2010). Transcription factor chromatin immunoprecipitation (Txn Factor ChIP) is also used and identifies regions of DNA where transcription factors (TF) are likely to bind. Transcription factors are proteins that bind to DNA and regulate gene expression by interacting with RNA polymerase (Wang et al., 2012). Lastly, the Vista human, mouse, rat (HMR)-Conserved Non-Coding Human Enhancers from Lawrence Berkeley National
Laboratory (LBNL) identifies distant-acting transcriptional enhancers in the human genome by coupling evolutionary conserved non-coding sequences with mouse enhancer assays (Pennacchio et al., 2006).

Pulling from the data presented in the previous sections, a total of 17 gene variants from 6 glutamate-system related genes with evidence of CLZ-interaction were selected for study inclusion (summarized in Table 2.1 & Table 3.1). Variants were selected based on previous investigation in neuropsychiatric phenotypes as cited in the literature, and/or potential functionality from information gathered from functional annotation websites. The first data chapter of this thesis investigated the association among eight gene variants in \textit{GRIN2B} with CLZ response. This gene encodes for the NR2B subunit of the NMDAR, contains 13 exons and is mapped to region 12p12 (Mandich et al., 1994; Endele et al., 2010). The second data chapter investigated the association among ten additional glutamate-system gene variants and response to CLZ. More specifically, the remaining ten variants included two variants in the mGluR2 gene, \textit{GRM2}, which maps to 3p21.1 (Joo et al., 2001), one variant in the AMPAR subunit GluR1 gene, \textit{GRIA1}, which maps to 5q31.1 (Puckett et al., 1991), three variants in EAAT2 encoded by \textit{SLC1A2} mapped to region 11p13-p12 (Li & Francke, 1995), three variants in GlyT1 encoded by \textit{SLC6A9} mapped to 1p33 (Jones et al., 1995), and lastly, one variants in the GAD1 enzyme gene, encoded by \textit{GAD1} that is mapped to region 2q.31 (Bu et al., 1992). The 17 glutamate system gene variants investigated in this study include: \textit{GRIN2B} (rs7301328, rs1072388, rs12826365, rs2284411, rs1806201, rs1806191, rs3764030, rs890); \textit{GRM2} (rs4067, rs2518461); \textit{SLC1A2} (rs4354668, rs4534557, rs2901534); \textit{SLC6A9} (rs12037805, rs1978195, rs16831558); \textit{GRIA1} (rs2195450); and \textit{GAD1} (rs3749034).
1.6 Study Rationale

1.6.1 Summary and Hypothesis

Treatment-resistant schizophrenia is a debilitating, costly and burdensome disorder that affects approximately 30-40% of patients diagnosed with SCZ. Disease prognosis is highly dependent upon successful early intervention strategies and stresses the utility of having the ability to predict likelihood of response before a drug is prescribed. Pharmacogenetics research aims to unravel the genetic underpinnings of drug response and to develop personalized treatment plans that are capable of guiding adjusted therapeutic doses, decreasing adverse drug reactions, improving treatment adherence and providing patients with a better quality of life.

This study was designed to test for associations among glutamate system gene variants and clinical response to CLZ in SCZ or schizoaffective (SA) disorder patients deemed resistant or intolerant to previous AP therapy. We hypothesize that putatively functional variants with the potential to alter glutamatergic neurotransmission may in some way interact with CLZ’s ability to achieve therapeutic effect, and may lead to variable response among study participants. In total, 17 glutamate system gene variants were selected for study inclusion, many of which were reported to be functional using ENCODE data.

By investing time, effort and resources in studying the relationship between putatively functional glutamate system gene variants and response to CLZ, we ultimately hope to contribute to the ever-evolving field of pharmacogenetics, and help make personalized medicine a better-established approach in psychiatric care.
Chapter 2

GENETIC ASSOCIATION ANALYSIS OF THE NMDA RECEPTOR SUBUNIT GENE GRIN2B WITH RESPONSE TO CLOZAPINE

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\textsuperscript{4}Department of Psychiatry, University of California, Irvine, CA, USA
\textsuperscript{5}Northwestern University Feinberg School of Medicine, Chicago, IL, USA
\textsuperscript{6}Department of Psychiatry, University of Toronto, Toronto, ON, Canada
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This chapter was modified from the following:


Key Words: GRIN2B, pharmacogenetics, clozapine response, glutamate, schizophrenia
2.1 ABSTRACT

Approximately 30% of patients with schizophrenia fail to respond to antipsychotic therapy and are classified as having treatment-resistant schizophrenia. Clozapine is the most efficacious drug for treatment-resistance and may deliver its superior therapeutic effects partly by modulating glutamatergic neurotransmission. Response to clozapine is highly variable and may depend on genetic factors as indicated by twin studies. This study investigated eight single nucleotide polymorphisms in the N-methyl-D-aspartate glutamate receptor subunit gene GRIN2B with response to clozapine. Standard TaqMan procedures were used to type rs7301328, rs1072388, rs12826365, rs2284411, rs1806201, rs1806191, rs3764030 and rs890 in 175 European schizophrenia/schizoaffective patients deemed resistant or intolerant to previous antipsychotic therapy. Response was assessed using change in Brief Psychiatric Rating Scale (BPRS) scores following six months of clozapine therapy. Categorical and continuous response was assessed using Pearson’s chi-squared test and analysis of covariance, respectively. No associations were observed between the variants and response to clozapine. A-allele carriers (AA and AG) of rs1072388 responded marginally better to clozapine therapy than GG-homozygotes, however the difference was not statistically significant ($p_{uncorrected}=0.067$, $p_{corrected}=0.440$, assuming 6.56 independent tests). Our findings do not support a role for these GRIN2B variants in altering response to clozapine in our sample.
2.2 INTRODUCTION

Treatment-resistant schizophrenia occurs in approximately 30% (20-40%) of patients treated with antipsychotic drugs (Borgio et al., 2007; Elkis & Meltzer, 2007; Solanki et al., 2009). TRS patients experience suboptimal response characterized by persistent positive symptoms that often result in long periods of hospitalization (McGlashan, 1988) and chronic severe disability. Treatment resistance remains an immense burden to both patients and their families and is associated with annual healthcare costs ranging 3-to-11 times higher than schizophrenia patients who respond to treatment ($66,360-$163,795 vs. $15,500-$22,300 per year). TRS is also associated with poorer quality of life that is approximately 20% lower than patients with SCZ who achieve remission (0.61 vs. 0.75) (Kennedy et al., 2014).

The atypical AP drug clozapine is particularly effective for treating the persistent symptoms experienced by patients with TRS. A landmark clinical trial conducted by Kane et al. showed that approximately 30% of TRS patients responded to CLZ versus only 4% of patients given the prototypical AP CPZ (p<0.001) (Kane et al., 1988). As an atypical AP, CLZ differs from typical APs in particular dimensions, such as decreased DRD2 receptor affinity (Altar et al., 1986) and lowered risk for adverse effects such as extrapyramidal symptoms and tardive dyskinesia (Pickar et al., 1992; Tandon and Fleischhacker, 2005). CLZ also has the ability to alter other neurotransmitter systems such as serotonin and glutamate (reviewed by Miyamoto et al., 2005).

A great deal of evidence suggests that CLZ augments glutamatergic neurotransmission (reviewed by Heresco-Levy, 2003). One pillar supporting this theory stems from observations that CLZ is capable of blunting the psychotomimetic effects of glutamate.
antagonists in SCZ patients (Malhotra et al., 1997). These antagonists, such as phencyclidine and ketamine, bind to the NMDAR and elicit schizophrenia-like symptoms in healthy controls and exacerbate psychotic symptoms in SCZ patients (Luby et al., 1959; Luisada, 1978; Javitt & Zukin, 1991; Lahti et al., 2001) – observations that form the basis of the ‘glutamate hypofunction hypothesis’ of SCZ. Collectively, these findings suggest that blockade and underactivity of glutamate signaling at the NMDAR contributes to the clinical presentation of SCZ. In turn, CLZ is theorized to offer therapeutic relief in part by augmenting glutamatergic signals.

Findings from preclinical models and functional studies support this theory. CLZ administration reverses PCP-induced psychotic-like behaviours such as hyper-locomotion (Freed et al., 1984; Sun et al., 2009; Zhao et al., 2012), social deficits (Corbett et al., 1995), enhanced immobility (Noda et al., 1995) and PPI deficits (Linn et al., 2003). Functional studies in both preclinical models and SCZ patients also indicate that CLZ alters glutamate receptor subunit expression (Fitzgerald et al., 1995; Meshul et al., 1996), binding (Giardino et al., 1997; Pilowsky et al., 2006), and density (Ossowska et al., 1999; Schmitt et al., 2003). In addition, CLZ appears to alter glutamate concentrations and activity of excitatory glutamate neurotransmission in different brain regions as exhibited by: micro-dialysis studies in rodents (Daly & Moghaddam, 1993; Yamamoto & Cooperman, 1994), increased amplitude of EPSPs in neuronal cell cultures (Banerjee et al., 1995; Arvanov et al., 1997; Kubota et al., 2000; Ninan et al., 2003; Kargieman et al., 2007), and increased serum glutamate levels in patients switched to CLZ (Evins et al., 1997). Taken together, these results provide evidence for CLZ’s ability to restore glutamate signaling – a factor that may distinguish CLZ as the most efficacious drug for treatment-resistant patients.
Clinical response to CLZ is highly variable (Bleehen, 1993; Davis et al., 2003) and an estimated 30-60% of TRS patients fail to respond to CLZ therapy (Kane et al., 1988; Lieberman et al., 1994). These patients are diagnosed with ultra treatment-resistant SCZ (URS) and various augmentation strategies for managing URS have proven inconclusive (Meltzer et al., 1989b; Lieberman et al., 1994; Remington et al., 2005). Because early intervention strategies with AP drugs attenuates disease prognosis, the ability to predict response prior to drug administration would have important clinical utility (Wyatt & Henter, 2001; Gunduz-Bruce et al., 2005; Perkins et al., 2005; Emsley et al., 2007). Given that a portion of the inter-individual variability in CLZ response is thought to be caused by genetic variation (Vojvoda et al., 1996; Horacek et al., 2001; Mata et al., 2001; Theisen et al., 2005; Hoyer et al., 2010), a number of pharmacogenetics studies investigating genetic variability in CLZ response have been conducted (reviewed by Kohlrausch, 2013). However, no predictive test for AP response utilizing this genetic information is currently in clinical use. The most notable gene variants investigated for association with CLZ response include those related to the dopamine, serotonin and to a lesser degree, glutamate neurotransmitter systems.

Glutamate is becoming increasingly popular in recent years due to novel therapies that target this neurotransmitter system (Heresco-Levy et al., 1996; Patil et al., 2007; Pinard et al., 2010; Hopkins, 2011). Additional advancements in SCZ genetics have also yielded promising genome-wide hits for glutamate genes in risk (GAIN Collaborative Research Group, 2007; Jia et al., 2012; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). More specifically, genome-wide significant associations with schizophrenia have been observed in glutamate system genes such as GRM3, GRIN2A, GRIA1 and GRIN2B. The GRIN2B gene (138252 [MIM]) codes for subunit 2B of the NMDAR and is of particular
interest. This gene contains 13 exons and is mapped to 12p12 (Mandich et al., 1994; Endele et al., 2010). Single nucleotide polymorphisms in GRIN2B have been investigated for associations with several neuropsychiatric disorders, including drug response phenotypes (Table 2.1). A small number of GRIN2B variants have also been studied in CLZ response – while no associations between rs1806201 (2664C/T) (Hong et al., 2001; Chiu et al., 2003) or rs1019385 (-200T/G) (Hwang et al., 2011) have been observed, the rs1805502 variant (T5988C) significantly predicted negative symptom change during CLZ therapy (Martucci & Kennedy, 2010). Associations between additional GRIN2B polymorphisms and CLZ response have not yet been investigated. This study examined for associations among eight GRIN2B variants and clinical response to CLZ monotherapy in a sample of treatment-resistant/intolerant patients.

2.3 METHODS

2.3.1 Subjects
One hundred and seventy-five patients meeting Diagnostic and Statistical Manual of Mental Disorders III-R or IV (DSM-III-R/IV) criteria for schizophrenia or schizoaffective disorder were included in this study (American Psychiatric Association, 1987, 1994). Patients were recruited from three clinical sites: Case Western Reserve University in Cleveland, Ohio (HY Meltzer, n=74), Hillside Hospital in Glen Oaks, New York (JA Lieberman, n=73), and the University of California at Irvine (SG Potkin, n=28). All patients were considered European based on self-reported ancestry. Informed consent was obtained from each study participant prior to enrollment in accordance with the Ethical Principles for Medical Research Involving
Human Subjects at the Centre for Addiction and Mental Health and with the Helsinki Declaration of 1975, as revised in 1989 (World Medical Association, 2013).

TRS was defined as failure to respond to two or more AP drug trials in the previous five years (involving drugs from two different chemical classes, with doses $\geq 1000$mg chlorpromazine equivalents for four to six weeks), accompanied by no period of good functioning within the preceding five years (Kane et al., 1988). Less than 15% of study participants met criteria for treatment intolerance, which was defined as the presence of moderate to severe TD and/or extreme EPS making treatment with therapeutic dosages intolerable (Lieberman et al., 1994). Before beginning CLZ treatment, patients underwent a two to four week wash-out period which involved no administration of pharmacotherapy unless clinically necessary. Following the washout period, all patients were treated with CLZ monotherapy, with mean dosages of 453 mg/d, for a period of six months or longer. Benzodiazepines were administered intermittently during the titration period. Throughout treatment, CLZ serum levels were monitored to ascertain adherence to the medication.

2.3.2 Response Measures

Baseline Brief Psychiatric Rating Scale (Overall & Gorham, 1962) scores were obtained at time of study enrollment. Following CLZ administration, response was evaluated after six months using two BPRS scoring methods. The first was a categorical responder/non-responder response measure that classified responders as individuals who experienced a $\geq 20\%$ decrease in BPRS total score. The second scoring method was a quantitative response measure that divided the BPRS items into three subcategories [positive (BPOS), negative (BNEG), and general (BPRS)]. Percent score reduction for each of these subscales was
calculated using the following equation: \[
\frac{\text{six month score} - \text{baseline score}}{\text{baseline score}} \times 100\%.
\]

2.3.3 SNP Selection

A literature search was conducted on PubMed for the following terms: “\text{GRIN2B}”, “NMDAR”, “\text{N-methyl-D-aspartate receptor}”, “clozapine” and “\text{pharmacogenetics}.” Literature pertaining to genetic association studies in psychiatric disorders and psychotropic drug response was reviewed to identify \text{GRIN2B} SNPs of interest. Variants previously investigated for an association with \text{CLZ} response or other psychiatric phenotypes were considered for inclusion. Altogether, eight \text{GRIN2B} polymorphisms were selected: rs1072388, rs12826365, rs1806191, rs2284411, rs3764030, rs7301328 (366G>C), rs1806201 (2664C>T), and rs890 (\textbf{Figure 2.1} & \textbf{Table 2.1}). These eight variants were assessed for potential functionality using the National Institute of Environmental Health Sciences Functional SNP Prediction (FuncPred) database (Xu & Taylor, 2009). Variants were deemed functional if alternate alleles were thought to have differential effects on gene transcription, translation, or splicing.

Variants rs1072388 and rs12826365 were the most significant \textit{p}-value SNPs from the Genetics Association Information Network (GAIN) schizophrenia GWAS from the European and African samples, respectively (GAIN Collaborative Research Group, 2007; Ayalew et al., 2012). Variant rs1806191 was previously investigated in SCZ risk and obsessive compulsive disorder (OCD) (Di Maria et al., 2004; Alonso et al., 2012), while variant rs2284411 had been investigated in attention deficit hyperactivity disorder (ADHD) (Dorval et al., 2007; Park et al., 2013). The promoter variant rs3764030 had previously been studied
in autism spectrum disorder (ASD) (Yoo et al., 2012) and is proposed to affect GRIN2B expression by altering transcription factor binding. Both rs7301328 and rs1806201 were investigated in a number of psychiatric phenotypes, including response to lithium (Szczepankiewicz et al., 2009) and CLZ (Hong et al., 2001). These SNPs both lie near intron-exon borders (within two base-pairs, specifically) and are proposed to regulate mRNA splicing. Lastly, rs890 had been investigated in SCZ risk (Di Maria et al., 2004), treatment-resistant depression (TRD) (Zhang et al., 2014), CLZ-induced OCD (CI-OCD) (Cai et al., 2013) and response to lithium (Szczepankiewicz et al., 2009). This variant lies in a micro RNA (miRNA) binding site at the 3’ untranslated region (UTR) of GRIN2B and is proposed to alter translation initiation.

