The use of adjuvant L-arginine in schizophrenia: A behavioural and neurochemical analysis

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Psychopharmacology

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University of Alberta

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ABSTRACT

Schizophrenia is a complex neurodevelopmental illness that often requires a combination of treatments to adequately control its symptoms. Despite the availability of several neuroleptic medications on the market, many patients with schizophrenia remain symptomatic. Research has shifted emphasis towards impaired glutamate signaling as a major contributor to the illness. Drug targets specific to the glutamate-nitric oxide (NO) pathway may offer a promising new approach to treatment.

The therapeutic effect of the amino acid L-arginine (Arg), the substrate for the NO-producing enzyme neuronal nitric oxide synthase (nNOS) was examined in this thesis. As an augmenting strategy, Arg was administered (at 6g per day) to patients with schizophrenia in an attempt to improve the efficacy of antipsychotic therapy. Responders to Arg therapy had a reduction in anxiety symptoms that were previously resistant to prescribed medication. The anxiolytic effect of Arg may be directly related to an increase in endogenous central NO production and the downstream induction of cyclic guanosine monophosphate (cGMP)-mediated signaling cascades. L-Arginine also proved to be a safe strategy in the treatment of schizophrenia and did not exacerbate any of the known side-effects that were already present as a result of ongoing antipsychotic therapy.

This thesis also investigated genetic influences on glutamate *N*-methyl-D-aspartate (NMDA) receptor functioning and treatment response to Arg therapy. The Neuronal Period-Arylhydrocarbon Nuclear Translocator

(ARNT)-Single-minded (PAS) domain containing 3 (*NPAS3*) gene codes for a protein that functions as a transcription factor. *NPAS3* and its related variants may be important for the development of functional postsynaptic density (PSD-95) scaffolding proteins that directly link the NMDA receptor to the nNOS enzyme in the brain. Variants associated with *NPAS3* and schizophrenia were examined to elucidate the integrity of the PSD-95 protein and used to predict the outcome of the effects of adjuvant Arg therapy. Two carriers of a *NPAS3* variant who did not respond to Arg treatment may have had compromised NMDA receptor function related to altered neurodevelopmental processes that occurred during early brain development. Participants were also genotyped for the catechol-O-methyltransferase (*COMT*) enzyme single nucleotide polymorphism (SNP) p.V158M to explore the relationship between this genetic marker and clinical outcomes. No significant gene-gene interaction between *NPAS3* and *COMT* was determined that would influence treatment response to Arg therapy.

Preclinical experiments were also included in this thesis to determine the behavioural and neurochemical mechanisms by which Arg was able to exert its therapeutic effect. Competing metabolic enzymes that utilize Arg as a substrate were investigated by measurement of related amino acids ornithine (Orn) and citrulline (Cit). The immediate potentiation of whole brain Cit levels following Arg administration suggested that NO is produced quickly and NOS enzymes require a much lower concentration of Arg than that of the arginase enzymes.

This thesis was also able to confirm the utility of phencyclidine (PCP) as a comprehensive pharmacological model of psychosis and the ability of antipsychotic drugs to reduce PCP-induced hyperlocomotor activity. The administration of Arg alone in a rodent PCP model had no significant effect on hyperlocomotor activity (a behavioural measure of the positive symptoms of psychosis) and it also did not enhance the locomotor-reducing effects of antipsychotic drugs. These results were consistent with the clinical trial data where no significant changes in positive symptoms were also found when augmenting antipsychotic treatment with Arg. Although Arg therapy was unable to potentiate antipsychotic effects in schizophrenia, work presented in this thesis does support the use of Arg as a tolerable and effective strategy that may target intrinsic anxiety in treatment-refractory schizophrenia.

PREFACE

This thesis is an original work done by Mary-Anne Bellwood MacKay. The clinical research project which is part of this thesis received research ethics approval from the University of Alberta Research Ethics Board, Project Name

"A RANDOMISED DOUBLE-BLIND CROSS-OVER, PLACEBO-CONTROLLED ADJUNCTIVE TREATMENT OF L-ARGININE ADDED TO TREATMENT AS USUAL (TAU) IN SCHIZOPHRENIA: AN EXPLORATORY THERAPEUTIC CLINICAL TRIAL PROTOCOL" REB approval No. MS3_Pro00002053 DECEMBER 14, 2007.

DEDICATION

I would like to dedicate this thesis to my father Ronald George Buchanan MacKay

ACKNOWLEGEMENTS

I would like to express my appreciation for all of the support and encouragement I have received from a number of people throughout my studies. First of all, to my supervisor, Dr. Serdar Dursun, who has been a tremendous mentor and teacher to me. I can never thank him enough for his exceptional guidance as a clinical research scientist. I have been so fortunate to work along side him and could not have asked for a better supervisor. I would also like to thank the members of my supervisory committee. To Dr. Glen Baker, for his ongoing support throughout my project and for his excellent guidance in the writing of this thesis. Also, to Dr. John Lind, for his words of encouragement over the years and his infectious enthusiasm for statistical methods. Thanks as well to Drs. Satyabrata Kar and Harold Robertson for taking the time to review my thesis as external examiners. To Gail Rauw, for spending all of the time you did with me in the lab and patiently teaching me the analytical techniques relevant to my thesis. Thanks to all at the Neurochemical Research Unit for making me feel so welcome. To Andy Greenshaw, your support and perspective made the milestones of graduate school seem much less intimidating. Also, thanks to Drs. Diane Cox and Georgina Macintyre for allowing me to work with you in your laboratory. Thanks also to Drs. P.J. White and Cory Czarnecki and all of the psychiatrists and nurses at Alberta Hospital Edmonton as well as the Addictions and Mental Health Clinic in Edmonton who helped support my efforts in recruiting for my study. A heartfelt thank you to all of the dedicated individuals that participated in the study and who were willing to investigate L-Arginine treatment for their illness. Finally, I am forever grateful to my family for their love and support. To my mum, I could not have completed this thesis without you. Thank you for being so kind and good to us. To my brother, his genuine love of science has always been an inspiration to me. He is the smartest fellow I know. And finally, to my friends, Lynn and Terence, for teaching me about true determination and to never give up.

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LIST OF ABBREVIATIONS

AIMS	Abnormal Involuntary Movement Scale
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AGMAT	agmatinase
Ala	alanine
ALT	alanine transaminase
AITF	Alberta Innovates Technology Futures
ALP	alkaline phosphatase
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	analyses of variance
ACE	angiotensin-converting enzyme
Arg1	arginase type I
Arg2	arginase type II
Arg	L-arginine
ADC	arginine decarboxylase
AGAT	arginine:glycine amidinotransferase
ASL	arginosuccinate lyase
ASS	arginosuccinate synthetase
Asp	aspartate

AST	aspartate transaminase
bHLH	basic helix-loop-helix
BBB	blood-brain barrier
BUN	blood-urea-nitrogen
BMI	body mass index
BPRS	Brief Psychiatric Rating Scale
CDRS	Calgary Depression Rating Scale for Schizophrenia
CaM	calmodulin
CPS1	carbamoyl phosphate synthetase 1
COMT	catechol-O-methyl transferase
CATS	cationic amino acid transporters
CNS	central nervous system
CPAT	Centre for Psychiatric Assessment and Therapeutics
CSF	cerebrospinal fluid
Cit	L-citrulline
CATIE	Clinical Anti-Psychotic Trials of Intervention Effectiveness
CGI	Clinical Global Impression Scale
CBC	complete blood count
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
dNTPs	deoxynucleoside triphosphates

DNA	deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual for Mental Disorders, Fifth Edition
DSM-IV TR	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revised
ddNTPs	dideoxynucleoside triphosphates
DETA	diethylenetriamine
DISC1	Disrupted in Schizophrenia 1 gene
DTNBP1	distrobrevin binding protein 1 gene
DA	dopamine
D2	dopamine receptor-2
DRD2	dopamine receptor D2 gene
DAT	dopamine transporter
DLPFC	dorsolateral prefrontal cortex
ECG	electrocardiogram
eNOS	endothelial nitric oxide synthase
ERBB4	erbB4
Epo	erythropoietin
EDTA	ethylenediaminetetraacetic acid
EEAT	excitatory amino acid transporter
EPS	extrapyramidal side-effects
FGAs	first-generation antipsychotic drugs

FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FG3	FlexiGene
GABA	gamma-aminobutyric acid
GABA-T	gamma-aminobutyric acid transaminase
GWAS	genome-wide association studies
Glu	glutamate
Gln	glutamine
GTN	glyceryl trinitrate
Gly	glycine
GlyT1	glycine transporter-1 inhibitor
GK	guanylate kinase-like
HPLC	high performance liquid chromatography
iNOS	inducible nitric oxide synthase
IL-8	interleukin 8
ICD-10	International Classification of Mental Disorders, 10th Edition
i.p.	intraperitoneal
IBLC	N-isobutyryl-L-cysteine
LTD	long-term depression
LTP	long-term potentiation

Lys	lysine
mRNA	messenger ribonucleic acid
mGluR	metabotropic Glu receptor
MEOH	methanol
Met	methionine allele
NMDA	*N*-methyl-D-aspartate
miRNA	micro-ribonucleic acid
MAP 6	microtubule-associated protein 6
MP	millipore
HCl	monohydrochloride
MIS	multi-innervated spine
MI	myocardial infarction
NRG1	neuregulin 1
nNOS	neuronal nitric oxide synthase
NPAS3	Neuronal Period-Arylhydrocarbon Nuclear Translocator (ARNT)-Single-minded (PAS) domain containing 3
NAD^+	nicotinamide-adenine dinucleotide
NADPH	nicotinamide-adenine dinucleotide phosphate
NADPHd	nicotinamide-adenine dinucleotide phosphate-diaphorase
NO	nitric oxide
NOS	nitric oxide synthase

L-NAME	*N*^{G}-nitro-L-arginine methyl ester
NPO	*non per os*
NSAIDS	nonsteroidal anti-inflammatory drugs
NORT	Novel Object Recognition Task
OPA	*o*-phthaldialdehyde
Orn	ornithine
OAT	ornithine aminotransferase
ODC	ornithine decarboxylase
ORN-T	ornithine transaminase
PD	Parkinson's disease
$ONOO^{-}$	peroxynitrite
PCP	phencyclidine
PCr	phosphocreatine
PDE-5	phosphodiesterase-5
PAS-HC	Photobeam Activity System-Home Cage
PEG	polyethylene glycol
PCR	polymerase chain reaction
PANSS	Positive and Negative Syndrome Scale
PSD	postsynaptic density
PSD-95	postsynaptic density 95kDa
PDZ	Postsynaptic density/Disc-large/Z0-1 homologous

PFC	prefrontal cortex
Pro	proline
^{1}H-MRS	proton magnetic resonance spectroscopy
P5C	l-Δ^{1}-pyrroline-5-carboxylate
RNA	ribonucleic acid
SGAs	second-generation antipsychotics
SSRI	selective serotonin reuptake inhibitor
Ser	D- and L-serine
SRR	serine racemase gene
5-HT	5-hydroxytryptamine (serotonin)
SERT	serotonin transporter
SBE	single-base extension
SNP	single nucleotide polymorphism
SPET	single photon emission tomography
SNP	sodium nitroprusside
sGC	soluble guanylate cyclase
STOP	stable tubule only polypeptide
S.E.M	standard error of the mean
TD	tardive dyskinesia
Taur	taurine
BH4	tetrahydrobiopterin

TSH	thyroid stimulating hormone
TCA	tricarboxylic acid
TNFα	tumor necrosis factor alpha
UKU	Udvalg for Kliniske Undersøgelser Side-Effect Rating Scale
UV	ultraviolet
Val	valine allele
VTA	ventral tegmental area
VGLUT	vesicular glutamate transporter

CHAPTER 1.

GENERAL INTRODUCTION

1.1 FOREWORD

The development of effective pharmacological treatments for schizophrenia continues to be a priority area of research. Schizophrenia is a progressive neurodevelopmental illness that may begin *in utero*, with symptom development usually emerging by the time of adolescence. The etiology of schizophrenia is not completely understood, but research has suggested that having a genetic vulnerability coupled with an early life insult to the brain during critical periods of neurogenesis may trigger its onset. Aberrant neurodevelopment and altered synaptic connectivity that drive the symptoms of schizophrenia have yet to be defined, and inadequate understanding of these processes has been critical to the lack of progress of pharmaceutical development to treat the illness. Factors involved in these neurodevelopmental processes may be important targets to consider for future therapeutics.

Nitric oxide (NO), a lipophilic gas and signaling molecule, may be a promising target for further development of antipsychotic therapies. Nitric oxide is essential for a number of important cellular processes throughout the body including those critical for normal brain development and synaptogenesis. Nitric oxide is generated in the brain by an interaction with a functional glutamate (Glu) *N*-methyl-D-aspartate (NMDA) receptor. Many of the genetic and environmental risk factors that have been identified in schizophrenia converge upon this receptor. Downstream signaling mechanisms that are regulated through a fully functional NMDA receptor, including the production of NO, may be disrupted in the illness.

Studies that have measured clinical response to direct pharmacologically-mediated elevation of NO levels in schizophrenia have been limited. As a result, drug development toward this particular molecular target has yet to be developed. The research described in this thesis investigated the potential therapeutic benefit of NO donation by L-arginine (hereafter referred to Arg, discussed in Chapters 2,3,4 and 6) in

schizophrenia. I examined the behavioural and neurochemical effects of NO synthesis in schizophrenia and also in an animal model of psychosis. The research investigated the clinical behavioural response to the amino acid Arg added to current antipsychotic therapy in adult human participants diagnosed with schizophrenia. It also measured the behavioural responsiveness and neurochemical effects of Arg supplementation alone and as an augmenting strategy to antipsychotic therapy in a phencyclidine (PCP) model of psychosis in rodents. No human or animal research to date has investigated the direct relationship of Arg-induced neuronal NO production in the brain as a possible augmenting strategy to improve antipsychotic effectiveness in schizophrenia. This research expands on previous studies that have investigated the role of the glutamate-NO pathway in animal models of psychosis, and aims to complement a recent clinical study as well as two case reports of the use of an adjuvant intravenous drug sodium nitroprusside (SNP)(a NO donor) in patients with schizophrenia. Drug targets specific to the glutamate-NO pathway may offer a promising new approach to treatment in schizophrenia, and the modulation of NO levels could be of therapeutic benefit to those who have developed resistance to current available treatments.

1.2 SCHIZOPHRENIA

1.2.1 Neurodevelopmental hypothesis

Schizophrenia was initially conceptualized as a neurodevelopmental disorder by the German psychiatrist Emil Kraepelin at the beginning of the 20th century. He described the syndrome as a dementia praecox, a term that highlighted the cognitive deficits of the illness (dementia) with the early onset of the disorder (praecox) (Kaplan and Sadock 2007). Today, schizophrenia is now widely accepted to be a progressive neurodevelopmental illness with its origins in very early brain development. Normal brain developmental trajectories rely on a number of very complicated cellular interactions that start shortly after conception and

continue well into adulthood. Subtle disease processes that occur in the brain *in utero* affect the formation of critical circuits in schizophrenia. Disrupted neuronal development is only later uncovered by the emergence of subtle symptoms, abnormal behaviour, and deficits in social, educational and occupational functioning that are uncovered at critical life milestones. In the case of schizophrenia, hints of altered neurodevelopment often first emerge during childhood. Children that have gone on to develop schizophrenia share similar characteristics of poor motor development such as delays in walking, sitting, and standing (Fish *et al.* 1992; Jones *et al.* 1994; Cannon *et al.* 2002a); abnormal movements and posturing of upper limbs and hands (Walker *et al.* 1994); and poor social skills including preferences for solitary play and anxiety in social situations (Jones *et al.* 1994).

1.2.2 Disease course

It is during the period of late adolescence that the first signs of psychosis usually start to emerge in those who will develop schizophrenia; this is a time when there is a significant amount of changes in both grey matter volumes and cortical circuit refinement involving synaptic pruning, reinforcement, and neuronal synchronization (Huttenlocher *et al.* 1979; Feinberg *et al.* 1982; Rakic *et al.* 1986). Adolescents who develop schizophrenia often exhibit sub-threshold psychotic symptoms such as behavioural withdrawal; various abnormalities in content, form, and flow of thinking; and emotional and concentration difficulties. These prodromal symptoms can go on for years before diagnosis occurs and antipsychotic medications are initiated (McGlashan 1996). Psychosis that emerges at the end of the prodromal period usually leads to a formal assessment and initiation of treatment for those with schizophrenia. Progressive brain changes (reduced prefrontal and temporal cortical volumes) do occur at an accelerated rate in psychotic adolescents (Thompson *et al.* 2001) and continue to progress following first-episode psychosis in both medicated (DeLisi *et al.* 1995; Cahn *et al.* 2002) and antipsychotic-naïve patients, with a

slowing of the cortical loss with the initiation of early antipsychotic treatment (Keshavan *et al.* 1998a; Lieberman *et al.* 2003). It is recommended that treatment should be started as early as possible following the development of psychotic symptoms (Addington *et al.* 2004). Most individuals with schizophrenia will go on to develop a chronic course of the illness that is characterized by periods of exacerbation and remission of psychosis. Recovery is highly dependent on compliance with antipsychotic medications, close therapeutic alliance to the treatment team, and ongoing social and vocational support. Even with the best treatments and supports, there is a sub-population of patients who will never achieve an adequate response to treatment and remain very ill. These patients have what is known as treatment-refractory or ultra-resistant disease (Kane *et al.* 1988; Mouaffak *et al.* 2006).