2.3.4 DNA Isolation and Genotyping

Venous blood samples were collected from study participants and sent to CAMH in Toronto, Canada where genomic DNA was isolated using the high salt method (Lahiri & Nurnberger, 1991). GRIN2B genotypes were determined using TaqMan allele specific single tube assays, the ABI Prism®7500 Sequence Detection System and the ABI allelic discrimination software according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA). The reaction mixture consisted of 20ng of genomic DNA, 2X TaqMan® Universal Master Mix and 40X SNP Genotyping Assay for a final reaction volume of 10 µL. The polymerase chain reaction (PCR) protocol consisted of a denaturation step for 10 mins at 95°C, followed by 50 cycles of amplification that consisted of denaturation (92°C for 15 s) and annealing (60°C for 1 min). Genotype calls were confirmed by two independent researchers and 10% of the total sample was re-genotyped to ensure genotyping accuracy. Discordant genotypes were set as missing in the statistical analysis.
2.3.5 **Statistical Analysis**

Quality-control (QC) was carried out using PLINK software v1.07 (Purcell et al., 2007). To pass filtering, SNPs were required to have a $\geq 90\%$ genotyping rate and a minor allele frequency (MAF) of $\geq 0.05$. Individuals with a genotype success rate less than 75% across all markers (samples producing genotypes for five SNPs or less) were also excluded from analysis. All analyses were carried out using the Statistical Package for the Social Sciences (SPSS) v20 software (IBM Corp, Armonk NY, USA) and PLINK.

Descriptive statistics were obtained for each clinical site. Mean age and baseline values of BPRS, BPOS and BNEG subscale scores across sites were compared using Student’s t-test or analysis of variance (ANOVA), while frequency counts for gender and response rates were compared using Pearson’s chi-square test ($\chi^2$-test). Genotype and allele frequencies in the responder/non-responder (R/NR) groups were compared using $\chi^2$-test or Fisher’s exact test (for cell counts less than 5). Differences in percent score reduction in BPRS, BPOS and BNEG subscales between genotype groups were compared using analysis of covariance (ANCOVA), co-varying for age and baseline scores.

Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) among SNPs were determined using Haploview v4.2 using the Solid Spine of LD to construct LD blocks (Figure 2.2) (Barrett et al., 2005). Haplotype analysis was carried out in UNPHASED v3.1.5 (Dudbridge, 2008). Haplotypes with $<5\%$ frequency were removed from analysis. Power calculations were conducted in Quanto v1.2.4 (Gauderman & Morrison, 2006). Correction for the effective number of independent markers was performed using the method described by Nyholt and resulted in a statistical significance threshold of $p<0.0076$ (Nyholt, 2004).
2.4 RESULTS

2.4.1 Sample Characteristics

Of the eight SNPs genotyped, seven met QC criteria and were included in subsequent association analyses. The genotype distribution for variant rs7301328 significantly deviated from HWE ($p=1.93 \times 10^{-7}$) and was therefore excluded. The remaining SNPs had a mean genotyping rate of 99.1%. Regarding study participants, 18 patients were removed due to low genotyping efficiency (<75% of markers were successfully genotyped). The remaining sample had a mean genotyping efficiency of 98.9%.

Following QC, 157 European treatment-resistant or intolerant patients were included in the study (Table 2.2). Our sample had over 80% power to detect an odds ratio (OR) as low as 2.00 (unmatched case-control design: n=157, non-responder frequency=48.4%, MAF=30.5%, $\alpha/2=0.05$) and down to 8.5% variance in the quantitative response variable (continuous design: n=85). The average minor allele frequency across the seven GRIN2B variants (excluding rs7301328) was used for power calculations. Patients between clinical sites did not differ in gender, categorical response rates, or BPRS/BPOS/BNEG baseline scores. The sites did differ, however, in mean age ($F(2,157)=7.10$, $P=0.001$). Post hoc analysis showed that the average age of the HY Meltzer sample was significantly lower than the SG Potkin sample. In order to combine samples for analysis, age and baseline BPRS/BPOS/BNEG scores were added as covariates to account for any disparities. Following QC, the final combined sample (n=157) consisted of 76.4% male patients and had a total mean age of 35.06 years (SD 8.1). Approximately half (51.6%) of the sample was
considered CLZ ‘responders’ (n=81). Quantitative response measures were available for a subset of patients in the BPRS (n=86), BPOS (n=84), and BNEG (n=85) subscales.

2.4.2 GRIN2B SNP and Haplotype Association Analysis

Genotype and allele frequencies for each of the seven GRIN2B variants were not significantly different between CLZ responder and non-responder groups or as measured by the quantitative percent score reduction in BPRS, BPOS and BNEG subscales (Table 2.3). Variant rs1072388 A-allele carriers (AA and AG) responded marginally better to CLZ therapy than GG-homozygotes, however this difference was not statistically significant (p<sub>uncorrected</sub>=0.067) (Figure 2.3). Haplotype association analyses confirmed allele and genotype findings with no association reported across all haplotypes tested (Table 2.4).

2.5 DISCUSSION

We report no significant associations among the seven GRIN2B variants investigated and response to CLZ in our sample of patients. Despite A-allele carriers of the intronic variant rs1072388 responding marginally better to CLZ therapy than GG-homozygotes (p<sub>uncorrected</sub>=0.067, genotype; p<sub>uncorrected</sub>=0.069, allele), this observation was statistically non-significant (p<sub>corrected</sub>=0.440, assuming 6.56 independent tests). To our knowledge, this is the first reported study to investigate associations of the remaining seven SNPs (rs1072388, rs3764030, rs12826365, rs2284411, rs1806191, rs890 and rs7301328) with CLZ response. The rs1806201 marker has previously been investigated in two other CLZ response samples (Hong et al., 2001; Chiu et al., 2003). Each aforementioned variant was selected based on previous investigation in CLZ response or other psychiatric phenotypes. After inclusion, the
potential functionality of these variants was assessed using the NIEHS Functional SNP Prediction Database (Xu & Taylor, 2009).

Our negative finding for rs1806201 (2664C/T) is in accordance with previous reports that this variant was not associated with CLZ response (Hong et al., 2001; Chiu et al., 2003). These studies did report a marginally higher mean CLZ dosage for patients carrying the CC genotype of rs1806201. Due to the inter-individual variability in CLZ metabolism and absorption, future studies may benefit from measuring serum CLZ levels as opposed to dosage in order to predict response. An optimal threshold for CLZ serum levels has already been established (≥450 ng/mL) and may be of clinical utility to identify non-responders (Lindenmayer & Apergi, 1996).

As a measure of genotyping quality, we tested for HWE to ensure genotype proportions observed in the study population coincided with those expected under equilibrium conditions (Hardy, 1908; Weinberg, 1908). The potentially functional variant rs7301328 deviated from HWE (p<0.05, data not shown). This deviation may have been caused by a number of reasons including systematic genotyping errors that mistyped heterozygotes as homozygotes or vice versa (Gomes et al., 1999; Hosking et al., 2004), stochastic variation, or due to the study population’s biological characteristics. The biological explanation posits that patient genotypes deviate from HWE because of the effect an allele has on a disease phenotype from which the sample was non-randomly selected (Wittke-Thompson et al., 2005; Xu & Taylor, 2009). The cause for deviation from HWE in our sample appeared to be genotyping error: no homozygous recessive genotypes were observed for either rs7301328 responder or non-responder groups, even though this variant has a MAF of 23% and therefore, was expected to
have a homozygous recessive count of approximately eight people out of 157. Because many genetic tests such as $\chi^2$ require HWE to be assumed, rs7301328 was removed from subsequent analyses (Sasieni, 1997). Retyping of rs7301328 with a newly designed assay remains a priority for future work.

This study had several limitations. The sample size is relatively small and may not have had sufficient power to detect the effect sizes of these genes. To increase statistical power in the future, it will be necessary to collect larger CLZ response samples. One promising sample is being collected by the CRESTAR consortium in Europe (CRESTAR, 2011). Sample heterogeneity may also have limited our findings. Patients were collected from three clinical sites that may have varied in methods and populations. Population ancestry was also based on self-report and in the future, a principle components analysis (PCA) on the study sample may provide more accurate ancestry profiles (Paschou et al., 2008; Price et al., 2008; Liu et al., 2011).

In addition, two environmental factors with the potential to alter CLZ metabolism were not controlled for in our study: cigarette smoking and caffeine consumption. Cigarette smoking induces the Cytochrome P450 hepatic enzyme CYP1A2 that is primarily responsible for CLZ metabolism (Bertilsson et al., 1994; Ghassabian et al., 2010). As a result, smokers are observed to have lower CLZ serum levels than non-smokers (Seppala et al., 1999; Palego et al., 2002), and therefore require higher CLZ dosages to achieve similar plasma levels as patient who do not smoke. In contrast, caffeine inhibits CYP1A2 and increases both CLZ plasma concentrations and the risk of developing side effects linked to toxicity (Odom-White & de Leon, 1996; Hagg et al., 2000). Future studies incorporating these environmental
factors may help identify more accurate predictors of response and develop dosage guidelines that are tailored to the individual patient.

Inter-study variability may also have limited comparison of results across studies. We evaluated treatment response following six months of CLZ therapy, while previous studies evaluated response after shorter periods, e.g. two months of treatment (Hong et al., 2001; Chiu et al., 2003). Differences in treatment length are particularly problematic because response to CLZ is variable and may take up to five months or longer in a subset of patients (Lieberman et al., 1994; Meltzer & Okayli, 1995b). Thus, patients deemed non-responders after a two month treatment period have the potential to be reclassified as responders following a longer trial of CLZ therapy. This disparity makes comparing results across studies problematic and reaching a consensus regarding treatment duration among researchers would make meta-analyses more efficient in the future.

To conclude, we report no associations between seven GRIN2B variants and CLZ response in our sample. Response to CLZ therapy is complex and likely depends on multiple gene systems and environmental factors. Therefore, investigation of additional glutamate variants and variants within related neurotransmitter systems is warranted for future studies. Even though conclusive predictive markers remain elusive thus far, continued research to identify such markers remains of high interest to improve clinical response and treatment outcomes.
Figure 2.1 Human GRIN2B Gene Diagram

Structure, location and size of the GRIN2B gene are noted along with the eight single nucleotide polymorphisms genotyped in this study. The coding region of GRIN2B is represented by darkly shaded boxes and the 5’ and 3’ untranslated regions are represented by lightly shaded boxes.
<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Mn&gt;Mj</th>
<th>Region, Position</th>
<th>NIEHS SNP FuncPred</th>
<th>MAF</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3764030</td>
<td>T&gt;C</td>
<td>Promoter</td>
<td>NIEHS: TFBS; Transition substitution</td>
<td>0.246</td>
<td>ASD: (Yoo et al., 2012)</td>
</tr>
<tr>
<td>(−489G&gt;A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs7301328</td>
<td>C&gt;G</td>
<td>Exon 2</td>
<td>NIEHS: ESS/ESE; Transversion substitution</td>
<td>0.229</td>
<td>SCZ: (Ohtsuki et al., 2001; Di Maria et al., 2004; Qin et al., 2005); TRS: (Chiu et al., 2003); TRD: (Zhang et al., 2014); Lithium Response: (Szczepankiewicz et al., 2009)</td>
</tr>
<tr>
<td>(366C&gt;G)</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>rs12826365</td>
<td>A&gt;G</td>
<td>Intronic</td>
<td>- Transition substitution</td>
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<td>GAIN-African American best p-value SNP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13948270</td>
<td></td>
<td></td>
<td>(Ayalew et al., 2012)</td>
</tr>
<tr>
<td>rs1072388</td>
<td>A&gt;G</td>
<td>Intronic</td>
<td>- Transition substitution</td>
<td>0.203</td>
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<td></td>
<td>(Ayalew et al., 2012)</td>
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<td>rs2284411</td>
<td>T&gt;C</td>
<td>Intronic</td>
<td>- Transition substitution</td>
<td>0.317</td>
<td>ADHD: (Dorval et al., 2007; Park et al., 2013)</td>
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<td></td>
<td>13866172</td>
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<td>NIEHS: ESS/ESE; Transition substitution</td>
<td>0.288</td>
<td>SCZ: (Nishiguchi et al. 2000; Ohtsuki et al., 2001; Di Maria et al., 2004; Qin et al., 2005); CLZ: (Hong et al., 2001); TRS: (Chiu et al., 2003); TRD: (Zhang et al., 2014)</td>
</tr>
<tr>
<td>(2664C&gt;T)</td>
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<td>13717508</td>
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<td></td>
</tr>
<tr>
<td>rs1806191</td>
<td>A&gt;G</td>
<td>Exon 13</td>
<td>- Transition substitution</td>
<td>0.462</td>
<td>SCZ: (Di Maria et al., 2004); OCD: (Alonso et al., 2012)</td>
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<td>13716638</td>
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<tr>
<td>rs890</td>
<td>A&gt;C</td>
<td>3′UTR</td>
<td>NIEHS: miRNA; Transversion substitution</td>
<td>0.494</td>
<td>SCZ: (Di Maria et al., 2004); Lithium response: (Szczepankiewicz et al., 2009); TRD: (Zhang et al., 2014); CI-OCD:(Cai et al., 2013)</td>
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<td>(409T&gt;G)</td>
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<td></td>
<td></td>
<td>(5072T&gt;G)</td>
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</tr>
</tbody>
</table>

NA: not available; Mn>Mj: minor>major allele; UTR: untranslated region; MAF: minor allele frequency in our sample (n=157); NIEHS: National Institute of Environmental Health Sciences (TFBS: transcription factor binding site; ESE/S: Exonic splicing enhancer/silencer; miRNA: microRNA binding site); References: GAIN: Genetic Association Information Network GWAS; ADHD: attention deficit hyperactivity disorder; ASD: autism spectrum disorder; CI-OCD: clozapine-induced OCD; CLZ: clozapine response; OCD: obsessive compulsive disorder; SCZ: schizophrenia; TRD: treatment-resistant depression; TRS: treatment-resistant SCZ.
Figure 2.2 GRIN2B Linkage Disequilibrium Map

Linkage disequilibrium among eight GRIN2B variants included in this study. The haplotype block was constructed with solid spine of LD using the standard (D'/LOD) colour scheme. White: D'<1, LOD<2; blue: D'=1, LOD<2; shades of pink/red: D'<1, LOD≥2; bright red: D'=1, LOD≥2. Values within each box represent correlation coefficient values as percentages ($r^2$) (Haploview v4.2).
### Table 2.2 Study Sample Demographics and Clinical Information

<table>
<thead>
<tr>
<th></th>
<th>Meltzer (n=64)</th>
<th>Lieberman (n=65)</th>
<th>Potkin (n=28)</th>
<th>Total (n=157)</th>
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<tbody>
<tr>
<td><strong>Age (mean±SD)</strong></td>
<td>32.67±8.1</td>
<td>35.66±7.9</td>
<td>39.14±6.6</td>
<td>35.06±8.1</td>
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<tr>
<td><strong>Gender</strong></td>
<td></td>
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<tr>
<td>Male n (%)</td>
<td>48 (75.0)</td>
<td>47 (72.3)</td>
<td>25 (89.3)</td>
<td>120 (76.4)</td>
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<tr>
<td>Female n (%)</td>
<td>16 (25.0)</td>
<td>18 (27.7)</td>
<td>3 (10.7)</td>
<td>37 (23.6)</td>
</tr>
<tr>
<td><strong>R/NR a n (%)</strong></td>
<td>31/33</td>
<td>37/28</td>
<td>13/15</td>
<td>81/76</td>
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<tr>
<td><strong>ΔBPRS scores (n=86)</strong></td>
<td>-10.64±13.0</td>
<td>NA</td>
<td>-6.82±11.8</td>
<td>0.192 b</td>
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<tr>
<td><strong>ΔBNEG scores (n=88)</strong></td>
<td>-1.19±3.5</td>
<td>NA</td>
<td>-0.57±4.2</td>
<td>0.470 b</td>
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<tr>
<td><strong>ΔBPOS scores (n=84)</strong></td>
<td>-3.43±5.45</td>
<td>NA</td>
<td>-3.25±6.65</td>
<td>0.896 b</td>
</tr>
</tbody>
</table>

SD: standard deviation; R/NR: responder/non-responder; BPRS: Brief Psychiatric Rating Scale; BNEG/BPOS: BPRS negative and positive subscales; NA: data not available; a Responder defined as having at least 20% reduction in Brief Psychiatric Rating Scale scores from baseline; b p-values from Student’s t-test or one-way ANOVA; c p-values from χ²-test.
Table 2.3 Association Analysis of GRIN2B Variants and Response to Clozapine

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>R/NR^a</th>
<th>ΔBPRS±SD^b</th>
<th>ΔBPOS±SD^b</th>
<th>ΔBNEG±SD^b</th>
<th>Allele</th>
<th>R/NR^a</th>
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<td>AA</td>
<td>4/1</td>
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<td>31/21</td>
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<td>-0.60±3.56</td>
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<tr>
<td></td>
<td>GG</td>
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<td>-0.96±3.80</td>
<td>G</td>
<td>121/121</td>
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<td></td>
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<td>GG</td>
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<td>G</td>
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<tr>
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<td>-13.45±13.72</td>
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HWE: Hardy-Weinberg equilibrium; R/NR: responder/non-responder; BPRS: Brief Psychiatric Rating Scale; BNEG/BPOS: BPRS negative and positive subscales; SD: standard deviation; ^p-values from χ^2-test; ^b-p-values from ANCOVA co-varied for age and baseline scores; ^c-p-values from Fisher’s Exact Test, category with low cell value (<5) collapsed with heterozygous group.
Figure 2.3 Genotype Analysis of GRIN2B Variant rs1072388 and Clozapine Response

A-allele carriers of GRIN2B variant rs1072388 responded marginally better to CLZ therapy than GG-homozygotes as measured by categorical responder/non-responder frequencies, however this observation was not statistically significant ($p_{uncorrected}=0.067$, $p_{corrected}=0.440$, assuming 6.56 independent tests).
Table 2.4 Haplotype Analysis of GRIN2B Variants and Response to Clozapine

<table>
<thead>
<tr>
<th></th>
<th>R/NR n (%)</th>
<th>ΔBPRS n (%)</th>
<th>ΔBPOS n (%)</th>
<th>ΔBNEG n (%)</th>
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<tr>
<td>Global p-valuea</td>
<td>p 0.188</td>
<td>p 0.843</td>
<td>p 0.529</td>
<td>p 0.680</td>
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<tr>
<td>A-G-C</td>
<td>27.2/12.3 (17.4/8.4)</td>
<td>15.3 (9.7)</td>
<td>14.2 (9.1)</td>
<td>15.0 (9.6)</td>
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<td>0.116</td>
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<td>A-G-T</td>
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<td>14.7 (9.3)</td>
<td>14.8 (9.5)</td>
<td>14.0 (9.0)</td>
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<td>0.652</td>
<td>0.556</td>
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<td>0.776</td>
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<tr>
<td>G-A-C</td>
<td>30/26 (19.2/17.8)</td>
<td>33 (20.9)</td>
<td>30 (19.2)</td>
<td>31 (19.9)</td>
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<td>G-G-C</td>
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<td>59.7 (37.8)</td>
<td>62.8 (40.3)</td>
<td>60.0 (38.4)</td>
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<td>0.513</td>
<td>0.724</td>
<td>0.740</td>
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<td>G-G-T</td>
<td>31.2/38.3 (20.0/26.2)</td>
<td>35.3 (22.3)</td>
<td>34.2 (21.9)</td>
<td>36.0 (23.1)</td>
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<th>R/NR n (%)</th>
<th>ΔBPRS n (%)</th>
<th>ΔBPOS n (%)</th>
<th>ΔBNEG n (%)</th>
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<tr>
<td>Global p-valuea</td>
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<td>p 0.431</td>
<td>p 0.293</td>
<td>p 0.230</td>
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<td>A-G-A</td>
<td>45/46 (28.1/30.3)</td>
<td>44 (26.2)</td>
<td>41 (25.0)</td>
<td>42 (25.3)</td>
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<td>0.803</td>
<td>0.0645</td>
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<td>G-A-A</td>
<td>10.8/11.6 (6.8/7.7)</td>
<td>13.88 (8.3)</td>
<td>13.95 (8.5)</td>
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<td>0.686</td>
<td>0.421</td>
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<tr>
<td>G-A-C</td>
<td>60.2/63.4 (37.6/41.7)</td>
<td>71.12 (42.3)</td>
<td>70.05 (42.7)</td>
<td>71.4 (43.0)</td>
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<td>0.380</td>
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<td>17.12 (10.2)</td>
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<td>16.4 (9.9)</td>
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*Haplotypes with frequencies <0.05 were excluded; a uncorrected p-values from UNPHASED version 3.1.5.
Chapter 3

PHARMACOGENETIC ANALYSIS OF GLUTAMATE SYSTEM GENE VARIANTS AND CLINICAL RESPONSE TO CLOZAPINE

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This chapter was modified from the following:


Keywords: GRM2, SLC1A2, SLC6A9, GRIA1, GAD1, pharmacogenetics, clozapine response, glutamate
3.1 ABSTRACT

Altered glutamatergic neurotransmission is implicated in schizophrenia etiology as well as in response to clozapine, the drug of choice for treatment-resistant schizophrenia. Response to antipsychotic therapy is highly variable, although twin studies suggest a genetic component. We investigated the association among ten glutamate system gene variants with clozapine response in 175 European schizophrenia or schizoaffective disorder patients deemed resistant or intolerant to previous pharmacotherapy. GRM2 (rs4067, rs2518461), SLC1A2 (rs4354668, rs4534557, rs2901534), SLC6A9 (rs12037805, rs1978195, rs16831558), GRIA1 (rs2195450) and GAD1 (rs3749034) were typed using standard genotyping procedures. Response was assessed following six months of clozapine treatment using change in Brief Psychiatric Rating Scale scores. Categorical and continuous response variables were analyzed using Pearson’s chi-squared test and analysis of covariance, respectively. No significant associations were observed following correction for multiple testing. Prior to correction, several nominally significant associations were seen for SLC6A9, SLC1A2, GRM2 and GRIA1. Most notably, CC-homozygotes of rs16831558 located in the glycine transporter 1 gene exhibited an allele dose-dependent improvement in positive symptoms compared to T-allele carriers ($p_{uncorrected}=0.008$, $p_{corrected}=0.08$). To clarify the role of SLC6A9 in clinical response to antipsychotic medication and clozapine in particular, this finding warrants further investigation in larger well-characterized samples.
3.2 INTRODUCTION

The glutamate system has emerged as an area of strong interest for both schizophrenia risk and response to antipsychotic medications (Shan et al., 2012). The glutamate hypothesis of SCZ first emerged from observations that administration of NMDAR antagonists such as PCP and ketamine elicit behaviours that mimic the symptoms of SCZ in healthy individuals and dramatically worsen symptoms in SCZ patients (Luby et al., 1959; Luisada, 1978; Javitt & Zukin, 1991; Lahti et al., 2001). Glutamate levels also appear to be altered in SCZ brains and dysregulation of glutamate receptors has been postulated to play an integral role in hyperdopaminergic states often thought to be the primary cause of psychosis (Moghaddam & Javitt, 2012).

CLZ is an atypical AP drug that is particularly effective for treating patients with TRS (Kane et al., 1988). Approximately 30% of patients fail two or more trials with AP drugs, and are subsequently diagnosed with TRS. CLZ is the one drug that has been approved for treatment of this particular subpopulation (Borgio et al., 2007; Elkis & Meltzer, 2007; Solanki et al., 2009). Response to CLZ is complex and thought to depend, at least in part, on genetic factors as indicated by twin and family studies (Vojvoda et al., 1996; Horacek et al., 2001; Mata et al., 2001; Hoyer et al., 2010). Like other APs, high inter-individual variability is also observed with CLZ treatment (Bleehen, 1993; Potkin et al., 1994; Davis et al., 2003), with up to 50% of eligible patients failing to respond (Kane et al., 1988; Lieberman et al., 1994). Studies in pharmacogenetics aim to identify gene variants with the potential to predict dosing, response and side effects prior to starting drug treatment (Müller et al., 2003). The implications of a genetic test incorporating such variants would have far-reaching clinical applications; given the fact that early and effective treatment has been associated with
favourable outcome (Gunduz-Bruce et al., 2005; Perkins et al., 2005). To date, the majority of PGx studies investigating CLZ response have focused on gene variants in dopamine and serotonin neurotransmitter pathways (reviewed by Kohlrausch, 2013).

As noted, there is mounting evidence to suggest that glutamatergic neurotransmission may also play a role in mediating response to CLZ (Potkin et al., 1999; reviewed by Heresco-Levy, 2003). The major observation supporting this hypothesis is that if CLZ is co-administered with NMDAR antagonists, their psychotomimetic effects are blunted (Malhotra et al., 1997). Glutamate concentrations and excitatory glutamatergic neurotransmission are also increased by CLZ administration, as shown by micro-dialysis studies in rodents (Daly & Moghaddam, 1993; Yamamoto & Cooperman, 1994), EPSPs in neuronal cell cultures (Banerjee et al., 1995; Arvanov et al., 1997; Kubota et al., 2000; Ninan et al., 2003; Kargieman et al., 2007), and serum glutamate levels in patients switched to CLZ (Evins et al., 1997).

CLZ is also posited to modulate various receptors, transporters and enzymes that are involved in glutamate signaling and consequently implicates single nucleotide polymorphism gene variants within these pathways as strong candidates for assessing susceptibility to CLZ non-response. In regard to glutamate receptors, CLZ administration has been observed to increase AMPAR density and GluR1 expression (Fitzgerald et al., 1995; Spurney et al., 1999). The GluR1 protein is encoded by the \textit{GRIA1} gene and has been mapped to 5q31.1 (Puckett et al., 1991). CLZ also increases mGluR2 signaling (Fribourg et al., 2011), which is encoded by the \textit{GRM2} gene, which maps to 3p21.1 (Joo et al., 2001). AMPA and mGluR glutamate receptors are involved in primary depolarization of glutamate-mediated neurotransmission and synaptic plasticity (Ichise et al., 2000; Santos et al., 2009).
With respect to transporter proteins, CLZ reportedly down-regulates expression of EAAT2 in rat cerebral cortex (Melone et al., 2001), which is encoded by the SLC1A2 gene that maps to region 11p13-p12 (Li & Francke, 1995). EAAT2 localizes to astrocytes in the mammalian CNS and is responsible for the greatest proportion of total glutamate reuptake from the synapse (Rothstein et al., 1996). Functional inactivation of EAAT2 raises extracellular glutamate levels – a biological phenomenon implicated in AP non-response (Egerton et al., 2012; de la Fuente-Sandoval et al., 2013; Demjaha et al., 2014). CLZ also increases synaptic glycine concentrations (Javitt et al., 2005). In neuronal tissue, the glycine transporter 1 chiefly determines the availability of glycine in the brain by mediating glycine reuptake into surrounding nerve terminals and glial cells (Kim et al., 1994). The SLC6A9 gene codes for GlyT1 and is mapped to 1p33 (Jones et al., 1995).

Lastly, CLZ has been shown to down-regulate promoter methylation of the GAD1 gene in mice (Dong et al., 2008). This gene is mapped to 2q.31 (Bu et al., 1992) and codes for the GAD1 enzyme, which catalyzes the decarboxylation of glutamic acid to GABA and carbon dioxide (Erlander et al., 1991). Interestingly, abnormal GABAergic function is also reported in patients with SCZ (Taylor & Tso, 2014). Based on this aforementioned body of evidence, the present study set out to investigate the contribution of variants within SLC1A2, SLC6A9, GRIA1, GRM2 and GAD1 to response to CLZ in a sample of treatment resistant or intolerant patients.
3.3 METHODS

3.3.1 Study Sample

Subjects included in this study were recruited from three clinical sites: Case Western Reserve University in Cleveland, Ohio (HY Meltzer, n=74); Hillside Hospital in Glen Oaks, New York (JA Lieberman, n=73); and University of California, Irvine (SG Potkin, n=28) (Total sample, n=175). All patients had a diagnosis of either SCZ or SA disorder according to the DSM-III-R/IV (American Psychiatric Association, 1987, 1994) and met criteria for either TRS or intolerance to standard pharmacotherapy. Treatment-resistant schizophrenia was defined as failure to respond to two or more antipsychotic trials with drugs from at least two different chemical classes at doses of ≥1000mg/d chlorpromazine equivalents for four to six weeks, accompanied by no period of good functioning in the preceding five years (Kane et al., 1988). A smaller portion of patients (<15%) met criteria for treatment intolerance defined as the presence of moderate to severe tardive dyskinesia or extreme sensitivity to extrapyramidal symptoms (Lieberman et al., 1994).

For this genetic study, written informed consent was obtained from all participants in accordance with the Ethical Principles for Medical Research Involving Human Subjects at the Centre for Addiction and Mental Health and with the Declaration of Helsinki, as revised in 1989 (World Medical Association, 2013). Participants underwent a two to four week washout period during which time no medication was administered unless clinically necessary. Patients were then placed on CLZ therapy for six months or longer with mean doses in the range of 450 mg/day. For the duration of the study, patients were seen weekly for blood draws to monitor white blood cell count as a precaution against clozapine-induced agranulocytosis.
3.3.2 Response Measures

CLZ response was assessed using both categorical and continuous response measures. Categorical response was assessed using change in Brief Psychiatric Rating Scale scores (Overall & Gorham, 1962) from baseline and following six months of CLZ therapy. Participants were considered responders if they experienced a 20% or more decrease in their BPRS scores. Continuous response was measured using percent score reduction for total BPRS, as well as for the BPOS and BNEG subscales, using the following calculation: \[ \frac{\text{six month score} - \text{baseline score}}{\text{baseline score}} \times 100\% \]. Using this formula, scores below zero indicated symptom improvement.

3.3.3 SNP Selection

In total, ten glutamate system gene variants were included in this study (Figure 3.1 & Table 3.1). Four variants were selected based on previous investigation in other neuropsychiatric phenotypes as cited in the literature, and the remaining six SNPs were selected from several functional annotation websites based on their potential to alter gene expression.

3.3.3.1 Identification of SNPs from the Literature

A literature search was conducted on PubMed using the following search terms: “GRM2”, “Metabotropic Glutamate Receptor 2”, “SLC1A2”, “Glutamate Transporter 1”, “GLT1”, “Excitatory Amino Acid Transporter 2”, “EAAT2”, “SLC6A9”, “Glycine Transporter 1”, “GlyT1”, “AMPA”, “GRIA1”, “GluA1”, “GLUR1”, “GAD1” and “Glutamate Decarboxylase 1.” Articles investigating genetic associations (genome-wide and candidate SNP studies) with neuropsychiatric phenotypes were reviewed. As well, articles investigating the functional significance (positron emission tomography ligand binding assays, electrophoretic
mobility shift assays, luciferase promoter assays, etc.) of variants within SLC1A2, SLC6A9, GRIA1, GRM2 and GAD1 were reviewed to identify glutamate system SNPs of interest. Four variants were selected based on previous investigations in other phenotypes: 1) SLC1A2 (rs4354668) in bipolar disorder (BP), response to lithium salts (Dallaspezia et al., 2012), EAAT2 expression in SCZ subjects (Ohnuma et al., 1998) and plasma glutamate concentrations (Mallolas et al., 2006); 2) SLC1A2 (rs4534557) in SCZ risk (Deng et al., 2004; Nagai et al., 2009); 3) GRM2 (rs4067) in major depressive disorder (MDD), BP, response to fluvoxamine, methamphetamine-induced psychosis and SCZ (Tsunoka et al., 2009, 2010); and 4) GAD1 (rs3749034) in childhood onset SCZ (COS), cortical gray matter volume (Addington et al., 2005) and GAD1 mRNA expression (Straub et al., 2007).

3.3.3.2 Identification of SNPs with Potential Functionality

The Broad Institute’s HaploReg database was used to identify glutamate system variants with potentially functional effects on gene expression (Ward & Kellis, 2012). The gene coding regions plus an additional 10kb upstream and 2kb downstream were submitted to the ‘Build Query’ search field on HaploReg. Gene regions (chrN:start-end) were obtained from the UCSC Genome Browser using Human Genome assembly version 19: GRM2 (chr3: 51,731,081-51,754,625), SLC1A2 (chr11: 35,270,752-35,461,610), SLC6A9 (chr1: 44,455,172-44,507,164) and GRIA1 (chr5: 152,849,175-153,088,732).

Seven criteria to measure functionality were provided for each SNP: 1) sequence conservation across mammals, 2) promoter histone marks, 3) enhancer histone marks, 4) DNAse hypersensitivity sites, 5) bound proteins, 6) expression quantitative trait loci (eQTL) data and 7) effect on regulatory motifs. SNPs with a minor allele frequency of at least 5% in the European (EUR) population that satisfied at least 3 of these 7 functionality criteria were
considered for study inclusion. Six variants satisfying these criteria were selected: *SLC1A2* (rs2901534), *SLC6A9* (rs12037805, rs1978195, rs16831558), *GRIA1* (rs2195450) and *GRM2* (rs2518461) (**Figure 3.2**). RegulomeDB (Boyle et al., 2012) and NIEHS Functional SNP Prediction (FuncPred) database scores (Xu & Taylor, 2009) were also obtained to further assess functionality.