1.2.3 Etiology

The precise mechanism responsible for the development of schizophrenia remains unknown. From the findings of studies conducted to date, it is likely a result of a complex interaction between many genes and multiple environmental risk factors. The combination of both genetic and environmental "hits" to the developing brain results in widespread abnormalities in neurons at the molecular, cellular, structural, and functional level. The widespread disruption of synapse and brain circuit connectivity leads to a heterogeneity of cellular pathological findings and also contributes to the multiple behavioural and functional domains of the illness.

1.2.4 Genetic risk factors

In some cases, schizophrenia is an inherited genetic illness. One of the most significant risk factors for developing schizophrenia is having a first degree relative with the illness, with higher concordance in monozygotic twins (35-58%) than in fraternal twins (9-27%), and an overall estimate of heritability to be around 80% (Shields and Gottesman 1972; Cardno *et al*. 1999; Sullivan *et al*. 2003). The brain is a vulnerable target for genetic

mutation as it relies on thousands of proteins and related processes to function properly. There have been several candidate genes that have been identified in schizophrenia such as catechol-O-methyl transferase (*COMT*) (Egan *et al*. 2001; Shifman *et al*. 2002); neuregulin 1 (*NRG1*) and its receptor erbB4 (*ERBB4*) (Stefansson *et al*. 2003; Norton *et al*. 2006); distrobrevin binding protein 1 or dysbindin (*DTNBP1*) (Straub *et al*. 2002); Disrupted in Schizophrenia 1 (*DISC1*), a gene with a balanced translocation between chromosome 1 and 11 with the breaking point at 1q42.1 (Millar *et al*. 2000); and many others. However, knowledge of their individual contribution to the development of the disease has been limited.

Genome-wide association studies (GWAS) have been useful for looking at copy number variants (additions and deletions) in different populations of schizophrenia worldwide to identify more rare mutations that may have a larger effect than what a single gene can contribute (Shi 2009b; Stefannson *et al*. 2009). The largest molecular GWAS to date was able to identify 108 independent genomic loci, with associations that included the dopamine receptor D2 gene (*DRD2*) along with many genes encoding a number of the Glu receptors including *GRM3, GRIN2A, AMPA1*, *GRIA1,* as well as the serine racemase gene (*SRR*) that encodes the enzyme that generates D-serine (from L-serine), a potent co-agonist at the NMDA receptor. These genetic associations are relevant to both dopaminergic and glutamatergic functioning in the illness, linking the two major competing neurotransmitter hypotheses of schizophrenia (SWGPGC 2014).

1.2.5 Environmental risk factors

Numerous environmental risk factors and their effect on liability for the development of schizophrenia have also been examined. Pre- and perinatal birth complications that have led to hypoxic-ischemic damage to the brain such as low birth weight (Geddes *et al*. 1999), bleeding during pregnancy, preeclampsia, asphyxia, and emergency caesarean sections have all been identified as increasing the risk for schizophrenia (Cannon 2002a).

Depending on the timing of the insult and the nature of the damage (chronic or acute hypoxia), this would have different effects on brain development and lead to a number of different pathological findings (typical of schizophrenia).

Maternal infections such as influenza, rubella, herpes simplex II virus, toxoplasmosis and cytomegalovirus have also been implicated as increasing the risk for schizophrenia (Brown *et al.* 2000a; Buka *et al.* 2001b; Torrey 1988; Torrey *et al.* 2007). Increased maternal cytokine response to any infection such as increased maternal levels of the proinflammatory cytokines interleukin 8 (IL-8) (Brown 2004a) and tumor necrosis factor alpha (TNFα) (Buka 2001a) is an immune reaction common to most infections, and these cytokines (and others) have been investigated for their role in disrupting early brain development and increasing the risk for schizophrenia (Gilmore and Jarskog 1997).

Prenatal nutritional deprivation has also been implicated as a risk factor for schizophrenia. Ecological studies that have investigated periods of famine in our history have determined that those individuals who were conceived during famine have a higher risk of developing schizophrenia (Susser and Lin 1992). Processes involved are thought to be related to a combination of poor maternal nutrition and stress that is experienced by surviving a famine (Susser and Lin 1992).

1.2.6 Diagnosis

There are no *in vivo* tests available to identify schizophrenia and so diagnosis is dependent on characteristic symptoms and behavior clusters outlined in the Diagnostic and Statistical Manual for Mental Disorders, Fifth Edition (*DSM-5*) which is widely used throughout North America (APA 2013). The International Classification of Mental Disorders, 10th Edition (*ICD-10*) (WHO 1992), the official international classification system, is used in most European countries to also guide diagnosis. The *DSM-5* diagnostic criteria for schizophrenia are divided into 6 separate criteria (A through F) that are

required to be met for diagnosis. Criterion A includes 5 items: Delusions, hallucinations, disorganized speech, disorganized or catatonic behavior, and negative symptoms (which include diminished emotional expression, avolition, and others). For diagnosis, at least two of the five symptoms within criterion A must be present for at least one month, and one of the two symptoms must be delusions, hallucinations, or disorganized speech. Criterion B outlines the level of functioning (social, occupational, educational) which needs to be markedly below the level achieved prior to the illness onset. Criterion C requires that the symptoms must persist for at least six months duration, during which the patient must experience at least one month of active symptoms. Criteria D, E, and F require the clinician to make a distinction between schizophrenia and schizoaffective disorder; to rule out psychosis due to substance abuse or a medical condition; and also to distinguish schizophrenia from autism spectrum disorder and other communication disorders (APA 2013).

1.3 ANTIPSYCHOTIC DRUG DEVELOPMENT

1.3.1 First-generation antipsychotic drugs

Historically, for many years since the introduction of the first-generation antipsychotic drugs (FGAs) in the 1950s, the dopamine system was thought to be the principal pathway involved in the pathology of schizophrenia. Based on the dopamine blocking properties of chlorpromazine and its ability to control acute psychotic symptoms, the hypothesis was generated that schizophrenia is a disorder of excess dopamine neurotransmission (Carlsson and Lindqvist 1963; Seeman and Lee 1975). Dopamine receptor-2 (D2) blocker antipsychotic drugs are very successful in many cases at reducing psychotic symptoms. Antagonism of D2 receptors within the mesolimbic dopaminergic circuits in the brain help to decrease delusional thoughts and hallucinations that are characteristic of the illness (Kane 2001). Unfortunately, the behavioural domains of schizophrenia include a wide-spectrum of symptoms beyond psychosis that

have been difficult to address pharmacologically. In particular, the D2 receptor antagonist drugs have not been successful at improving the negative, cognitive, and affective symptoms that are critical to individual social and occupational functioning. The FGAs are also not without serious side-effects. Dopamine-2 receptor antagonist drugs are not selective to dopamine blockade within the mesolimbic pathway. These drugs also reduce dopamine transmission within the mesocortical, nigrostriatal, and tuberoinfundibular dopamine pathways in the brain. Reduced dopamine transmission within these circuits contributes to the negative, cognitive, extrapyramidal, and endocrine side-effects (such as hyperprolactinemia) that are frequently experienced by patients taking these drugs (Kane 2001). Interactions with other neurotransmitter receptors such as α_1–adrenergic receptors, histaminic H_1 receptors, and muscarinic M_1 acetylcholine receptors also cause additional side-effects, increasing the burden of therapy. The lack of efficacy, and extra burden of extrapyramidal side-effects (EPS), warranted the development of better tolerated drugs with fewer side-effects and improved clinical response (Kane 2001; Jones and Pilowsky 2002; Remington 2003; Miyamoto *et al.* 2012).