### 3.3.4 DNA Isolation and Genotyping

Venous blood samples were collected from study participants and sent to our Centre (CAMH, Toronto, Canada) where genomic DNA was extracted using the high salt method (Lahiri & Nurnberger, 1991). Genotyping was performed using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Final PCR reaction mixtures consisted of 20ng of genomic DNA and 2μL of 2X TaqMan® OpenArray® Genotyping Master Mix. Samples were loaded onto a QuantStudio Digital PCR Plate using QuantStudio 12K Flex AccuFill System and run on the QuantStudio 12K Flex Instrument as per the manufacturer’s instructions. Genotype calls were visualized using the DigitalSuite Software and were validated by two independent researchers blind to genotyping conditions. To ensure genotyping accuracy, 10% of the samples were re-genotyped and conflicting genotypes were set as missing for analysis.

### 3.3.5 Statistical Analysis

Quality-control of genotyping data was carried out using PLINK v1.07 (Purcell et al., 2007). In order to pass QC, SNPs were required to have ≥90% genotyping rate and a MAF of ≥0.05, and to satisfy HWE (p>0.005). In addition, individuals with genotyping efficiency less than
80% across all markers (samples producing genotypes for seven SNPs or less) were excluded from analysis.

Descriptive statistics were carried out using SPSS v20 (IBM Corp, Armonk NY, USA). Continuous variables were analyzed using Student’s t-test or ANOVA, and categorical variables were analyzed using $\chi^2$-test. Genotype and allele frequencies in R/NR groups were compared using $\chi^2$-test or Fisher’s Exact for cell counts less than 5. Differences in percent score reduction in BPRS, BPOS and BNEG subscales among genotypic groups were compared using ANCOVA, with age and baseline scores added as covariates.

HWE and LD among SNPs were determined using Haploview v4.2 and the Solid spine of LD to construct LD blocks (Figure 3.3) (Barrett et al., 2005). Haplotype analysis was carried out using UNPHASED v3.1.5 and $p$-values were corrected using permutation (10,000 tests) (Dudbridge, 2008). Haplotypes with a frequency of <0.05 were removed from subsequent analyses. Power calculations were performed using Quanto v1.2.4 (Gauderman & Morrison, 2006). Multiple testing correction for genotype and allele tests was performed using the Nyholt method and resulted in a revised statistical significance threshold of $p<0.005$ (Nyholt, 2004).

3.4 RESULTS

3.4.1 Sample Characteristics

Following QC procedures, twelve individuals were removed due to low genotyping efficiency (<80% of markers were successfully genotyped). The remaining sample had a mean genotyping efficiency of 98.9%. All SNPs met QC criteria and had a mean genotyping rate of 99.3%. In total, 163 European treatment-resistant/intolerant patients were included in
the statistical analysis (Table 3.2). Our sample had over 80% power to detect an OR as low as 2.1 (unmatched case-control design, n=163, non-responder frequency=47.2, MAF=26.5%, $\alpha/2=0.05$) and down to 8.5% of the variance in the quantitative response variable (continuous design, n=91). The average minor allele frequency across all ten glutamate system variants was used for power calculations.

For the three clinical sites, no statistically significant differences were observed for gender, response rates, or percent score reduction in BPRS, BPOS and BNEG scales. A statistically significant difference was observed, however, for age of study participants across clinical sites ($p=0.001$). Post hoc analysis revealed that patients originating from the SG Potkin sample were significantly older than patients from the HY Meltzer sample. Baseline BPRS, BPOS and BNEG values were also correlated with their respective percent score reductions.

In order to combine the three samples for analysis, both age and baseline scores were included as covariates in the ANCOVA analysis of continuous response. The combined sample (n=163) was 74.8% male and had a mean age of 35.1 years (SD 8.1). Following six months of CLZ therapy, patients experienced a mean percent score reduction of -9.1 points (SD 12.4), -1.1 (SD 3.6) and -3.2 (SD 5.7) from baseline for the BPRS, BNEG and BPOS scales, respectively. In addition, 52.8% of patients experienced a $\geq 20\%$ decrease in BPRS scores and were subsequently classified as responders.

3.4.2 Genotype and Allele Association Analysis

Following correction for multiple testing, no significant differences between responder and non-responder groups or change in percent scores using BPRS, BPOS and BNEG scales were observed for genotype and allele frequencies for each of the ten glutamate system gene
variants (Table 3.3). A number of nominally significant associations were observed; however, none of these findings were significant following correction for multiple testing.

Prior to correction, SLC6A9 rs16831558 C-allele carriers exhibited an allele dose-dependent reduction (improvement) in BPOS subscale scores following six months of CLZ therapy ($p_{uncorrected}=0.008$, $p_{corrected}=0.08$) (Figure 3.4). CC-homozygotes of rs16831558 also exhibited greater reduction in BPRS total scores ($p_{uncorrected}=0.045$, $p_{corrected}=0.421$). In addition, greater reduction in BNEG subscale scores were observed for five variants: SLC1A2 rs4534557 homozygotes (GG and CC) ($p_{uncorrected}=0.015$, $p_{corrected}=0.140$), GRIA1 rs2195450 A-allele carriers ($p_{uncorrected}=0.017$, $p_{corrected}=0.162$), SLC1A2 rs4354668 TT-homozygotes ($p_{uncorrected}=0.026$, $p_{corrected}=0.243$), GRM2 rs2518461 GG-homozygotes ($p_{uncorrected}=0.037$, $p_{corrected}=0.346$), and SLC1A2 rs2901534 CC-homozygotes ($p_{uncorrected}=0.047$, $p_{corrected}=0.440$).

3.4.3 GRM2 and SLC6A9 Haplotype Analysis

No haplotype blocks within GRM2 rs4067-rs2518461 were significantly different between responder/non-responder groups, or regarding continuous response measures (Table 3.4). Initially, the haplotype block rs16831558-rs12037805-rs1978195 T-T-A within SLC6A9 appeared significantly associated with change in BPOS scores following CLZ treatment ($p_{uncorrected}=0.001$, $p_{corrected}=0.006$, permutation corrected, individual haplotype effect). However, further investigation revealed that the positive association was driven solely by rs16831558. When the SLC6A9 rs12037805-rs1978195 haplotype was analyzed independently with the rs16831558 variant added as a conditioning marker, this two-marker haplotype was no longer significant (Table 3.5).
3.5 DISCUSSION

This investigation explored the association between ten glutamate system gene variants and clinical response to CLZ. No significant associations were observed following correction for multiple testing in our sample of patients. To our knowledge, this is the first reported study to investigate the role of SLC1A2 (rs4354668, rs4534557, rs2901534), SLC6A9 (rs12037805, rs1978195, rs16831558), GRIAI (rs2195450), GRM2 (rs4067, rs251) and GAD1 (rs3749034) in clinical response to CLZ. Four of these variants were previously investigated in other neuropsychiatric phenotypes and the remaining six SNPs were previously unstudied and were selected using HaploReg.

Several nominally significant associations were observed for SLC6A9, SLC1A2, GRM2 and GRIAI with CLZ response before correction for the number of independent tests. Of interest was the association between C-allele carriers of SLC6A9 rs16831558 and percent score reduction in the BPOS subscale ($p_{uncorrected}=0.008$). Individuals carrying two copies of the C-allele showed a 4.25% decrease in the severity of positive symptoms, those carrying one copy experienced no symptom change, and individuals with two copies of the T-allele experienced a worsening of their positive symptoms as indicated by BPOS scores that were approximately 3.00% higher than at baseline. This finding became non-significant following correction ($p_{corrected}=0.08$), but given our relatively small sample size, this nominally significant finding for SLC6A9 rs16831558 deserves further investigation in larger CLZ response samples.

The SLC6A9 locus is particularly complex, with 16 different mRNA transcripts and 14 different splice variants (Thierry-Mieg & Thierry-Mieg, 2006). The rs16831558 variant
reported herein is located approximately 5kb upstream of the \textit{SLC6A9} start-site. This variant has a RegulomeDB functional score of 2b (‘likely to affect binding’) and is predicted to lie within an enhancer histone mark, a DNAse I hypersensitivity site, and to affect regulatory motifs. Therefore, characterization of this SNP’s potential functionality using mobility shift and luciferase promoter assays remains a priority for future work.

As was mentioned in the introduction, GlyT1 has recently been the focus of several novel therapies for SCZ (Harvey & Yee, 2013). Five phase III clinical trials investigating bitopertin, a synthetic GlyT1 inhibitor, as an adjunct to conventional AP therapy for persistent negative symptoms or partial responders have been conducted, although the results have been non-significant thus far (ClinicalTrails.gov identifiers: NCT01192867, NCT01192906, NCT01192880, NCT01235520, and NCT01235559). Because GlyT1 regulates extracellular glycine concentrations in the vicinity of NMDARs (Smith et al., 1992), GlyT1 is able to regulate NMDAR function (Kuryatov et al., 1994; Laube et al., 1997). By blocking glycine reuptake from the synapse, GlyT1 inhibitors cause an increase in NR1 glycine site occupancy and NMDAR activity, ultimately reversing the NMDAR hypofunction that is thought to contribute to SCZ etiology.

Following the same rationale, various NR1 glycine site agonists including glycine, D-serine and D-cycloserine have been investigated as adjuncts to conventional AP therapy for the treatment of persistent negative and cognitive symptoms in SCZ (reviewed by Javitt, 2004). Even though glycine agonist drugs are not designed to improve the positive symptoms seen in TRS patients, observations of their use in clinical studies to improve negative symptoms have shed light on a possible action of CLZ on the glycine system: NR1 glycine site agonists are generally well received when paired with most APs, however, provide little therapeutic
benefit and may in fact worsen symptoms when administered to patients taking CLZ (Potkin et al., 1999; Singh & Singh, 2011). This incompatibility between NR1 glycine site agonists and CLZ may be due to CLZ’s pre-existing ability to potentiate NMDAR-mediated neurotransmission through an as-of-yet-unknown mechanism (Lane et al., 2006). Several mechanisms have been posited, for instance, CLZ may already increase synaptic glycine levels (Javitt et al., 2005) or may already act as a partial agonist at the NR1 glycine site of the NMDAR (Schwieler et al., 2008). Alternatively, CLZ’s ability to alter dopamine activity and achieve therapeutic effect may depend in part on availability of the NMDAR glycine site (Schwieler & Erhardt, 2003). Future studies clarifying the role of glycine transporter gene variants in the mechanism of CLZ’s action are necessary.

There are some limitations inherent in our study that must be mentioned. For instance, our small sample size may not have had sufficient statistical power to detect the effect sizes of these gene variants, and going forward the collection of larger samples will help determine the contribution of gene variants to CLZ response. One such sample is currently being collected by the CRESTAR consortium in Europe (CRESTAR, 2011) with the goal of identifying markers involved in CLZ response and side effects. Heterogeneity within our study sample may also have confounded association findings. Participants were collected from three clinical sites that may have differed in population substructure. In addition, patients with a diagnosis of either SCZ or SA disorder were eligible for study inclusion.

Incomplete outcome data may also have limited our findings. Individuals who ceased treatment prior to study completion invariably have an affect on response rates and may have confounded our ability to identify genetic variants linked to treatment response. Unfortunately, rates for treatment discontinuation in AP drug trials are estimated to be as
high as 40%, with common reasons for discontinuation being noncompliance, lack of efficacy and the occurrence of adverse side effects (Lieberman et al., 1994; Meltzer & Okayli, 1995b; Kinon et al., 2006). In certain patients, response to CLZ may take up to five months or longer and suggests that a portion of participants who discontinue treatment because of lack of efficacy most likely do so prematurely. The high rates of discontinuation during AP drug trials stresses the need for understanding inter-individual variability in drug response phenotypes and emphasizes the utility associated with identifying clinically relevant predictive markers.

To conclude, we report no significant associations among ten glutamate system gene variants and response to CLZ in our sample following correction for multiple testing. A nominally significant association within the glycine transporter 1 gene variant rs16831558 was observed prior to correction, and deserves further examination in larger well-characterized CLZ response samples.
**Figure 3.1 Glutamate System Gene Diagrams**

Schematic representation of *GRM2, SLC1A2, SLC6A9, GRIA1* and *GAD1* genes indicating chromosomal location and gene size. Single nucleotide polymorphisms genotyped in this study are shown. Dark boxes represent coding exons and light boxes represent 5’ and 3’ untranslated regions (UTRs). Gene diagrams were constructed using NCBI Reference Sequence from *Homo sapiens*, transcript variant 1. Alternatively spliced variants exist for all five genes and can be viewed on NCBI AceView (Thierry-Mieg & Thierry-Mieg, 2006).
Figure 3.2 HaploReg Results for Functional Glutamate System Gene Variants

To be considered for study inclusion, variants were required to have a minor allele frequency of at least 5% and to satisfy at least 3 of 7 functionality criteria. Chr: chromosome; pos: position; LD: linkage disequilibrium; $r^2$: correlation coefficient; D': Hedrick’s multi-allelic D'; Ref: reference allele; Alt: alternate allele; AFR/AMR/ASN/EUR freq: African/ad Mixed American/Asian/European minor allele frequency; GERP/SiPhy cons: genomic evolutionary rate profiling/SiPhy mammalian conservation algorithms; DNAse: DNAse I hypersensitivity site; eQTL: expression quantitative trait loci; dbSNP fun annot: dbSNP functional annotation (Ward & Kellis, 2012).
Figure 3.3 GRM2, SLC6A9 and SLC1A2 Linkage Disequilibrium Maps

Haplotype blocks were constructed using Solid spine of LD and standard colour scheme. Bright red represents Hedrick’s multi-allelic $D'=1$ and logarithm of the odds (LOD) $\geq 2$; blue represents $D'=1$ and LOD$<2$; shades of pink/red represents $D'<1$ and LOD$\geq 2$; white represents $D'<1$ and LOD$<2$. Correlation coefficient values as percentages ($r^2$) are displayed within each box (Haploviev v4.2).
<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP ID</th>
<th>Mn&gt;Mj</th>
<th>Region, Position</th>
<th>Functional Characterization</th>
<th>MAF</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRM2</td>
<td>rs4067</td>
<td>A&gt;G</td>
<td>Promoter 51738256</td>
<td>HaploReg: EHM; NIEHS: TFBS, miRNA; Transition substitution</td>
<td>0.163</td>
<td>MDD, BP, fluvoxamine response: (Tsunoka et al., 2009); METH-IP, SCZ: (Tsunoka et al., 2010)</td>
</tr>
<tr>
<td>GRM2</td>
<td>rs2518461</td>
<td>A&gt;G</td>
<td>Promoter 51738101</td>
<td>HaploReg: EHM, DNAse, MC; NIEHS: TFBS, miRNA; RegulomeDB score: 2a; Transition substitution</td>
<td>0.071</td>
<td>NA</td>
</tr>
<tr>
<td>SLC1A2</td>
<td>rs4354668 (-181A&gt;C)</td>
<td>G&gt;T</td>
<td>5’UTR Promoter 35440976</td>
<td>HaploReg: Cons, PHM, EHM, DNAse, PB, MC; NIEHS: TFBS; RegulomeDB score: 2b; Transversion substitution</td>
<td>0.457</td>
<td>BP, lithium response: (Dallaspezia et al., 2012); SCZ: (Ohnuma et al., 1998); Gene expression: (Mallolas et al., 2006)</td>
</tr>
<tr>
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<td>rs4534557</td>
<td>G&gt;C</td>
<td>Intron 35413964</td>
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<td>0.396</td>
<td>SCZ: (Nagai et al., 2009)</td>
</tr>
<tr>
<td>SLC1A2</td>
<td>rs2901534</td>
<td>C&gt;G</td>
<td>Intron 35364569</td>
<td>HaploReg: PHM, EHM, DNAse, PB, MC; Transversion substitution</td>
<td>0.211</td>
<td>NA</td>
</tr>
<tr>
<td>SLC6A9</td>
<td>rs12037805</td>
<td>C&gt;T</td>
<td>Intron 44497249</td>
<td>HaploReg: Cons, PHM, EHM, DNAse, PB, MC; NIEHS: TFBS; Transition substitution</td>
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<td>NA</td>
</tr>
<tr>
<td>SLC6A9</td>
<td>rs1978195</td>
<td>G&gt;A</td>
<td>2.1kb upstream 44499242</td>
<td>HaploReg: PHM, EHM, DNAse, PB, MC; NIEHS: TFBS; Transition substitution</td>
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<td>NA</td>
</tr>
<tr>
<td>SLC6A9</td>
<td>rs16831558</td>
<td>T&gt;C</td>
<td>5kb upstream 44502163</td>
<td>Haploreg: EHM, DNAse, MC; RegulomeDB Score: 2b; Transition substitution</td>
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<td>NA</td>
</tr>
<tr>
<td>GRIA1</td>
<td>rs2195450</td>
<td>A&gt;G</td>
<td>Intron 152871009</td>
<td>Haploreg: Cons, PHM, EHM, MC; NIEHS: TFBS; Transition substitution</td>
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<td>NA</td>
</tr>
<tr>
<td>Gene</td>
<td>SNP</td>
<td>Allele</td>
<td>Region</td>
<td>Functionality</td>
<td>MAF</td>
<td>Disease</td>
</tr>
<tr>
<td>-------</td>
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</tr>
<tr>
<td>GAD1</td>
<td>rs3749034</td>
<td>A&gt;G</td>
<td>5'UTR</td>
<td>Haploreg: Cons, PHM, DNase, PB, MC</td>
<td>0.253</td>
<td>SCZ: (Addington et al., 2005; Lundorf et al., 2005; Straub et al., 2007)</td>
</tr>
<tr>
<td></td>
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<td>Exon 1</td>
<td>NIEHS: TFBS, Transition substitution</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>171673475</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