1.3.2 Second-generation antipsychotic drugs

Clozapine has been a major advancement in the pharmacological treatment of schizophrenia, especially for treatment-resistive cases. Clozapine was the first medication of the atypical or second-generation antipsychotics (SGAs) to be developed that has been beneficial for both of the positive and negative symptoms of the illness. Pharmacological properties of having high affinity for D4 receptors, rather than D1 or D2 receptors, and high affinity for serotonin 5-hydroxytryptamine-2A (5-HT2A) receptors, meant that clozapine was classified as 'atypical' compared to the FGAs. The ability to allow more regulated dopamine transmission (Kapur and Seeman 2001) and antagonize serotonin 5-HT2A transmission in the striatum (Meltzer *et al*. 1989) has reduced the incidence and clinical reports of EPS

and tardive dyskinesia (TD). Unfortunately, clozapine is not without serious side-effects of its own. Despite the low incidence of EPS and TD, it can cause significant metabolic side-effects and cardiac toxicity. Agranulocytosis is also a serious side-effect of clozapine, so systematic monitoring while on the drug is required and regulated by all manufacturers who produce it (Clozapine Monograph 2012).

Other second-generation antipsychotics were developed to be more effective and better tolerated than typical antipsychotic drugs and safer than clozapine. They share the D2 receptor blocking pharmacological properties of the FGAs, but have a much lower affinity for the D2 receptors in the striatum (a fast dissociation) as compared to the affinity to these receptors in the ventral tegmental area (VTA) (regional specificity) that contribute to the control of the positive symptoms and the reduction of EPS (Kane 2001; Jones and Pilowsky 2002; Remington 2003; Miyamoto *et al.* 2012). Like clozapine, SGAs also have a greater affinity for 5-HT2A receptors relative to the D2 receptors, leading to increases in dopamine release in the frontal cortex as well as the striatum, providing a possible mechanism for the reduction in EPS and the suggested improvement of the negative and cognitive symptoms (Jones and Pilowsky 2002).

Unfortunately, these drugs have not been more beneficial clinically than the first-generation medications. The Clinical Anti-Psychotic Trials of Intervention Effectiveness (CATIE) study (Lieberman *et al.* 2005) examined whether SGAs were any more effective than perphenazine, a FGA or typical antipsychotic. There was no significant difference in symptom remission between the typical and atypical medications tested, and many patients also showed no improvements in cognitive function or negative symptoms on the atypical medications. The study also reported a 74% discontinuation rate within the first 18 months of commencement due to unwanted side-effects (Lieberman *et al.* 2005).

1.3.3 Third-generation antipsychotic drugs aripiprazole and brexpiprazole

Third-generation antipsychotic drugs that combine both antagonism with partial agonism at D2 receptors along with agonism at serotonin 5-HT(1A) receptors (5-HT1A) have been developed to stabilize dopamine concentrations and reach a much broader range of symptoms. In areas of the brain where there is an increase in extracellular dopamine (mesolimbic areas) the partial agonist properties of these drugs compete with dopamine and cause partial postsynaptic antagonism, decreasing psychotic symptoms. The ability to modulate dopamine within the striatum would also reduce the risk of EPS. In areas of the brain where extracellular dopamine concentrations are low (prefrontal cortex), the drugs can occupy additional presynaptic D2 autoreceptors and cause partial activation, releasing dopamine (Tamminga and Carlsson 2002; Lieberman 2004). Partial agonist activity at 5-HT1A receptors, a property of clozapine and other SGAs as well, also offer some improvements in anxiety, depression, cognitive and negative symptoms, and an added benefit of also decreasing the risk for EPS.

1.4 GLUTAMATE

1.4.1 Glutamate synthesis

Glutamate, an amino acid neurotransmitter, is the major excitatory neurotransmitter in the mammalian brain. Glutamate plays an important role throughout the lifespan and is critically involved in neuronal migration and survival processes during early development (Konradi and Heckers 2003); ongoing synaptic plasticity (long-term potentiation [LTP] and long-term depression [LTD]); and neuronal viability (Garthwaite and Boulton 1995). Glutamate is synthesized within the brain from glucose and α-ketoglutarate as well as from glutamine (Gln). Because of its potential to induce excitotoxic damage via Ca^{2+} influx into cells, Glu must be contained and regulated by various mechanisms within the brain. Glutamate released by the presynaptic neuron into the synaptic cleft is quickly removed by

surrounding astrocytes once depolarization of the postsynaptic neuron has occurred. Astrocytes enclose glutamatergic synapses to contain the diffusion of Glu from one synapse to the next, a natural mechanism designed to prevent toxicity (Clements *et al.* 1992). Released Glu is transported by excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) into astrocytes and synthesized into Gln by the glial-specific enzyme glutamine synthetase. Glutamine is then released into the extracellular space and transported again back into the neuron by EAAT3 and EAAT4 and transformed back to Glu by phosphate-activated glutaminase, an enzyme that is contained within the mitochondria of the neuron. Glutamate is then finally repackaged into the vesicles via a vesicular glutamate transporter (VGLUT) (Hediger 1999) as shown in Figure 1-1.

1.4.2 Glutamate receptors

Receptors involved in glutamatergic transmission are divided into two categories, ionotropic and metabotropic receptors. Ionotropic receptors are further classified and differentiated based upon their pharmacological affinities and sensitivity to the ligands NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainate (Dingledine *et al.* 1999). The metabotropic Glu receptors bind either Glu or quisqualate as a ligand and are distinguished into 3 groups based upon G protein second messenger coupling: Group I metabotropic receptors mGluR1 and mGluR5 and Group II metabotropic receptors mGluR2 and mGluR3, both stimulate phospholipase C while Group III metabotropic receptors mGluR4 and mGluR6-8 inhibit adenylate cyclase (Dingledine *et al.* 1999; Traynelis *et al.* 2010).

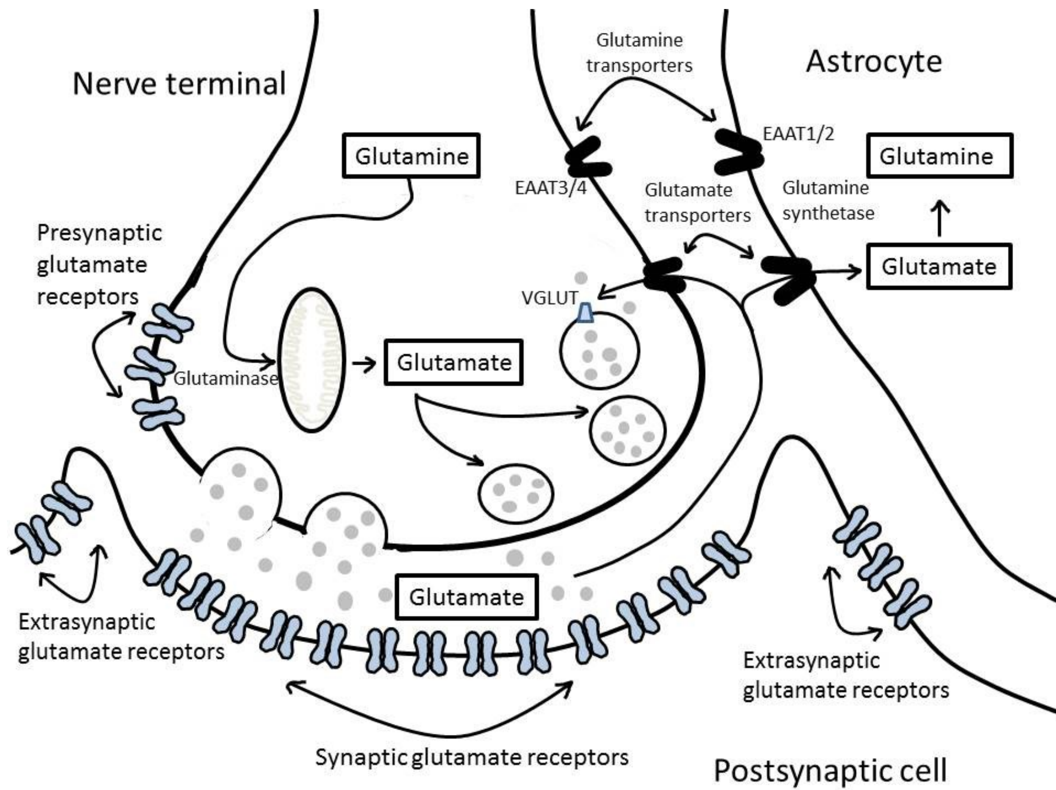

Figure 1-1. Schematic diagram of glutamate synthesis in brain. EAAT: excitatory amino acid transporter; VGLUT: vesicular glutamate transporter. (Adapted from Bern *et al*. 2009).