NA: not available; Mn>Mj: minor>major allele; UTR: untranslated region; MAF: minor allele frequency in our sample (n=163); NIEHS: National Institute of Environmental Health Sciences (TFBS: transcription factor binding site; miRNA: microRNA binding site); Haploreg Functional Database (2a/2b: likely to affect binding; Cons: evolutionarily conserved variant; P/E-HM: promoter/enhancer associated histone mark; PB: protein bound; MC: motifs changed); References (BP: bipolar disorder; MDD: major depressive disorder; METH-IP: methamphetamine-induced psychosis; SCZ: schizophrenia).
<table>
<thead>
<tr>
<th></th>
<th>Meltzer (n=66)</th>
<th>Lieberman (n=69)</th>
<th>Potkin (n=28)</th>
<th>p-value</th>
<th>Total (n=163)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean±SD)</td>
<td>32.70±7.78</td>
<td>35.70±8.27</td>
<td>39.15±12.78</td>
<td>0.001^b</td>
<td>35.07±8.10</td>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td>0.150^c</td>
<td></td>
</tr>
<tr>
<td>Male n (%)</td>
<td>48 (72.7)</td>
<td>49 (71.0)</td>
<td>25 (89.3)</td>
<td></td>
<td>122 (74.8)</td>
</tr>
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<td>Female n (%)</td>
<td>18 (27.3)</td>
<td>20 (29.0)</td>
<td>3 (10.7)</td>
<td></td>
<td>41 (25.2)</td>
</tr>
<tr>
<td>R/NR^a n (%)</td>
<td>33/33 (50.0/50.0)</td>
<td>40/29 (58.0/42.0)</td>
<td>13/15 (46.4/53.6)</td>
<td>0.496^c</td>
<td>86/77 (52.8/47.2)</td>
</tr>
<tr>
<td>ΔBPRS scores (N=94)</td>
<td>-10.05±12.67</td>
<td>NA</td>
<td>-6.82±11.78</td>
<td>0.253^b</td>
<td>-9.09±12.44</td>
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<td>ΔBNEG scores (N=93)</td>
<td>-1.32±3.40</td>
<td>NA</td>
<td>-0.57±4.19</td>
<td>0.365^b</td>
<td>-1.10±3.65</td>
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<td>ΔBPOS scores (N=91)</td>
<td>-3.13±5.30</td>
<td>NA</td>
<td>-3.25±6.65</td>
<td>0.925^b</td>
<td>-3.16±5.71</td>
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</tbody>
</table>

SD: standard deviation; R/NR: responder/non-responder; BPRS: Brief Psychiatric Rating Scale; BNEG/BPOS: BPRS negative and positive subscales; NA: not available; ^aResponders defined as having at least 20% or more reduction in Brief Psychiatric Rating Scale scores from baseline; ^b p-values from Student’s t-test or one-way ANOVA; ^c p-values from χ²-test.
Table 3.3 Associations Among Glutamate System Gene Variants and Clozapine Response

<table>
<thead>
<tr>
<th>Gene SNP</th>
<th>Genotype</th>
<th>R/NR</th>
<th>ΔBPRS±SD</th>
<th>ΔBPOS±SD</th>
<th>ΔBNEG±SD</th>
<th>Allele</th>
<th>R/NR</th>
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<tr>
<td>GRM2</td>
<td>AA</td>
<td>1/4</td>
<td>-6.67±12.66</td>
<td>0.67±8.14</td>
<td>-3.00±4.36</td>
<td>A</td>
<td>26/30</td>
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<tr>
<td>rs4067</td>
<td>AG</td>
<td>23/22</td>
<td>-8.14±10.78</td>
<td>-2.57±6.72</td>
<td>-1.14±3.91</td>
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<tr>
<td>0.7838</td>
<td>GG</td>
<td>62/51</td>
<td>-9.49±13.05</td>
<td>-3.52±5.27</td>
<td>-1.00±3.58</td>
<td>G</td>
<td>148/124</td>
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<tr>
<td></td>
<td>P</td>
<td>0.497c</td>
<td>0.666</td>
<td>0.223</td>
<td>0.688</td>
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<td>0.276</td>
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<tr>
<td>GRM2</td>
<td>AA</td>
<td>1/0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>A</td>
<td>12/12</td>
</tr>
<tr>
<td>rs2518461</td>
<td>AG</td>
<td>10/12</td>
<td>-11.30±12.53</td>
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<tr>
<td>0.6025</td>
<td>GG</td>
<td>75/65</td>
<td>-8.86±12.55</td>
<td>-2.76±5.80</td>
<td>-1.26±3.66</td>
<td>G</td>
<td>162/142</td>
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<tr>
<td></td>
<td>P</td>
<td>0.657c</td>
<td>0.535</td>
<td>0.335</td>
<td>0.037</td>
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<tr>
<td>SLC1A2</td>
<td>GG</td>
<td>17/14</td>
<td>-11.29±11.61</td>
<td>-3.56±5.54</td>
<td>-1.25±3.30</td>
<td>G</td>
<td>80/67</td>
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<tr>
<td>rs4354668</td>
<td>GT</td>
<td>44/39</td>
<td>-7.57±11.85</td>
<td>-3.02±5.17</td>
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<tr>
<td>0.7605</td>
<td>TT</td>
<td>25/24</td>
<td>-10.94±13.58</td>
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<td>-2.19±4.35</td>
<td>T</td>
<td>94/87</td>
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<tr>
<td></td>
<td>P</td>
<td>0.944</td>
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<td>SLC1A2</td>
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<td>17/11</td>
<td>-12.24±10.82</td>
<td>-2.60±4.43</td>
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<td>69/57</td>
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<tr>
<td>rs4534557</td>
<td>CG</td>
<td>33/35</td>
<td>-6.63±11.26</td>
<td>-3.91±5.82</td>
<td>0.20±2.88</td>
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<tr>
<td>0.1155</td>
<td>CC</td>
<td>35/31</td>
<td>-9.50±14.29</td>
<td>-2.51±6.14</td>
<td>-1.86±4.31</td>
<td>C</td>
<td>103/97</td>
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<tr>
<td></td>
<td>P</td>
<td>0.548</td>
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<tr>
<td>SLC1A2</td>
<td>CC</td>
<td>5/1</td>
<td>-16.00±8.88</td>
<td>-3.80±4.32</td>
<td>-4.33±3.98</td>
<td>C</td>
<td>39/28</td>
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<td>rs2901534</td>
<td>CG</td>
<td>29/26</td>
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<td>-3.71±5.69</td>
<td>-1.71±3.24</td>
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<tr>
<td>0.8233</td>
<td>GG</td>
<td>52/50</td>
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<td>-0.31±3.68</td>
<td>G</td>
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<td>P</td>
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<td>SLC6A9</td>
<td>TT</td>
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<td>5.67±4.04</td>
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<td>2.67±1.53</td>
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<td>35/42</td>
<td>-6.52±12.79</td>
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<td>T</td>
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<td>P</td>
<td>0.203</td>
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**Note:** HWE indicates Hardy-Weinberg Equilibrium.
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<th>Region</th>
<th>Allele</th>
<th>Count</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>Category</th>
<th>p-value</th>
<th>HWE: Hardy-Weinberg equilibrium; R/NR: responder/non-responder; BPRS: Brief Psychiatric Rating Scale; BNEG/BPOS: BPRS negative and positive subscale; SD: standard deviation; ( a ) ( p )-values from ( \chi^2 )-test; ( b ) ( p )-values from ANCOVA with baseline scores and age added as covariates; ( c ) ( p )-values from Fisher’s Exact Test in which category with low cell value (&lt;5) collapsed with heterozygous group; ( * ) ( p )-value from Welch ANOVA test due to significant Levine’s test; Bolded ( p )-values indicate nominally significant findings obtained prior to correction (&lt;0.05).</th>
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<td>SLC6A9</td>
<td>GG</td>
<td>17/8</td>
<td>-10.27±9.27</td>
<td>-2.00±4.761</td>
<td>-1.75±4.31</td>
<td>G</td>
<td>73/57</td>
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<td>rs1978195</td>
<td>AG</td>
<td>38/41</td>
<td>-10.74±12.36</td>
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<td>0.7521</td>
<td>AA</td>
<td>30/26</td>
<td>-6.39±14.46</td>
<td>-1.23±5.86</td>
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<td>A</td>
<td>99/93</td>
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<td>-19.50±18.27</td>
<td>-6.50±2.89</td>
<td>-4.00±5.48</td>
<td>A</td>
<td>46/36</td>
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<td>rs2195450</td>
<td>AG</td>
<td>30/28</td>
<td>-8.70±11.92</td>
<td>-3.45±5.81</td>
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<td>0.4035</td>
<td>GG</td>
<td>48/44</td>
<td>-8.42±11.91</td>
<td>-2.81±5.88</td>
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<td>G</td>
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<td>0.428</td>
<td>0.794</td>
<td>0.986</td>
<td>0.349</td>
<td></td>
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<td>GAD1</td>
<td>AA</td>
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<td>-6.75±9.54</td>
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<td>-2.00±3.93</td>
<td>A</td>
<td>44/36</td>
<td></td>
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<td>rs3749034</td>
<td>AG</td>
<td>34/24</td>
<td>-10.47±13.95</td>
<td>-3.33±6.16</td>
<td>-1.70±4.33</td>
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<td>0.6878</td>
<td>GG</td>
<td>44/47</td>
<td>-8.33</td>
<td>-3.04±5.74</td>
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<td>G</td>
<td>124/118</td>
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<td>0.428</td>
<td>0.794</td>
<td>0.986</td>
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C-allele carriers of *SLC6A9* variant rs16831558 experienced an allele dose-dependent reduction in BPOS subscale scores following six months of CLZ therapy (\(p_{uncorrected}=0.008\), \(p_{corrected}=0.08\), assuming 9.36 independent tests), however, this finding did not remain significant following correction for multiple testing.
Table 3.4 Haplotype Analysis of *GRM2* Variants and Response to Clozapine

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>R/NR n (%)</th>
<th>ΔBPRS n (%)</th>
<th>ΔBPOS n (%)</th>
<th>ΔBNEG n (%)</th>
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<tr>
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<td>p 0.711a</td>
<td>p 0.719a</td>
<td>p 0.190a</td>
<td>p 0.133a</td>
</tr>
<tr>
<td>A-G</td>
<td>27 (15.0)/30 (18.1)</td>
<td>28 (16.3)</td>
<td>27 (15.9)</td>
<td>27 (15.7)</td>
</tr>
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<td>0.441</td>
<td>0.695</td>
<td>0.132</td>
<td>0.567</td>
</tr>
<tr>
<td>G-A</td>
<td>12(6.7)/12(7.2)</td>
<td>10 (5.8)</td>
<td>10 (5.9)</td>
<td>10 (5.8)</td>
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<td>0.837</td>
<td>0.506</td>
<td>0.236</td>
<td>0.055</td>
</tr>
<tr>
<td>G-G</td>
<td>141(78.3)/124(74.7)</td>
<td>134 (77.9)</td>
<td>133 (78.2)</td>
<td>135 (78.5)</td>
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<td>0.425</td>
<td>0.456</td>
<td>0.517</td>
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*Haplotypes with frequency <0.05 were excluded; †uncorrected *p*-values from UNPHASED version 3.1.5.
Table 3.5 Haplotype Analysis of SLC6A9 Variants and Response to Clozapine

<table>
<thead>
<tr>
<th></th>
<th>rs16831558-rs12037805-rs1978195*</th>
<th>rs12037805-rs1978195*†</th>
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<tbody>
<tr>
<td></td>
<td>R/NR n (%)</td>
<td>ΔBPRS n (%)</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>C-C-G</td>
<td>54/35 (34.2/24.0)</td>
<td>44 (28.6)</td>
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<td>0.051</td>
<td>0.066</td>
</tr>
<tr>
<td>C-T-A</td>
<td>70/67 (44.3/45.9)</td>
<td>71 (46.1)</td>
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<td>0.781</td>
<td>0.404</td>
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<tr>
<td>C-T-G</td>
<td>16/23 (10.1/15.7)</td>
<td>21 (13.6)</td>
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<td>0.167</td>
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<tr>
<td>T-T-A</td>
<td>18/21 (11.4/14.4)</td>
<td>18 (11.7)</td>
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<td>0.016</td>
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</table>

*Haplotypes with frequency <0.05 were excluded; a uncorrected p-values from UNPHASED version 3.1.5; †rs16831558 included as ‘conditioning marker’ to two-marker haplotype.
Chapter 4

DISCUSSION

4.0 OVERVIEW

This thesis work investigated the association of 17 glutamate system gene variants with clinical response to CLZ. No significant associations for genotype, allele, or haplotype analyses were observed across all genes following correction for multiple testing. Prior to correction, several nominally significant associations were observed, the most important of which was for the glycine transporter 1 gene variant rs16831558 and change in BPRS positive symptom subscale scores. In this section we discuss main findings, explore their implications, assess study limitations and conclude with future directions.

4.1 SUMMARY OF FINDINGS

4.1.1 Association of GRIN2B with Clozapine Response

The first chapter of this thesis investigated the association of eight GRIN2B variants with response to CLZ. To our knowledge, this is the first reported study to investigate associations among rs7301328, rs1072388, rs3764030, rs12826365, rs2284411, rs1806191 and rs890 with CLZ response, while the rs1806201 variant had previously been investigated in two other response studies (Hong et al., 2001; Chiu et al., 2003). Variant rs7301328 significantly deviated from Hardy-Weinberg equilibrium and was therefore excluded from analysis ($p_{HWE}=1.93\times10^{-7}$). The implications regarding deviation from HWE are discussed in more detail in Study Limitations Section 4.3.5.
For all \textit{GRIN2B} SNPs investigated, no significant associations were observed for allele, genotype, or haplotype analyses prior to and after correction for multiple testing. A-allele carriers of variant rs1072388 responded marginally better to CLZ therapy than GG-homozygotes, however this finding was not statistically significant ($p_{\text{uncorrected}}=0.067$, $p_{\text{corrected}}=0.440$) (Chapter 2, \textbf{Figure 2.3}). Our negative association findings for \textit{GRIN2B} variant rs1806201 was consistent with previous reports that also found no association between this variant and CLZ response in two independent samples of Chinese TRS patients (Hong et al., 2001; Chiu et al., 2003).

\subsection*{4.1.2 Association of Glutamate System Variants with Clozapine Response}

The second chapter investigated the association of ten glutamate system gene variants and response to CLZ. To our knowledge, this is the first reported study to investigate the association of \textit{GRM2} (rs4067, rs2518461), \textit{SLC1A2} (rs4354668, rs4534557, rs2905134), \textit{SLC6A9} (rs12037805, rs1978195, rs16831558), \textit{GRIA1} (2195450) and \textit{GAD1} (rs3709054) with clinical response to CLZ. No significant genotype, allele, or haplotype associations were reported across all SNPs following correction for multiple testing.