The three ionotropic receptors are ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the brain (Dingledine *et al.* 1999). Ionotropic receptors share common structural features and are organized into tetrameric assemblies composed of more than one type of subunit. All of the ionotropic receptor subunits are transmembrane proteins with 1 N-terminal (amine-terminal) extracellular ligand-binding domain, 3 transmembrane domains, and an intracellular carboxyl-terminal domain. Following the first transmembrane domain there is a re-entrant pore (P)-loop that serves to form the ion channel. The 2nd and 3rd transmembrane domains are linked by a large extracellular loop. Polypeptides within the N-terminal domain (S1 domain), and the extracellular loop between transmembrane domains 2 and 3 (S2 domain) are the location of the Glu binding sites (Dingledine *et al.* 1999; Traynelis *et al.* 2010).

The ionotropic receptor subunits can combine in homomeric or heteromeric fashion, to make a variety of multimeric channels that are expressed in distinct regions in the adult central nervous system (CNS) (Dingledine *et al.* 1999). All of the ionotropic receptor subunits are subject to alternative messenger ribonucleic acid (mRNA) splicing and post-transcriptional editing and as a result have high structural and functional diversity (Dingledine *et al.* 1999). The AMPA receptor is composed of four receptor subunits (GluR1-GluR4) that are encoded by 4 separate genes (*GRIA1-GRIA4*) respectively (McBain and Mayer 1994). The GluR2 subunit determines the permeability of heteromeric AMPA receptors to Ca^{2+} via an Arg substitution for Gln on the GluR2 corresponding site of GluR1, GluR3, and GluR4 subunits. Arginine has a positive charge and so heteromers with the GluR2 subunit will be impermeable to Ca^{2+}. Adding to the complexity, all AMPA subunits can present as two alternative spliced forms, flip and flop. Flip variants are expressed primarily during neuronal development, and flop variants are expressed after birth. Flip and flop splice variants influence the

rate and extent of desensitization of AMPA receptors as well as their response to allosteric modulators (Kew and Kemp 2005).

Kainate receptors are composed of subunit families GluR5-GluR7 and KA-1 and 2 and are coded by corresponding genes GluR5-GluR7 (*GRIK1-GRIK3*) and KA-1 and 2 (*GRIK4 and GRIK5*) (Dingledine *et al.* 1999; Traynelis *et al.* 2010). Kainate receptors KA-1 and 2 combine with members of GluR5-7 to form functional heteromeric receptors. Both of the AMPA and kainate receptors are linked to sodium-potassium channels that open in the presence of low affinity Glu. This results in ion flow that lasts less than 1 msec, causing a short-lasting depolarization of the postsynaptic neuron (Javitt and Zukin 1990).

1.5 NMDA RECEPTOR COMPLEX

The NMDA receptor is structurally more complex than the other ionotropic receptors and is referred to as the NMDA complex. NMDA receptors are distributed widely throughout the brain with increased density throughout the cortex and subcortical structures. The NMDA receptor includes seven subunits NR1, NR2A-D and NR3A and NR3B. All of the subunits are encoded by separate genes, namely *GRIN1*, *GRIN2A-D*, and *GRIN3A* and *GRIN3B* (Dingledine *et al.* 1999; McBain and Mayer 1994; Traynelis *et al.* 2010). Functional NMDA receptors require the heteromeric assembly of 2 NR1 subunits in combination with 2 NR2 and/or NR3 subunits. To become fully activated, the receptor requires binding of two independent glycine sites on NR1 and two independent Glu sites on NR2 (Kew and Kemp 2005).

An essential feature of the NMDA receptor is the intrinsic voltage-dependent block of Mg^{2+} within the channel itself at resting membrane potential. Binding and activation of AMPA and kainate receptors by Glu from presynaptic neurons (and Glu binding to NR2 subunits and glycine or D-serine binding to NR1 NMDA subunits) allow the depolarization of the membrane and removal of Mg^{2+} from the NMDA channel pore, allowing the

influx of Na^+, Ca^{2+}, and K^+ into the cell and completing depolarization of the postsynaptic neuron. Synaptic NR2A-containing NMDA receptors utilize D-serine as a co-agonist and may have neuroprotective properties, whereas extrasynaptic NR2B-containing NMDA receptors require glycine as a co-agonist and may have neurodegenerative effects (Vizi *et al*. 2013) Adding to this complexity, pharmacological binding sites for a variety of other compounds on the NMDA receptor ion channel complex can activate, inhibit, or enhance the functioning of the receptor.

NMDA receptors are embedded within the postsynaptic membrane along with a variety of cytoskeletal and signaling proteins collectively referred to as the postsynaptic density (PSD) (Sheng and Pak 2000). The postsynaptic density 95kDa (PSD-95) family of PSD/Disc-large/Z0-1 homologous (PDZ) scaffolding proteins are classified according to their molecular weight and are encoded by four genes *PSD-95/ SAP-90* (synapse-associated protein 90), *PSD-93/chapsin-110*, and *SAP-102* and *SAP-97* (Kim and Sheng 2004). They all contain three PDZ domains, an SRC homology 3 (SH3) domain, and a guanylate kinase-like (GK) domain (Kim and Sheng 2004). PDZ domains allow protein-binding at C-terminal tails of other proteins, and so they are able to assemble large molecular complexes (Kim and Sheng 2004). PSD-95 is directly linked to the NMDA receptor by a PDZ domain within the C-terminal tail of the NR2 subunit and the PDZ domains of specific intracellular proteins including neuronal nitric oxide synthase (nNOS), the enzyme responsible for NO production in the brain. The opening of the postsynaptic NMDA receptor upon depolarization initiates NO signaling cascades through Ca^{2+} activation of nNOS (Brenman *et al.* 1996), as shown in Figure 1-2.

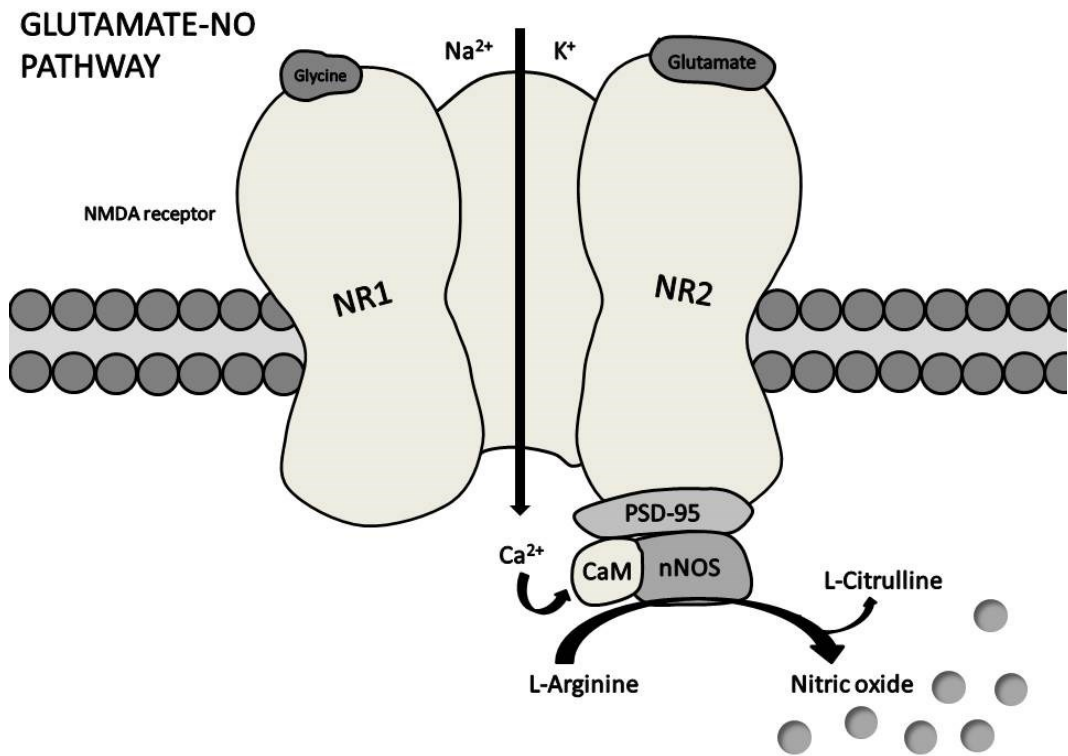

Figure 1-2. Schematic diagram of the glutamate-NO pathway. CaM: Calmodulin; nNOS: neuronal nitric oxide synthase; PSD-95: postsynaptic density protein; NR1: NMDA receptor subunit 1; NR2: NMDA receptor subunit 2.

1.6 NITRIC OXIDE

Nitric oxide, a gaseous signaling molecule, is classified as a neuromodulator or second messenger due to the lack of a membrane bound receptor, unlike what is essential for a conventional neurotransmitter. Nitric oxide is produced by a family of enzymes known as nitric oxide synthases (NOS). Three separate isoforms of the NOS enzyme exist, and they are encoded by three separate genes (Knowles and Moncada 1994). The endothelial form of the enzyme (eNOS) is located in endothelial cells. Nitric oxide produced by this particular isoform mediates vasodilation and inhibits platelet aggregation. In the immune system, the inducible form of the enzyme (iNOS) is produced by macrophages in response to inflammation, and the neuronal form (nNOS) is responsible for neuronal signaling (Bredt and Snyder 1992; Garthwaite and Boulton 1995). There are 4 splice variants of nNOS (α, β, γ, and μ), but the dominant form in the brain is nNOSα (Brenman *et al.* 1996).

All three of these NO-producing enzymes are large heme-containing enzymes that have an oxygenase domain (N-terminal) and a reductase domain (C-terminal) separated by a calmodulin binding motif. The reductase domain binds the cofactor nicotinamide-adenine dinucleotide phosphate-diaphorase (NADPHd) and also contains high-affinity binding sites for other cofactors like flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The oxygenase domain which binds Arg contains a tetrahydrobiopterin (BH4) binding site and a cytochrome P-450-type heme active site (Zhou and Zhu 2009).

Glutamate NMDA receptors have functional connections via the PSD-95 protein to the NO system in the brain. The binding of Glu to the NMDA receptor initiates the depolarization of the neuron, allowing Na^+ and Ca^{2+} to enter the cell. The influx and binding of Ca^{2+} to calmodulin activates nNOS. To produce NO, electrons donated from NADPH are transferred from the reductase domain to the oxygenase domain of the enzyme via FMN and FAD. The NOS enzyme then catalyzes the oxygenation of Arg to form L-citrulline

(Cit) and NO. Once NO is produced, it diffuses out of the cell into neighboring target cells, binds to soluble guanylate cyclase (sGC), which increases the production of the second messenger cyclic guanosine monophosphate (cGMP). Increases in cGMP activate various kinases, which phosphorylate proteins and promote the desired physiological response in the target cell (Bredt and Snyder 1992; Brenman and Bredt 1997).

1.7 DYSREGULATED GLUTAMATE IN SCHIZOPHRENIA

1.7.1 Glutamate hypofunction hypothesis

The idea that Glu transmission may be disrupted in schizophrenia was initially generated in the late 1950s with the experience of the non-competitive NMDA receptor antagonist drug and dissociative anesthetic PCP. In initial toxicology studies to determine the potential of PCP as a human anaesthetic agent, the administration of PCP in animals produced both stimulant and sedative properties depending on the animal species tested (Chen 1973). Subsequent experiments in primates revealed that at lower doses PCP induced behavioural withdrawal and apathy in the animals. At higher doses, PCP induced a catatonia-like syndrome (Chen 1973). Following toxicology safety testing in animals, PCP was marketed under the brand name Sernyl®, a name chosen to describe the serenity-like effect it had on the behaviour of normally aggressive monkeys. It was then used as an anaesthetic agent in humans undergoing surgery.

Altered sensorium and delirium were common side-effects of PCP that were experienced post-surgery, including cognitive and negative symptoms that mimicked schizophrenia (Luby *et al.* 1959; Domino and Luby 2012). Researchers became very interested in PCP as a pharmacological model for schizophrenia due to its ability to reproduce the wide-range of symptoms. In healthy volunteers PCP and ketamine can produce psychotomimetic symptoms (Krystal *et al.* 1994), and exacerbate acute psychosis in stabilized patients with schizophrenia (Lahti *et al.* 1995b; Malhotra *et al.* 1997). The discovery that PCP was an antagonist of the NMDAR (Zukin and Zukin 1979),

along with an influential study that reported an overall lower amount of Glu in the cerebrospinal fluid (CSF) of patients with the illness (Kim *et al.* 1980), led to the Glu hypofunction hypothesis of schizophrenia. The structural mechanism that causes NMDA receptor hypofunction in schizophrenia leads to a series of downstream effects on the Glu system in the brain. Increased Glu release from presynaptic neurons and binding to AMPA and kainate receptors may be a compensatory effect of the NMDA blockade (Olney and Farber 1995; Thornberg and Saklad 1996; Coyle *et al*. 2012; Moghaddam and Javitt 2012; Laruelle 2014).

1.7.2 Glutamate hyperfunction hypothesis

A key finding that excessive Glu may be released onto non-NMDA receptors as a result of NMDA dysfunction was confirmed by Deakin et al. (1989). In post-mortem ligand-binding studies in patients with schizophrenia, dense glutamatergic innervation and synapses in areas of the frontal cortex were found (Simpson *et al.* 1998). *In vivo* microdialysis experiments in animals have also showed increased extracellular levels of Glu in the prefrontal cortex (PFC) in rats following administration of low dose ketamine (Moghaddam *et al.* 1997). Further animal studies used agonists of mGlu2/3 autoreceptors that regulate the presynaptic release of Glu to reverse NMDA receptor antagonist-induced behaviours (Moghaddam and Adams 1998).

By measuring increased metabolic activation in PFC areas following NMDA receptor antagonist administration, imaging studies have also suggested increased cortical Glu outflow in schizophrenia (Vollenweider *et al.* 1997; Holcomb *et al.* 2005). Pharmacological clinical trials using drugs that can reduce Glu release in patients with schizophrenia have also led to similar conclusions. Dursun et al. (1999) initially demonstrated improvement in clinical outcomes with lamotrigine, a Glu release inhibitor, as an augmenting strategy to clozapine treatment in schizophrenia, and several

others have replicated this finding (Tiihonen *et al.* 2003; Kremer *et al.* 2004; Zoccali *et al.* 2007).

More recent studies have examined the efficacy between the mGlu2/3 agonist pomaglumetad methionil (also known as LY2140023) and atypical antipsychotics (Patil *et al.* 2007; Kinon *et al.* 2011). LY2140023 also is able to inhibit presynaptic Glu release. Initially, there were promising results of comparable efficacy of LY2140023 to olanzapine for reducing positive and negative symptoms with fewer metabolic and motor side-effects (Patil *et al.* 2007). However, in two subsequent trials, LY2140023 did not separate from placebo at improving the positive and negative symptoms of the illness (Kinon *et al.* 2011).

1.7.3 Cortical glutamate disinhibition

Glutamate is an excitatory neurotransmitter but it also plays a key role in regulating inhibitory tone in the brain by binding to NMDA receptors on other neurotransmitter pathways (Olney and Farber 1995). NMDA receptor hypofunction can result in disinhibition of excitatory pathways by understimulation of inhibitory gamma-aminobutyric acid (GABA) neurons (Olney and Farber 1995). In animal models, activity of neocortical and hippocampal pyramidal neurons is regulated by fast-spiking GABA interneurons (Homayoun and Moghaddam 2007b). Without tonic inhibition, excitatory inputs onto cortical and limbic neurons lead to increased activation.

This concept explains increased Glu release as well as the reciprocal effects of glutamatergic drive onto dopaminergic pathways, linking the two systems in schizophrenia. Descending cortical pyramidal neurons can normally inhibit dopaminergic neurons in the VTA through stimulation of an inhibitory GABA interneuron. When functioning properly, tonic inhibition of dopamine release from the mesolimbic pathway is controlled. Hypofunctioning NMDA receptors in the VTA are unable to inhibit release of dopamine from mesolimbic dopamine neurons and cause subsequent

dopamine hyperactivity in the area and symptoms of psychosis. NMDA receptors that regulate mesocortical dopamine pathways do not synapse onto GABA interneurons; they synapse directly onto dopamine neurons in the VTA that project to the cortex, exciting these neurons. Hypoactivity of NMDA receptors in this pathway would explain the cognitive, negative and affective symptoms of the illness (Stahl 2007).

1.7.4 Ligand synthesis

Glutamate levels in the central nervous system have been measured directly in CSF samples from patients with schizophrenia, as well as by proton magnetic resonance spectroscopy (^{1}H-MRS) brain imaging. Kim et al. (1980) first reported that in a group of 20 medicated patients with schizophrenia, levels of Glu were reduced in patients' CSF as compared to healthy controls. Other investigators have had conflicting results regarding Glu levels in CSF of patients with schizophrenia (Gattaz *et al.* 1982; Gattaz *et al.* 1985), suggesting that there may be more complex disturbances in Glu production. In drug naïve patients with schizophrenia, levels of CSF Glu and Gln were found to be no different from healthy controls; however the ratio of Gln to Glu was higher in the CSF of patients in comparison to the control group, suggesting that the Gln/Glu cycle may be dysregulated (Hashimoto *et al.* 2005a). As Gln is synthesized and stored within glia, communication between neurons and glia may be disrupted in the illness.