Prior to correction, several nominally significant associations were observed. Briefly, a greater reduction in BPRS negative symptom subscale scores was observed for five variants: \textit{SLC1A2} rs4534557 homozygotes (GG and CC) ($p_{\text{uncorrected}}=0.015$, $p_{\text{corrected}}=0.140$); \textit{GRIA1} rs2195450 A-allele carriers ($p_{\text{uncorrected}}=0.017$, $p_{\text{corrected}}=0.162$), \textit{SLC1A2} rs4354668 TT-homozygotes ($p_{\text{uncorrected}}=0.026$, $p_{\text{corrected}}=0.243$), \textit{GRM2} rs2518461 GG-homozygotes ($p_{\text{uncorrected}}=0.037$, $p_{\text{corrected}}=0.346$), and \textit{SLC1A2} rs2901534 CC-homozygotes ($p_{\text{uncorrected}}=0.047$, $p_{\text{corrected}}=0.440$).
The most interesting association observed before correction was for *SLC6A9* rs16831558: C-allele carriers experienced an allele dose-dependent reduction in BPOS subscale scores following six months of CLZ therapy ($p_{uncorrected}=0.008$, $p_{corrected}=0.08$) (Chapter 3, Figure 3.4). More specifically, individuals carrying two copies of the C-allele showed a 4.25% decrease in severity of positive symptoms, those carrying one copy experienced no symptom change, and individuals carrying two copies of the T-allele experienced a 3.00% increase in positive symptoms compared to baseline. The rs16831558 SNP is a previously unstudied variant that was identified using HaploReg functional annotation database (Benson et al., 2013). Located in the promoter region of *SLC6A9*, this variant lies in an enhancer/promoter-associated histone mark and is proposed to affect both *SLC6A9* transcriptional activity and protein expression by altering transcription factor binding. This variant’s functional potential and biological plausibility, in combination with the nominally significant association finding reported herein makes rs16831558 an ideal candidate to be further investigated in larger CLZ response samples and for characterization using functional assays.

### 4.2 IMPLICATIONS OF FINDINGS

The main findings of this work suggest that genetic variation in the glycine transporter 1 gene may confer risk for clozapine non-response. This section begins with a discussion on the biological significance of the glycine neurotransmitter system in relation to SCZ etiology and treatment. CLZ’s ability to interact with the glycine system will then be noted. This section concludes by exploring a possible mechanism through which the *SLC6A9* promoter variant rs16831558 may regulate glycine transporter expression, and lead to differential drug effects.
4.2.1 Glycine in Schizophrenia Etiology and Treatment

Glycine is a necessary co-agonist at the NR1 subunit of the NMDAR. Through a coincidence detection type mechanism, the NMDAR will not fire without the simultaneous binding of both glycine and glutamate (Johnson & Ascher, 1987; Mayer et al., 1989; Thomson, 1990). Therefore, control of glycine levels at glutamatergic synapses is an important regulatory component for the activation of excitatory neurotransmission. As was mentioned in the introduction, the glycine transporter 1 protein is localized to glutamatergic neurons and controls the reuptake of glycine from the synapse (Kleckner & Dingledine, 1988; Bergeron et al., 1998). Changes in GlyT1 activity alter NMDAR-mediated neurotransmission as evidenced by increased NMDAR excitatory post-synaptic currents following GlyT1 antagonism (Bergeron et al., 1998) and enhanced NMDAR function in GlyT1 knockout mice (Gabernet et al., 2005).

Because glycine is necessary for NMDAR activation, abnormalities in the glycine system may contribute to the hypoglutamatergic states implicated in SCZ etiology. Several abnormalities related to glycine biology have been reported in patients with SCZ. In comparison to healthy controls, AP naïve patients have decreased plasma levels of glycine and low glycine levels have been shown to correlate with more severe negative symptoms (Sumiyoshi et al., 2004; Neeman et al., 2005). Binding assays with 3H-glycine in postmortem brain tissue also show patients experience increased NR1 glycine site binding in sensory and motor cortices (Ishimaru et al., 1992), and may indicate postsynaptic compensation for glutamatergic hypofunction.

Since glycine abnormalities may contribute to the hypoglutamatergic states that are implicated in SCZ’s etiology, glycine has been targeted as a plausible mechanism for novel
treatments. Glycine was also recognized as having antipsychotic potential upon the discovery that pretreatment with glycine or glycine-like compounds reverse PCP-induced hyperactivity in preclinical models (Javitt et al., 1997). The glycine-modulating compounds that exist at present include the glycine transporter inhibitors, such as bitopertin (RG1678) developed by Roche, as well as the use of adjunct glycine or glycine-like compounds for the treatment of persistent negative symptoms. Glycine-modulating compounds share a common mechanism of action to achieve therapeutic effect: glycine transporter inhibitors antagonize GlyT1 function to restore normal levels of glycine at the synapse, while glycine compounds directly stimulate NMDAR function.

4.2.2 Interaction of Clozapine with the Glycine System

CLZ is theorized to achieve therapeutic effect, in part, by acting on the glycine system. Scientific evidence supporting this theory is as follows: 1) CLZ treatment significantly restores plasma glycine to levels seen in healthy controls (Neeman et al., 2005; Yamamori et al., 2014); 2) baseline glycine levels have the ability to predict negative symptom response during CLZ treatment (Sumiyoshi et al., 2004); and 3) glycine/glycine-like compounds have no effect or worsen patient symptoms when combined specifically with CLZ (Goff et al., 1999; Tsai et al., 1999; Evins et al., 2000). The mechanism through which CLZ alters glycine levels is largely unknown, however, one study has suggested that CLZ may do so by interfering with glycine reuptake from neuronal synapses (Javitt et al., 2005).

Alternative mechanisms have been postulated to explain how glycine compounds interfere with CLZ’s therapeutic effect. One group of researchers propose that CLZ may directly interact with the NMDAR by binding to the NR1 glycine site (Schwieler et al., 2004; Schwieler et al., 2008), however, no direct evidence supporting this theory currently exists,
for instance, from co-immunoprecipitation assays. An alternative hypothesis suggests that
CLZ may depend on NR1 glycine site availability to achieve therapeutic effect (Schwieler &
Erhardt, 2003). Despite the mechanism, these findings suggest that response to CLZ depends
in part on plasma glycine levels and/or availability of the NR1 glycine site.

4.2.3 Promoter Variant Confers Differential Response to Clozapine

We observed that individuals carrying two copies of the rs16831558 C-allele showed a
4.25% improvement in positive symptom severity, those carrying one copy experienced no
symptom change, and patients with two copies of the T-allele experienced a 3.00%
worsening of their positive symptoms from baseline. The rs16831558 promoter variant is
proposed to alter GlyT1 expression through altering transcription factor binding. Future work
entails conducting luciferase promoter assays to test if the C-allele has greater transcriptional
activity over the T-allele. Functional characterization of this SLC6A9 promoter variant is
discussed in more detail in Future Directions Section 4.4.1.

If rs16831558 is found to have an affect on GlyT1 expression, we propose that variation in
rs16831558 may lead to differential response to CLZ. This proposition stems from previous
research indicating that CLZ response is contingent upon glycine levels: baseline levels of
glycine inversely correlate with negative symptom response during CLZ treatment
(Sumiyoshi et al., 2004) and increasing levels of NR1 agonists through the addition of
exogenous glycine/glycine-like compounds during CLZ treatment leads to lack of response
or worsening of symptoms (Goff et al., 1999; Tsai et al., 1999; Evins et al., 2000). Therefore,
variants with the ability to alter the quantity or quality of glycine at neuronal synapses may
also affect CLZ efficacy. We now propose a mechanism through which the glycine
transporter 1 gene promoter polymorphism rs16831558 may regulate GlyT1 expression, and
subsequently lead to differential response to CLZ. The mechanism is as follows: if the C-allele is found to increase SLC6A9 transcriptional activity, individuals carrying the CC-genotype should exhibit increased GlyT1 expression, leading to lower levels of synaptic glycine and a more favourable response to CLZ. In contrast, T-allele carriers should exhibit normal-to-low GlyT1 expression, resulting in normal-to-high levels of endogenous synaptic glycine, which may either have no effect or may interfere with CLZ’s ability to achieve therapeutic effect. Further characterization of the rs16831558 variant will be needed to give credence to this proposed mechanism.

The clinical implications of predictive biomarkers that are capable of distinguishing responders from non-responders prior to treatment are far reaching and would serve as an invaluable tool for optimizing treatment outcomes for patients with SCZ. Several examples of promoter variants conferring risk for non-response exist in other areas of medical research and provide hope for the clinical application of predictive variants in psychiatric care. For instance, the vitamin K epoxide reductase complex subunit 1 gene (VKORC1) promoter variant -1639G>A significantly correlates with Warfarin dose (Carlquist et al., 2008). A luciferase promoter assay was used to show the G-allele of this variant is associated with a 50% increase in VKORC1 transcriptional activity as compared to the A-allele (Yuan et al., 2005). One additional example demonstrating an interaction between gene promoter sequence variants and drug treatment response is in asthma (Drazen et al., 1999).

Recent literature has confirmed that polymorphisms in drug targets have the ability to alter patient sensitivity to treatment and change the pharmacodynamics of treatment response. Continued research in pharmacogenetics that aims to increase our understanding of the complex interplay between genetic variants that control the pharmacokinetic and
pharmacodynamic factors involved in drug effects is an important step on the road to personalizing treatment. An overview of the future of personalized medicine in psychiatry is discussed in more detail in Personalized Medicine in Psychiatry Section 4.4.5.

4.3 LIMITATIONS AND CONSIDERATIONS

Several limitations associated with our study must be considered before interpreting our findings. This section explores the importance of study heterogeneity, inter-study variability, statistical power, correction for multiple testing, deviation from Hardy-Weinberg equilibrium and incomplete outcome data, as they relate to our findings.

4.3.1 Study Heterogeneity

Study heterogeneity refers to the variability inherent in a study and can be caused by differences in study design (methodological diversity), and variation among participants, interventions and outcomes (clinical diversity) (Hanson & Levin, 2013). Heterogeneity in association studies is particularly problematic because it may lead to spurious associations or the inability to detect true associations between marker loci and phenotypes of interest. Steps are often taken to limit the heterogeneity in a sample so that results may be interpreted with the least amount of confounding.

The first source of heterogeneity in our sample was caused by methodological and clinical diversity. Participants were collected from three clinical sites that may have differed in study methodology and populations. Combining individuals with different ancestral backgrounds into a single group for analysis can lead to population stratification where false positive associations arise because of population substructure rather than true associations (Cardon & Palmer, 2003). Ad-mixed populations particularly confound drug response phenotypes
because response tends to vary according to a patient’s ethnicity (Frackiewicz et al., 1997; Wilson et al., 2001).

Patients included in our study were considered to be European based on self-reports. Even though the accuracy between self-reported ancestry and actual ancestry is fairly consistent (agreement rate of 75.1%) (Choudhry et al., 2007; Lee et al., 2010), the use of a principle components analysis to experimentally determine ancestry profiles remains a priority for future work. Briefly, PCA utilizes a panel of SNPs that are known to differ in allele frequencies across populations of different geographical regions. Ancestry is then determined by comparing genotyping results to reference panels from well-characterized datasets such as genetic data from the HapMap Project (Paschou et al., 2008). PCA analysis is also useful for detecting population stratification (Liu et al., 2011). Ideally, genetic data from admixed populations should be stratified prior to analysis to maintain as much homogeneity as possible.

Heterogeneity related to disease diagnosis and differences between SCZ and SA disorder may also have increased variation in our sample. For instance, these disorders have different clinical presentations: SA disorder is characterized as periods of psychosis with concurrent symptoms meeting criteria for a Major Mood Episode, while in comparison, SCZ is characterized by less frequent/prominent depressed or manic mood states (American Psychiatric Association, 2013). As well, SA disorder patients tend to experience higher rates of response and better clinical outcomes than patients with SCZ (Harrow et al., 2000). Taken together, these differences suggest that SCZ and SA disorder may differ in their underlying biology (Abrams et al., 2008; American Psychiatric Association, 2013; Cosgrove & Suppes, 2013). The propensity of SA disorder patients to respond more favourably to treatment may
have increased our CLZ response rate, and combining these two disorders into a single sample for analysis may limit detection of causal variants that contribute to response.

Lastly, our sample consisted of patients who were placed on CLZ for different reasons. CLZ is a 3rd line treatment for SCZ patients who are diagnosed as having TRS either because they failed to respond to previous treatments with pharmacotherapy (treatment resistance) or because they developed intolerable side effects during treatment (treatment intolerance) (Moore et al., 2007). Patients diagnosed with treatment resistance experience suboptimal response characterized by persistent psychotic symptoms, while those experiencing intolerance cease treatment due to intolerable side effects such as TD. Clinical evidence suggests treatment intolerant and resistant patients respond differently when treated with CLZ: treatment intolerant patients tend to experience higher rates of response than patients who are resistant (Claghorn et al., 1987; Owen et al., 1989; Lieberman et al., 1994). In fact, higher baseline levels of EPS have been shown to correlate with a favourable response to CLZ in treatment intolerant patients (Pickar et al., 1992; Lieberman et al., 1994). The implications for combining treatment resistant and intolerant patients into a single group for association analyses in unknown, and ideally patients should be stratified to maintain as much homogeneity as possible.

4.3.2 Inter-Study Variability

Inter-study variability limits comparison of results across studies and also complicates the process of combining data for meta-analysis. Meta-analyses are important because they aim to reconcile the contribution of common variants to disease risk, when genetic associations are unclear from individual studies (Lohmueller et al., 2003). To increase comparability of results across studies, variables such as inclusion criteria, duration of treatment and clinical
outcomes should be selected with consideration to readily accepted standards in the literature.

Many definitions for TRS exist in the literature and these inconsistencies impede interpretation of results across studies (Suzuki et al., 2011). Studies using less conservative definitions for TRS are likely to report higher rates of response. This is because the likelihood of response decreases after each subsequent failed AP drug trial. Herein, we defined TRS as failure to respond to two or more adequate trials with AP drugs from at least two different chemical classes (with doses of ≥1000mg chlorpromazine equivalents for 4-6 weeks), and no period of good functioning in the previous five years (Kane et al., 1988). This definition of TRS is consistent with definitions existing in the literature (Canadian Psychiatric Association, 2005; Kreyenbuhl et al., 2010; McIlwain et al., 2011; Kennedy et al., 2014) and accurately reflects CLZ’s current prescribing policy as a 3rd line treatment (Elkis & Meltzer, 2007; Moore et al., 2007).

The duration of treatment that constitutes an adequate trial with CLZ also varies across studies (Rosenheck et al., 1999). High inter-individual variability in AP response has been observed during treatment and response has been reported to occur as soon as one week (Pickar et al., 1992; Stern et al., 1994) and as late as one year (Meltzer, 1992; Wilson, 1996) following treatment initiation. In addition, length of CLZ treatment has been found to correlate with treatment outcomes: approximately 30% of patients respond in the first six weeks (Kane et al., 1988; Lieberman et al., 2008) and an additional 20% after three months (Claghorn et al., 1987; Lindstrom, 1988). These findings suggest that studies assessing response after only one or two months may do so prematurely for a subset of patients. Our study assessed CLZ response after a six month trial of CLZ to ensure response rates were
accurate and accounted for ‘late responders’. Agreement upon the length of time that constitutes an adequate CLZ trial will help maintain consistency among studies and aid in the interpretation of results across studies.

The last source of variability in our study stems from differences in the assessment of clinical outcomes. Various definitions for response exist in the literature; however, most studies assessing response use change in either the PANSS or the BPRS (reviewed by Suzuki et al., 2012). The difference between these two scales is negligible and their scores are easily interchangeable (Overall & Gorham, 1962; Kay et al., 1987). Herein, response was assessed using change in BPRS scores from baseline following six months of CLZ therapy. Categorical response was measured using responder/non-responder frequencies and CLZ responders were classified as those individuals who experienced a $\geq 20\%$ decrease in BPRS scores, while continuous response was measured using percent change in scores controlled for baseline (Kane et al., 1988). This assessment of clinical outcome was in line with similar studies evaluating CLZ response from the literature (Hong et al., 2001; Chiu et al., 2003). Despite widespread use, symptom rating scales have been criticized for their inability to reflect a patient’s personal, social and cognitive functioning – factors that hold greater weight in regards to how a patient will function in society. To ensure the clinical utility of response studies, it may be worthwhile to construct a more ‘patient focused’ response assessment method that takes factors beyond symptom change into consideration (Mortimer, 2007).

4.3.3 Statistical Power

The power of a statistical test is defined as the probability of correctly rejecting the null hypothesis of ‘no association’ when the null hypothesis is false, or said in another way, the ability to detect a true effect when it is present. Power is equal to one minus the probability
of a type II error \((1-\beta)\). Type II errors are otherwise known as false negatives and occur when true effects are not detected even though they are present. The power of an association study is influenced by a number of factors including sample size, statistical significance criteria \((\alpha)\) and the magnitude of the desired effect size to be detected (Evans & Purcell, 2012). Power tends to be highest for studies with large sample sizes, less stringent significance levels and larger effect sizes.

A common limitation of genetic association studies is insufficient statistical power. Many pharmacogenetics studies investigating response are underpowered due to limited sample sizes. Patients placed on CLZ are particularly hard to come by because of the strict guidelines surrounding its prescribing and the need for mandatory blood monitoring to protect against the life-threatening adverse effects of CIA (Warnez & Alessi-Severini, 2014). Averaged between the two manuscripts, our sample of 160 patients with categorical (responder/non-responder) response data and 88 patients with continuous response data, had over 80% power to detect an odds ratio (OR) as low as 2.05 and down to 8.5% of the variance in the quantitative response measure (unmatched case control design: non-responder frequency=47.8%, MAF=28.5%, \(\alpha/2=0.05\)) (Gauderman, 2002; Gauderman & Morrison, 2006).

Similar to the phenotype of SCZ, AP response is a complex trait that follows a polygenic mode of inheritance. However, unlike risk for SCZ, drugs administered to a patient for treatment follow a relatively narrow pathway through the body to the brain where they bind to a limited number of target receptors. AP drugs thereby actively provoke the phenotype of response by enacting change in the symptoms of the patient. Therefore, drug response phenotypes should exhibit larger gene effects than seen when studying SCZ diagnosis due to
the *a priori* selection of genes based on the known pharmacologic mechanism of the drug. A recent example demonstrating this phenomenon is the large effect size of the melanocortin receptor 4 gene (*MC4R*) (RR=3.5 or greater, n=139) for predicting antipsychotic-induced weight gain (Malhotra et al., 2012; Chowdhury et al., 2013). Therefore, our sample of patients likely had sufficient statistical power to detect the effect sizes of genes contributing to response.

For other genes with very small effects, the future collection of larger CLZ response samples will help unravel their contribution to complex phenotypes, such as drug response. A larger CLZ response sample is currently being collected by the CRESTAR consortium in Europe with the aim of developing pharmacogenetic biomarkers for treatment response and side effects (CRESTAR, 2011). To illustrate the effect of sample size on power: a sample size of 373 cases and 392 controls would have sufficient statistical power (80%) to detect an odds ratio as low as 1.5 (unmatched case-control design, MAF=15%, \(\alpha/2=0.05\)) (Gauderman, 2002; Gauderman, Morrison, 2006). Samples of this size, once available, will aid in the discovery of novel genetic markers with the ability to predict response to CLZ.

### 4.3.4 Multiple Testing

Genetic studies often test multiple alleles for association with a desired phenotype and invariably inflate the type I error rate (\(\alpha\)), or likelihood of observing a false-positive finding. To account for multiple comparisons, a more stringent threshold of significance is applied. The Bonferroni method is a commonly used correction tool that divides the significance criteria by the number of independent tests that were conducted (\(k\)) (Bonferroni, 1936). This method is considered conservative for correlated tests such as when SNPs are in linkage disequilibrium, as this method assumes that tests are independent. To account for these
correlations, an alternative method was developed by Nyholt that computes a new value for the number of independent tests that considers linkage among variants (Nyholt, 2004).

For the first data chapter of this thesis, the Nyholt method was used to compute a corrected significance threshold of 0.008, based on 6.56 independent tests among seven *GRIN2B* variants. The Nyholt method was also used for the second data chapter to compute a corrected significance threshold of 0.005, based on 9.36 independent tests among the ten glutamate system gene variants. If all tests across the two manuscripts were combined, a study-wise corrected threshold of 0.003 based on 15.92 tests would be computed. With these significance threshold adjustments in place, no polymorphism typed in our study would reach statistical significance.

Our correction criteria considered the number of SNPs to equal to the number of tests being performed, however, alternative methods for correction do exist. A less stringent method is a gene-wise correction, in which *k* is considered to be equal to the number of SNPs present within a single gene (*≥*2) (as used in Kalsi et al., 2010). To illustrate, a gene-wise correction for the three SNPs located in *SLC6A9* would result in a corrected significance threshold of 0.019 based on 2.70 tests and would have rendered the association between rs16831558 and CLZ response statistically significant (*p*uncorrected=0.008). Opinions differ on what level of stringency should be used for correction and some researchers may consider this gene-wise correction to be insufficient. On the other hand, a considerably more stringent approach is to correct for the total number of intragenic SNPs in the human genome. This correction is analogous to meeting statistical criteria for a genome wide association study, in which *p* equal to $10^{-8}$ (Risch & Merikangas, 1996). Many believe this correction to be overly stringent and argue that a genome wide correction would leave the majority of association studies
underpowered (Neale & Sham, 2004; Patnala et al., 2013). A final method to consider corrects for the total number of statistical tests that are performed and in our case, would include all allele, genotype and haplotype analyses performed for each variant (Lewis & Knight, 2012).

The Bonferroni and Nyholt methods of correction are considered family-wise error rate (FWER) procedures that seek to reduce the probability of observing even one false discovery. FWER correction methods are disadvantageous for studies in which many tests are performed because the significance threshold becomes increasingly small and often result in insufficient statistical power to detect true effects (Perneger, 1998). Because the consequences of false discoveries in complex traits like AP response are less severe than for single gene disorders (van den Oord & Sullivan, 2003), less conservative methods of correction may be applied with caution. A more recently developed less conservative method is the false discovery rate (FDR) approach. Like the Bonferroni and Nyholt methods, FDR also determines adjusted $p$-values for each test ($q$-values), however, does so by controlling for the number of false positive discoveries in tests for which the null hypothesis was rejected as opposed to taking all tests into consideration. This method results in greater statistical power to find true effects while controlling for the type 1 error rate (Benjamini & Hochberg, 1995).

### 4.3.5 Deviation from Hardy-Weinberg Equilibrium

Hardy-Weinberg equilibrium is a quality-control measure used to ensure observed genotyping frequencies in a study population coincide with those expected under equilibrium conditions where alleles segregate randomly (Hardy, 1908; Weinberg, 1908). HWE is calculated using $\chi^2$-test where observed genotype frequencies in the study population are
compared to those expected under HWE (Weir, 1996). One variant in our sample, **GRIN2B rs7301328**, significantly deviated from HWE. HWE threshold values were set at the multiple-testing adjusted significance levels calculated for each study using the Nyholt method. This SNP was removed from subsequent analyses because chi-square tests require SNPs to be in HWE (Sasieni, 1997).

Several reasons may have accounted for why this variant deviated from HWE such as: genotyping errors that mistype heterozygotes as homozygotes or vice versa (Gomes et al., 1999; Hosking et al., 2004), stochastic variation, or due to the underlying biological characteristics of the study population (Nielsen et al., 1998). Deviations may also be a symptom of disease association (Balding, 2006).

### 4.3.6 Incomplete Outcome Data

Incomplete outcome data is generated when a patient discontinues treatment and is usually as result of noncompliance, lack of efficacy, the occurrence of adverse effects, or because the patient was lost to follow-up. Patients who leave a study prematurely contribute to inaccurate estimations of response rates in response studies and confound the ability of researchers to identify genetic markers that may have predictive potential. Noncompliance is particularly common among patients with SCZ and has been linked to relapse, re-hospitalization and poor outcome (Centorrino et al., 2001). Paranoia, cognitive dysfunction and bad judgment are potential reasons to explain why patients may not adhere to their medication regimens (Lacro et al., 2002). Fortunately, routine blood monitoring for patients taking CLZ allows treating physicians to both ascertain compliance and ensure CLZ serum levels have reached optimal threshold that is necessary to achieve response (≥450 ng/mL) (Lindenmayer & Apergi, 1996).
Discontinuation due to lack of efficacy and adverse reactions also similarly effect response rates. Lack of efficacy to a medication may indicate that drug levels are too low, while toxicity and adverse effects suggest drug levels are too high, and in both cases a dose adjustment may be necessary. As would be expected for a phenotype that has high inter-individual variability, optimal doses for achieving maximum therapeutic effect vary greatly among patients. Doses within the therapeutic window for the majority of patients may be inadequate or excessive for a subset of patients and may lead to undesirable treatment outcomes (Ma & Lu, 2011). The high rates of discontinuation in AP drug trials stresses the importance of understanding this inter-individual variability in drug response phenotypes and for identifying predictive markers that may have clinical utility.

4.4 FUTURE DIRECTIONS

This section explores future research initiatives assuming time and resources were unlimited. The first initiative would characterize the potential functionality of the glycine transporter 1 gene variant rs16831558 using a luciferase promoter assay. The second initiative would investigate the role of gene-gene and gene-environment interactions in drug response phenotypes. Lastly, the third initiative would investigate the contribution of novel rare variants in drug response phenotypes using next generation sequencing (NGS) platforms. This section concludes with a look at the emerging field of personalized medicine in psychiatry.

4.4.1 Characterization of Glycine Transporter 1 Promoter Variant

The GlyT1 promoter variant rs16831558 emerged as the highest priority SNP from our study. As mentioned previously, CC-homozygotes exhibited a notable improvement in their
positive symptom scores compared to T allele carriers following six months of CLZ therapy ($p_{uncorrected}=0.008$, $p_{corrected}=0.08$, assuming 9.36 independent tests). This novel variant is located approximately 5kb upstream of the SLC6A9 gene and is proposed to be functional according to data generated by ENCODE (ENCODE Project Consortium, 2012).

Four functional characteristics have been cited for this variant: 1) rs16831558 is localized to a region on chromosome 1 where GATA-binding factor 1 (GATA1) binds to the DNA (Hu et al., 2011). GATA1, otherwise known as erythroid transcription factor, has been shown to play a role in cell growth and cancer (Ferreira et al., 2005); 2) rs16831558 is localized to a T-box element DNA-motif where T-box transcription factor 18 (TBX18) binds to the DNA (Matys et al., 2006; Pique-Regi et al., 2011). TBX18 has been shown to interact with GATA4 and other transcription factors in gene promoter regions to modulate transcriptional activity (Farin et al., 2007); 3) rs16831558 is found within a DNAse hypersensitivity site in 13 different cell lines from ENCODE, including a medulloblastoma neuron precursor cell line (Medullo_D341) and cerebellum tissue from Caucasian donors (Cerebellum_OC) (ENCODE Project Consortium, 2012). DNAse-sequencing methods map DNAse hypersensitivity sites to locate regions of open chromatin where gene regulatory elements such as promoters, enhancers and silencers are likely to reside (Song & Crawford, 2010); and lastly, 4) rs16831558 is localized to a region of DNA designated as ‘enhancer’ in tissues from various brain regions (Bernstein et al., 2010; Ernst & Kellis, 2012). Collectively, this evidence provides sufficient rationale to investigate rs16831558’s potential to regulate transcription using, for example, a luciferase promoter assay.

Luciferase promoter assays allow researchers to study changes in mammalian gene expression in vitro by measuring the strength of a luminescent signal that is created when a
reporter gene that encodes the luciferase protein is transcribed (Fan & Wood, 2007). Conducting a luciferase promoter assay for rs16831558 would first involve designing two promoter DNA fragments: one containing the C-allele and the other containing the T-allele. These fragments would then be cloned into two different luciferase reporter vectors upstream from the luciferase complementary DNA (cDNA), which has had its promoter elements removed. These vectors would then be transfected into a neuronal cell-line to assess the differential ability of each promoter variant to drive expression of the luciferase protein. Expression levels are then measured by comparing the luminescent signal generated by each rs16831558 promoter construct to basal levels of luminescent intensity generated by promoter-less vector controls (Solberg & Krauss, 2013).

Certain aspects of the study methodology must be considered before designing a luciferase promoter assay for rs16831558. For instance, the cell-line chosen for the in vitro experiment is of particular importance: selecting a cell-line that mimics or is closest to the tissue of interest will yield results that more accurately represent that variant’s function in vivo. Neuroblastoma (NB) cell lines derived from neural crest cells are often used in neurobiology studies (Shastry et al., 2001). Because luciferase promoter assays are performed in vitro, they do not account for certain cellular and environmental factors that are present in vivo. Furthermore, sites relatively far away from the gene of interest may in reality have the ability to effect gene expression. This is possible due to looping and folding of the longer stretches of DNA in its native state, which is a 3D chromatin structure. Therefore, the results obtained from in vitro studies may only partially reflect the actual ability of the cloned promoter DNA construct to regulate gene expression in vivo. Consequently, additional studies to further
characterize this GlyT1 variant would be necessary. If this variant is found to be functional, further characterization in transgenic mice, for instance, may be indicated.

### 4.4.2 Gene-Environment Interactions

Gene-environment interactions occur when the effect of an individual’s genotype on a particular phenotypic outcome depends on the level of exposure to an environmental factor (Clayton & McKeigue, 2001). The contribution of gene-environment interactions to risk phenotypes is often overlooked for reasons such as lack of environmental data or bias in estimating the effect of exposure (Arranz & Munro, 2011). Gene-environment interactions also involve additional tests that invariably inflate the type I error rate (Peduzzi et al., 1995; Peduzzi et al., 1996). Despite these limitations, the combined effect of genetic and environmental factors such as cigarette smoking, caffeine consumption and concomitant medication use have been shown to contribute to variation in drug response phenotypes (de Leon, 2004; Caspi & Moffitt, 2006; Arranz & Munro, 2011). Therefore, future work characterizing gene-environment interactions in CLZ response may be useful for personalizing CLZ treatment and developing targeted interventions for patients at high risk of non-response and adverse side effects.

The first gene-environment interaction worth exploring in CLZ response investigates the joint effect of cigarette smoking and variation in the *GRIN2B* gene. The rationale for investigating this interaction is as follows: 1) genetic variation in *GRIN2B* is associated with early onset cigarette smoking and smoking initiation (Vink et al., 2009; Grucza et al., 2010); 2) nicotine administration in rats increases *GRIN2B* expression in particular brain regions (Wang et al., 2007); and 3) by-products that are generated from smoking tobacco induce CLZ metabolism and result in lower CLZ serum levels in patients who smoke versus those
who do not (Seppala et al., 1999; Meyer, 2001; Palego et al., 2002). Patients who carry a particular \textit{GRIN2B} genotype that predisposes them to smoking behaviours may be more likely to experience sub-optimal CLZ response due to sub-therapeutic serum levels and/or alteration of NMDAR subunit expression. Identification of a gene-environment interaction between cigarette smoking and high-risk genotypes in \textit{GRIN2B} may have clinical applications. Should an interaction be identified, for instance, clinicians may recommend that patients with risk genotypes reduce their cigarette smoking behaviours, or clinicians may prescribe higher CLZ dosages to patients who smoke.

Caffeine consumption may also interact with glutamate system genes and alter response to CLZ. The following observations support investigating this interaction: 1) caffeine consumption enhances glutamate release and excitatory neurotransmission in particular brain regions by binding to adenosine receptors on glutamatergic neurons (Dunwiddie, 1980; Dunwiddie et al., 1981; Solinas et al., 2002; Salmi et al., 2005); and 2) during acute administration, caffeine has been shown to inhibit clozapine metabolism and lead to higher CLZ serum levels in patients who consume caffeine versus patients who do not (Hagg et al., 2000; de Leon, 2004). Therefore, higher serum CLZ levels in patients who drink coffee, in combination with caffeine’s ability to mediate glutamate signaling may have multiplicative effects on drug response and may increase the risk of developing adverse side effects. Akin to the first interaction, understanding the relationship between caffeine intake and CLZ response may have clinical applications. Following Hagg et al., clinicians may initially prescribe lower CLZ dosages to patients who consume high amounts of caffeinated beverages, or recommend that patients with risk genotypes either limit caffeine consumption
or make attempts to maintain consistency in the amount of caffeine consumed per day (Hagg et al., 2000).

4.4.3 Gene-Gene Interactions

Gene-gene interaction, or epistasis, is the dynamic interaction among different genetic variants that occurs when one allele modifies the effect of alleles at other loci, or when two or more loci interact to create a new phenotype (Phillips, 2008). Epistatic interactions are thought to account for a portion of the inter-individual variability that is observed in polygenic traits, such as AP response (Long & Langley, 1999; Sadee, 2012; Zuk et al., 2012). Treatment outcomes likely depend on the multiplicative effect of several genes that are involved in drug action, such as enzymes involved in metabolism, membrane transporters, receptors and target proteins (Sadee, 2013). However, compared to the large number of genes involved in disease phenotypes such as SCZ, CLZ response likely depends on a lesser number of genes to determine treatment outcomes as evidenced by pharmacogenetics studies (Johnson et al., 2011; Ma & Lu, 2011). Therefore, effective biomarker panels requiring only a limited number of genes may be able to capture much of the genetic variation contributing to drug response (Sadee, 2013). The clinical utility of such predictive tests provides sufficient rationale for the investigation of three potential epistatic interactions involving glutamate genes with response to CLZ.