Glutamate levels in the brain may fluctuate during prodromal and maintenance phases of the illness. Investigators have utilized proton ^{1}H-MRS imaging in populations that are at a high genetic risk of developing schizophrenia, as well as in first-episode patients and in those with longstanding illness to understand these Glu changes. Tibbo et al. (2004) reported high Glu+Gln levels (a combined measure of both amino acids often referred to in studies as Glx) in the right medial frontal cortex in adolescents exhibiting no acute psychopathology of illness but at high genetic risk for developing schizophrenia (Tibbo *et al.* 2004). No differences in Glu

metabolites in high-risk adolescents have also been reported (Keshavan *et al.* 2009).

Glutamate levels measured in the cortex and subcortical structures in untreated adolescents with prodromal symptoms of schizophrenia as well as drug naïve first-episode patients have also been reported to be elevated (Theberge *et al.* 2002; Olbrich *et al.* 2008; Bustillo *et al.* 2010; de la Fuente-Sandoval *et al.* 2011). In drug treated first-episode patients, those who remained symptomatic after one course of antipsychotic medication had higher Glu+Gln levels than those patients who had achieved remission (Egerton *et al.* 2012). In contrast, in patients with chronic illness, Glu+Gln levels have been reported to be decreased compared to controls (Theberge *et al.* 2003; Ohrmann *et al.* 2007). Although Glu+Gln concentrations in the brain normally decrease with age (Kaiser *et al.* 2005), patients with schizophrenia in the very earliest stages of their illness seem to have the highest levels of Glu+Gln in most areas of the brain, including cortical and subcortical locations. Subtle increases in Glu production may explain prodromal and acute phase symptom expression and may also be responsible for the progressive widespread neuronal dysfunction that is characteristic of this illness.

1.7.5 NMDA receptor expression

Post-mortem studies have been used to determine NMDA receptor density and subunit expression in cortical and limbic regions of the brain in schizophrenia. Brain areas rich in NMDA receptors and that have been studied the most include the cortex, the temporal lobe (including the superior temporal gyrus and hippocampus), the thalamus, and basal ganglia (for reviews see Pietraszek 2003; Kristiansen 2007). Densities of NMDA receptors have been measured using tritium [^{3}H] labelled ligands that are targeted for different sites on the receptor. Receptor binding has also been inconsistent, with varied amounts of NMDA densities reported depending on the area of the brain examined and specific NMDA binding site targeted. The

Glu binding site tracers [^{3}H]glutamate and its antagonist [^{3}H]CGP 39653 revealed no change in the frontal cortex, temporal cortex, hippocampus, thalamus, or basal ganglia when used in patients with schizophrenia as compared to age-matched controls (Grimwood *et al.* 1999; Kerwin *et al.* 1990; Gao *et al.* 2000; Ibrahim *et al.* 2000; Meador-Woodruff *et al.* 2001).

However, in studies where the glycine binding site was targeted with [^{3}H]glycine or its antagonists [^{3}H]L-689,560 and [^{3}H]MDL-105,519, increased NMDA receptor density was detected in the prefrontal cortex, parietal cortex, and superior temporal cortex (Ishimaru *et al.* 1992; Grimwood *et al.* 1999; Nudmamad and Reynolds 2001), with lower densities in the thalamus (Ibrahim *et al.* 2000) and no change in densities in the basal ganglia (Meador-Woodruff *et al.* 2001) of patients with schizophrenia.

Similar findings were reported with the polyamine site antagonist [^{3}H]ifenprodil (Grimwood *et al.* 1999; Ibrahim *et al.* 2000; Meador-Woodruff *et al.* 2001). Binding of the intrachannel PCP-sensitive site with [^{3}H]MK-801 also showed increased NMDA densities in the parietal and temporal cortex with no change in prefrontal cortex (Ishimaru *et al.* 1992) and no change in the thalamus (Ibrahim *et al.* 2000) or the basal ganglia (Meador-Woodruff *et al.* 2001; Noga *et al.* 1997). Finally, the use of [^{3}H] TCP on the PCP-sensitive site showed increased NMDA density in the frontal cortex, with no change in the temporal cortex or amygdala (Simpson *et al.* 1992).

In addition to agonist-binding studies, altered NMDA receptor subunit gene transcript and protein expression levels have also been measured for specific NMDA receptor subunits in schizophrenia. Mixed results have also been found in individual NMDA receptor subunit expression. In the dorsolateral prefrontal cortex (DLPFC), occipital cortex, and superior temporal gyrus, studies have reported an increase in NR1 transcript expression (Dracheva *et al.* 2001; Le Corre *et al.* 2000). A decrease in NR1 transcript expression has also been reported in the same regions (Sokolov 1998; Humphries *et al.* 1996), and no change in total NR1 protein levels have

also been reported in the orbital frontal cortex or superior temporal cortex (Toro and Deakin 2005; Nudmamud-Thanoi and Reynolds 2004).

NR2 transcript expression in the PFC and occipital cortex has been reported to be increased in patients with schizophrenia (Dracheva *et al.* 2001), with a high amount of the NR2D receptor subunit expression in the PFC in particular (Akabarian *et al.* 1996). NMDA receptor subunit transcript expression in the hippocampus has also been varied, with reports of an increase in expression of NR2B transcripts (Gao *et al.* 2000; Law and Deakin 2001) and no change in NR2A transcripts (Gao *et al.* 2000).

In the thalamus, different levels of NMDA subunit expression have been found depending on age and progression of illness. In a younger population with schizophrenia, an increase in expression of NR2B transcripts has been found (Clinton and Meador-Woodruff 2004). In older subjects, transcripts for NR1, NR2B and NR2C have been decreased (Ibrahim *et al.* 2000). Also, subunit protein expression varies within the thalamic nuclei studied, as Clinton (2006) reported an increase in NR2B protein expression in the dorsomedial but not ventral thalamic nuclei. As with the binding studies, subunit expression in schizophrenia seems to be incredibly diverse and varies considerably depending on the area of the brain and subunit under study.

NMDA receptors have also been examined in schizophrenia by *in vivo* neuroimaging studies. NMDA binding has been measured by the use of [^{123}I]CNS-1261, a high affinity single photon emission tomography (SPET) receptor tracer that selectively binds to the intra-channel PCP/ketamine/MK-801 site on the NMDA receptor (Stone *et al.* 2006). In a study that compared patients with schizophrenia to healthy controls, a deficit in NMDA receptor binding was found in the hippocampus of medication-free patients compared to both healthy controls and to patients receiving typical antipsychotic treatment. Antipsychotic treatment, particularly with clozapine, seemed to attenuate this deficit (Bressan *et al.* 2005; Pilowsky *et al.* 2006).

1.8 NITRIC OXIDE AND SCHIZOPHRENIA

The exact role that NO may play in the pathophysiology of schizophrenia remains unknown. From the various studies that have been published, there are conflicting reports about whether there is an increased or decreased synthesis of NO in the illness (for reviews see Akyol *et al.* 2004; Bernstein *et al.* 2005; Bernstein *et al.* 2011; Maia-de-Oliveira *et al.* 2012). The controversy stems from the ability of NO to be able to induce both cell death and cell survival mechanisms in neurons (for review see Calabrese *et al.* 2007).

1.8.1 NO and neurotoxicity

Certainly it is well established that in neurodegenerative disorders, like Alzheimer's disease (AD) and Parkinson's disease (PD), excess production of NO promotes stress and damages neurons. NO has the ability to react with the free radical superoxide anion (O^{2-}), a product of a one-electron reduction of O_2. Under conditions of inflammation or excitotoxicity, NO produced via iNOS or nNOS can form peroxynitrite ($ONOO^-$), an ion with very strong oxidant properties (Pacher *et al.* 2007). The oxidant properties of $ONOO^-$ can damage a wide array of molecules in cells by a process of protein nitration. In the case of neurons, $ONOO^-$ removes an electron from the lipid bi-layer of the neuronal membrane and causes membrane lipid peroxidation (Pacher *et al.* 2007). In AD and PD, membrane lipid peroxidation is a well-documented finding (Calabrese *et al*. 2007). However in schizophrenia, the evidence for this particular mechanism is not as robust. Nitric oxide can also be neurotoxic by the activation of cyclooxygenase (COX) (the enzyme that is responsible for the production of prostaglandins and other prostanoids) in brain cells under pro-inflammatory conditions as previously discussed. A few recent studies have used nonsteroidal anti-inflammatory drugs (NSAIDS) such as aspirin and Cox 2 inhibitors as adjuvant therapies in clinical trials of schizophrenia, which have

demonstrated an improvement in symptoms (Akhondzadeh *et al*. 2007; Laan *et al*. 2010).