The first gene-gene interaction worth exploring in CLZ response involves two genes that were involved in this study: the NMDAR NR2B subunit gene \textit{GRIN2B}, and the GlyT1 gene \textit{SLC6A9}. Three observations provide support for investigating this gene-gene interaction: 1) Differential expression of either NR2B and GlyT1 are capable of altering NMDAR activation (Flint et al., 1997); 2) NR2B and GlyT1 co-localize to glutamatergic synapses and
may physically interact (Cubelos et al., 2005); and 3) NMDAR activation is thought to account for a portion of CLZ’s therapeutic effects (Banerjee et al., 1995; Arvanov et al., 1997; Kubota et al., 2000; Ninan et al., 2003; Kargieman et al., 2007). Collectively, these findings suggest that differential expression of NR2B and GlyT1 proteins by potentially functional genetic variants may interact to augment NMDAR neurotransmission, and subsequently, response to CLZ. A proposed gene-gene interaction analysis would investigate the putatively functional SLC6A9 variant rs16831558 that was genotyped in this study, and the GRIN2B variant rs1805502 that was previously associated with negative symptom change during CLZ therapy (Martucci & Kennedy, 2010).

An epistatic interaction may also exist for the mGluR2 gene GRM2 that was investigated in this study, and the 5-HT2A gene, HTR2A. The rationale for investigating this interaction is as follows: 1) In particular brain regions, translation of the 5-HT2A receptor is necessary for normal mGluR2 expression (Gonzalez-Maeso et al., 2008); 2) mGluR2 has been shown to form a special hetero-complex with 5-HT2A through specific transmembrane helix domains (Gonzalez-Maeso et al., 2008); 3) CLZ binds to the 5-HT2A component of this complex and up-modulates mGluR2-elicited signaling by 40% (Fribourg et al., 2011); and 4) The 5-HT2A-dependent antipsychotic-like behavioural effects of CLZ require the expression of mGluR2 (Fribourg et al., 2011). Taken together, these findings suggest that CLZ may act on the mGluR2-5-HT2A complex to achieve symptom change in SCZ patients and that altered expression of both HTR2A and GRM2 may have an additive affect on response. Of high priority is the functional HTR2A variant rs6314 previously associated with CLZ response (Nothen et al., 1995; Masellis et al., 1995; Burnet & Harrison, 1995; Arranz et al., 1995) and the potentially functional GRM2 SNP rs2518461 identified in this study.
The final interaction worth considering for future work involves *GRIN2B* and the 5-HT1A gene, *HTR1A*. Support for this interaction is as follows: 1) CLZ is a partial agonist at the 5-HT1A receptor (Newman-Tancredi et al., 1996); 2) CLZ facilitates formation of a synergistic triad between 5-HT1A, the NMDAR, and CaMKII (Purkayastha et al., 2012); and 3) This triad is necessary for CLZ-mediated increases in EPSPs in PFC pyramidal neurons (Purkayastha et al., 2012). Genetic variation in both *HTR1A* and *GRIN2B* may have the potential to alter triad formation and, as a result, alter CLZ’s ability to elicit excitability in certain brain regions. The functional *HTR1A* promoter variant rs6295 previously implicated in depression and antidepressant response (Lemonde et al., 2003; Lemonde et al., 2004; Parsey et al., 2006) is of high interest and deserves investigation with *GRIN2B* SNPs of interest.

There are a number of challenges associated with analysing gene-gene and gene-environment interactions in drug response phenotypes. One challenge of modelling such relationships is the increasing dimensionality that results from a large number of possible interactions (Moore, 2004; Motsinger et al., 2007). As a result, the application of traditional parametric tests to analyse interaction data has limited use and often increases the risk of type I and type II errors, while at the same time decreasing power (Moore, 2004). For these reasons, more advanced statistical approaches have been developed such as the multifactor dimensionality reduction (MDR) method (Ritchie et al., 2001). MDR reduces dimensionality by dividing high-risk and low-risk interactions into two separate groups for analysis, and then selects the model(s) best able to accurately predict response/non-response status in independent testing sets generated through cross-validation. Models with the highest cross-validation consistency are used in permutation testing to identify models with statistically significant results not
expected by chance alone. Often the most challenging aspect of interaction analysis involves interpreting the biological plausibility of the results, and follow-up studies using cell-culture or other in vitro experiments can be useful aids to interpretation (Ritchie & Motsinger, 2005).

### 4.4.4 Next Generation Sequencing

Next generation sequencing (NGS) is a high-throughput DNA sequencing technology that has revolutionized the way scientists extract genomic data from biological systems. NGS utilizes a similar concept to Sanger sequencing whereby bases in a small fragment of DNA are sequentially identified from fluorescent signals emitted as each fragment is resynthesized (Sanger et al., 1977). However, NGS carries out this process on a grander scale by sequencing millions of DNA fragments at once and results in a quicker, less expensive method for sequencing whole genomes (Shendure & Ji, 2008).

One particular application of NGS that is of interest to disease risk and medication response is targeted sequencing, wherein a subset of genes is sequenced, as opposed to the whole genome. Targeted sequencing allows researchers to achieve high sequence coverage, obtain sequencing results for many individuals, and makes it possible to identify rare variants that were missed using previous methods. Rare, in this case, refers to variants that occur less than once per 100 individuals. Rare variants have given rise to what is known as the ‘common disease-rare variant’ hypothesis, which argues that multiple different rare mutations in overlapping genomic locations and/or biochemical pathways drive susceptibility to risk phenotypes, and are likely to contribute to a portion of the missing heritability in complex traits (Schork et al., 2009; Altmann et al., 2013).
NGS technologies have already identified excess rare variants and deletions in glutamate-related synaptic genes in SCZ and autism (Kelleher et al., 2012; Myles-Worsley et al., 2013; Kenny et al., 2014). Regarding drug treatment, NGS has mostly been used in cancer research to identify rare and common variants in response to chemotherapeutic agents (Ross & Cronin, 2011; Tran et al., 2013; Ong et al., 2014). A current review of the literature does not reveal any major discoveries using NGS technologies to identify genetic predictors of response to AP therapy. However, the practical applications of such discoveries cannot be understated. Discovery of rare variants and predictive markers would allow clinicians to distinguish between responders and non-responders and identify patients who are at high risk of failing treatment. The information derived from NGS may also be used to guide development of novel drugs that target aberrant gene systems in the non-responder group. Therefore, future research involving targeted sequencing of glutamate system genes is recommended to help uncover rare variants involved in CLZ response.

To briefly illustrate the methodology behind NGS, a hypothetical workflow for sequencing a gene of interest using the Ion Torrent Personal Genome Machine (PGM) Sequencer (Life Technologies) in our CAMH laboratory is proposed. The four steps are as follows: 1) Library preparation: The first step is to create a library of DNA fragments that are flanked by Ion Torrent adapters. Adapters can be added to DNA fragments by one of two ways – ligating the adapter to the PCR product, or by incorporating the adapter sequence automatically during PCR using specially designed primers; 2) Template preparation: The second step involves coating special ‘Ion Sphere’ particles with DNA fragments via their adapter sequences, followed by clonal amplification of the library fragments by emulsion PCR (emPCR); 3) Sequencing: The Ion Sphere particles coated with library fragments are then
deposited onto the sequencing chip, placed into the Ion Torrent machine, and the sequencing run is begun, 4) Data Analysis: The final step of the work flow analyses sequencing data. Data is transferred to the appropriate servers, and then run through signal processing and base-calling algorithms to re-construct DNA sequences. Sequencing data is then imported into analysis programs such as the Partek® Genomics Suite™. One additional point to consider during study design is the depth of coverage. Depth refers to the number of times the region of interest is re-sequenced. Rare variants and somatic mutations that are present at low frequencies require higher depths of coverage to ensure the mutation calls are made with sufficient statistical confidence (Life Technologies, 2011).

Challenges associated with NGS technology must also be mentioned. Because this technology produces vast amounts of data in a relatively short period of time, future research initiatives will need to focus on how to organize and interpret the sheer volume of genomic data that is produced. In addition, the ability to detect rare variants depends on the availability of large sample sizes and stresses the need for a collaborative effort in the research community. Ideally, a common database will be developed where researchers investigating AP response phenotypes can upload their DNA samples and/or sequencing results, with the shared goal of advancing medical research within this field. Ethical issues also abound and stress the need for legislation to protect citizens from discrimination based on their genome (Mathieu et al., 2013). Several nations have passed legislation barring insurance companies and employers from discriminating against citizens based on their DNA sequence. The United States of America passed the Genetic Information Nondiscrimination Act of 2008 (GINA) (United States of America, 2008), while the United Kingdom passed the Equality Act of 2010 (United Kingdom of Great Britain, 2010). Canada passed Bill C-445 in
2011, which amended the Canadian Human Rights Act to include protection of Canadian citizens from discrimination based on their ‘genetic characteristics’ (Parliament of Canada, 2011). Shortly afterwards, Bill S-201 was passed by Canadian legislature, otherwise known as the Genetic Nondiscrimination Act (Parliament of Canada, 2013), to provide Canadian citizens with further protection against discrimination based on their DNA. Despite these limitations, NGS has already revolutionized the field of genetics research and is predicted to lead to many groundbreaking discoveries in the future.

### 4.4.5 Personalized Medicine in Psychiatry

Personalized medicine in psychiatry is a relatively new field with the goal of tailoring drug treatment to suit an individual’s personal genetic makeup. The realization that inter-individual variability in drug response had a genetic underpinning was a pivotal discovery on the path to personalized medicine (Garrod, 1909; Beutler et al., 1955; Evans et al., 1960). Furthermore, launch of the Human Genome Project (Lander et al., 2001) and the development of more cost-effective genome sequencing methods have made genetic information more easily accessible to the general population. New studies in pharmacogenetics that investigate how drugs and genetics interact are also of high importance for the developing field of personalized medicine.

The field of personalized medicine in psychiatry is evolving and seeing the development of genetic panels and algorithms used as aids in medication prescribing. A number of pharmacogenetic tests are available on the market and have been reviewed elsewhere (Arranz & Munro, 2011). One genetic test worth mentioning is the GeneSight® test developed by AssureRX Health Inc. This test incorporates genotype data for four different CYP enzymes, as well as the serotonin transporter 1 (SLC6A4) and HTR2A genes. Studies investigating the
utility of the test have reported improved outcomes should the test be used in routine clinical care (Hall-Flavin et al., 2013).

Unfortunately, a scientifically validated test for CLZ response has not yet emerged. Approximately 15 years ago, researchers Kerwin and Arranz along with their colleagues published findings on a genetic panel of six polymorphisms that were capable of predicting CLZ response with a 76.6% success rate in their sample of 200 Caucasian SCZ patients \((p=0.001)\) (Arranz et al., 2000). The six variants identified were located in neurotransmitter-receptor related genes including two \(HTR2A\) variants (102T/C and His452Tyr), two serotonin 2C receptor gene \((HTR2C)\) variants (-330-GT/-244-CT and Cys23Ser), the serotonin transporter promoter \((5\text{-HTTLPR})\), and the histamine H2 receptor gene \((HRH2)\) variant \((-1018\text{-G/A})\). These six polymorphisms, when combined, had a retrospective sensitivity of 95% for identifying patients with ‘satisfactory response’. This report was the first of its kind attempting to predict CLZ response using genetic markers. Unfortunately, other authors were unable to replicate these findings (Arranz et al., 2000; Schumacher et al., 2000). A commercialized test was developed based on Kerwin and Arranz’s findings by a UK company known as LGC (formerly known as the Laboratory of the Government Chemist that was privatized in 1996). Unfortunately, no prospective studies assessing the clinical and economic implications of this test have been conducted.

Despite the lack of success in developing a predictive test for CLZ response, advancements in other areas of pharmacogenetics such as the ability to predict drug side effects, offer hope for the future. Relatively recently, our colleagues at CAMH in Toronto developed a gene panel consisting of 5 gene markers that is able to explain 27% of the variance associated with developing antipsychotic induced weight gain (AIWG) (Tiwari et al, unpublished). AIWG is
a debilitating side effect of second-generation antipsychotics such as CLZ and OLZ that frequently results in obesity, metabolic syndrome and premature morbidity. The ability to predict the likelihood of developing such negative side effects will allow physicians to make more informed decisions regarding medication choice.

The benefits of personalized medicine to psychiatric care cannot be understated: outcomes from personalized treatment may allow physicians the ability to define diseases more precisely and to distinguish subtypes/groups of SCZ patients that are based on a drug’s limited group specificity. In addition, the increased availability of genome sequencing data may greater our understanding of the underlying pathophysiological basis of SCZ and lead to the development of novel treatments that are more effective for treating specific disease subtypes (reviewed by Insel & Scolnick, 2006). For instance, research has suggested that patients suffering from TRS may present with a different underlying etiology than patients who respond to conventional dopamine-blocking antipsychotic medications (Egerton et al., 2012; de la Fuente-Sandoval et al., 2013; Demjaha et al., 2014). Demarcation between individuals likely to respond and those who are not at first episode would enable physicians to select a drug, once developed, that is more specific to this subgroup of patients.

There are several issues surrounding the use of genetic testing in personalized medicine that must be mentioned. For instance, drug response likely results from a complex interaction between genes, brain and environmental factors such as a patient’s lifestyle, age, gender, diet, ethnicity and amount of social support. Therefore, it is imperative that researchers investigating pharmacogenetic processes sufficiently acknowledge the uncertainties pertaining to their research and report their findings with caution (Evers, 2009). As well, the contribution of epigenetic interactions must also be considered regarding nonresponse to
medications. Additional concerns include increased drug costs associated with targeted drug development and an increased risk of pharmacological exclusion due to ethnicity or under-represented genetic groups. As with all novel areas of research, appropriate infrastructure to handle challenges associated with using pharmacogenomic drugs in psychiatric care will also need to be implemented. Additional aspects that will need to be addressed are reliability of genotyping, equal access to testing, as well as clinician and patient education (Costa e Silva, 2013).

Given the advancements in genetic technology and the interest in developing genetic panels capable of response prediction, the future of personalized medicine in psychiatry looks promising. As with all new areas of research, educating physicians and patients, developing the appropriate infrastructure and anticipating ethical issues will need to be considered as the field of personalized medicine continues to evolve. To highlight the prospective benefits, personalized medicine in psychiatry has the potential to guide clinician choice regarding therapeutic dose, decrease the occurrence of adverse drug reactions, and improve overall disease prognosis. Results from pharmacogenetics studies and personalized treatment may also guide development of novel treatments and identify more homogeneous disease subclasses based on the propensity of certain individuals to respond to select medications.

4.5 CONCLUDING REMARKS

Glutamate signaling pathways are emerging as top candidates in SCZ risk and as targets for novel therapies for the treatment of SCZ. As the field of psychiatric genetics continues to expand with the advent of more cost effective methods for analysing genomic data, personalized medicine in psychiatry is becoming more of a reality.
Herein, we investigated the association of 17 glutamate system gene variants and clinical response to CLZ. We reported no significant associations after correction for multiple testing. Prior to correction, several nominally significant associations were observed. The most noteworthy preliminary finding was for the glycine transporter 1 gene variant rs16831558: patients carrying the C-allele exhibited a marked improvement in positive symptoms compared to T-allele carriers following six months of CLZ therapy. Our findings provide further credence to the ‘glutamate hypofunction hypothesis’ of SCZ and support future research initiatives to investigate the potential functionality of rs16831558 and further explore this variant in larger CLZ response samples.
REFERENCES


Bleuer E. (1911). *Dementia praecox oder gruppe der schizophrenien (dementia praecox or the group of schizophrenias)*. Leipzig, Germany: Franz Deuticke.


Li ML, Hu XQ, Li F & Gao WJ. (2015). Perspectives on the mGluR2/3 agonists as a therapeutic target for schizophrenia: Still promising or a dead end? *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 60*: 66-76.


Santos SD, Carvalho AL, Caldeira MV & Duarte CB. (2009). Regulation of AMPA receptors and synaptic plasticity. *Neuroscience, 158*(1): 105-125.


Schwieler L & Erhardt S. (2003). Inhibitory action of clozapine on rat ventral tegmental area dopamine neurons following increased levels of endogenous kynurenic acid. *Neuropsychopharmacology, 28*(10): 1770-1777


Seppala NH, Leinonen EV, Lehtonen ML & Kivisto KT. (1999). Clozapine serum concentrations are lower in smoking than in non-smoking schizophrenic patients. *Pharmacology & Toxicology, 85*(5): 244-246.