1.8.2 NO and neuroprotection

Nitric oxide is also known for its neuroprotective properties that are produced through various downstream cellular mechanisms. Nitric oxide-mediated *S*-nitrosylation, a process where a NO group is transferred onto a cysteine on proteins, can directly protect against NMDA receptor excitotoxicity and formation of $ONOO^-$. Hyperstimulation of nNOS via NMDA receptor activation will produce NO that will *S*-nitrosylate NR1 and NR2 subunits and eventually decrease the influx of Ca^{2+}, preventing toxicity and promoting neuronal survival. The process of NO-mediated *S*-nitrosylation can also inhibit caspase 3 activity, the enzyme that is primarily responsible for inducing apoptosis in neurons (Calabrese *et al.* 2007).

1.8.3 NO in animal models of psychosis

Most of the NO-related studies that have been published in schizophrenia have relied on the measurement and manipulation of various biomarkers and NO metabolites that are involved in NO synthesis or catabolism as indirect measures of its presence in the illness. Preclinical studies have been helpful to determine the behavioural and cellular effects of NO production in PCP animal models of schizophrenia. The use of NOS inhibitors (drugs used to decrease NO production) and NO donors (drugs used to increase NO production) has provided some clues about NO's contribution to PCP-induced psychosis.

Increased hyperlocomotion, stereotyped behaviour, and ataxia are all behavioural manifestations of PCP effects in rodents. Several studies have shown that a decrease in NO production (by blocking NOS activity with NOS inhibitors) can enhance these locomotor effects (Noda *et al.* 1995; Noda *et al.* 1996; Bujas-Bobanovic *et al.* 2000a). Others, however, have reported the exact opposite effect at similar doses of PCP and various NOS inhibitors (Johansson *et al.* 1997; Johansson *et al.* 1999; Klamer *et al.* 2004; Klamer *et*

al. 2005). The conflicting results may be explained by ataxia that is produced by PCP. Ataxia is a reliable measure of drug intoxication related to cerebellar impairment (Sturgeon *et al*. 1979). Ataxia present in PCP-treated animals that was further potentiated by the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) impaired the animals' ability to engage in hyperlocomotor activity and stereotyped behaviours (Bujas-Bobanovic *et al.* 2000a). Failure to account for ataxia may lead to opposing conclusions in studies where locomotor activity and stereotyped behaviors are measured alone (Johansson *et al.* 1997; Johansson *et al.* 1999; Klamer *et al.* 2004; Klamer *et al.* 2005).

The use of NO donors, such as SNP, molsidomine, and the NO precursor Arg have also antagonized locomotor effects and cognitive deficits of PCP and MK-801 in animal models (Bujas-Bobanovic *et al.* 2000b; Pitsikas *et al*. 2006; Pitsikas 2009; Noda *et al.* 1995). Sodium nitroprusside pretreatment was also shown to prevent the emergence of psychosis-like behavior induced by ketamine (Maia-de-Oliveira *et al.* 2015). Taken together, these studies suggest that NO is directly implicated in the mechanism of action of PCP, and the modulation of the glutamate-NO pathway may have therapeutic benefit in preventing and correcting the behavioural and cognitive manifestations of NMDA receptor pathophysiology in PCP- and ketamine-induced psychosis and schizophrenia.

In another study, Kajitani *et al*. (2010) were able to enhance performance deficits by darbepoetin alpha, an erythropoetin analog, in a STOP (stable tubule only polypeptide) null mouse model of schizophrenia by increasing production of NO. Erythropoetin (Epo) is a glycoprotein that stimulates erythropoesis, exhibits neuroprotective properties, increases extracellular concentrations of NO, and improves cognitive functioning in schizophrenia. In this particular model of schizophrenia, polymorphisms in the gene-encoding microtubule-associated protein 6 (MAP 6) generate a gene product known as STOP, a Ca^{2+} calmodulin regulated microtubule-associated protein present in neurons and glial cells. Mice lacking a functional copy of

STOP display synaptic abnormalities and cognitive deficits similar to those seen in schizophrenia. In this experiment, activation of voltage gated Ca^{2+} channels by the darbapoetin analog increased extracellular NO in the animals and reversed performance deficits in the Novel Object Recognition Task (NORT).

1.8.4 NO in clinical studies

There have also been various clinical studies suggesting that signaling within the glutamate-NO-cGMP pathway may be disrupted in schizophrenia. Although NO gas is difficult to measure directly, the amount of the NO metabolites nitrite and nitrate in both plasma and CSF has been considered as an indirect index of *in vivo* NO production. Reduced levels of NO metabolites in plasma of drug-free patients with schizophrenia as compared to normal controls have been reported (Nakano *et al.* 2010). Nitric oxide metabolite levels measured both pre- and post-treatment with risperidone have also been reported to be lower in patient groups (Lee and Kim 2008), and treatment with risperidone can significantly increase NO metabolite plasma levels after 6-8 weeks of therapy (Nakano *et al.* 2010; Lee and Kim 2008), suggesting that an increase in NO production may contribute to the therapeutic effects of antipsychotic medications. Plasma NO metabolite levels have also been reported to be significantly lower in schizophrenic patients with prominent negative symptoms when compared to schizophrenic patients without such symptoms (Suzuki *et al.* 2003), and positively correlated with the Positive and Negative Syndrome Scale (PANSS) negative symptom scores prior to antipsychotic treatment (Nakano *et al.* 2010). Similar reductions in NO metabolites have also been reported in CSF of patients with schizophrenia as compared to controls (Ramirez *et al.* 2004; for review see Maia-de-Oliveira *et al.* 2012). Reduced NO production in schizophrenia is also thought to be related to reduced cGMP levels in CSF (Gattaz *et al.* 1983), an effect that has also been reversed with antipsychotic treatment (Gattaz *et al.* 1984; Ebstein *et al.* 1976).

In post-mortem histological samples, disrupted cellular migration and functioning involving NO-producing neurons has been identified in those patients who had schizophrenia. Altered distribution of cells containing NADPHd have been found in frontal and temporal lobes of patients with schizophrenia (Akabarian *et al.* 1993a; Akabarian *et al.* 1993b). Abnormalities have also been found in NOS-containing neurons in the striatum (Lauer *et al.* 2005), and a decrease of NOS activity, but not protein expression, was also found to be significantly reduced in the PFC of patients with schizophrenia (Xing *et al.* 2002). These findings support the involvement of NO during neuronal migration and suggest that a decrease in production of NO in the cortical areas could perhaps contribute to altered neuronal development, disrupted circuitry, hypofrontality, and poor cognitive functioning that are all characteristic of the illness (Andreasen *et al.* 1992; Lewis and Levitt 2002; McGlashan and Hoffman 2000).

A recent study has also implicated the PSD-95 protein within the glutamate-NO-cGMP system that directly links the NR2 subunit of the NMDA receptor to the nNOS enzyme in schizophrenia (Brennand *et al.* 2011). In this study, fibroblast cells taken from 6 patients with schizophrenia were reprogrammed into human induced pluripotent stem cells and then further differentiated into disease-specific neurons for the illness. These neurons had reduced neuronal connectivity, reduced neurite numbers, and reduced glutamate receptor expression, also suggesting that several neuronal components that are necessary to produce adequate levels of NO in the brain are disrupted in the illness.

1.8.5 NO-based therapeutics

Studies that have measured clinical response to pharmacologically-mediated NO neurotransmission in schizophrenia have been limited, and as a result, drug development directed toward this particular molecular target has yet to be adequately pursued. Phosphodiesterase-5 (PDE-5) inhibitor drugs like sildenafil have been used as an augmenting strategy in

schizophrenia to enhance cGMP signaling mechanisms downstream of NMDA receptors (Akhondzadeh *et al.* 2011). Phosphodiesterase-5 specifically degrades cGMP. By inhibiting the PDE-5 enzyme, the degradation of cGMP is protected and downstream effects of this second messenger are achieved. In schizophrenia, this strategy acts to increase cGMP levels without directly affecting the NMDA receptors, bypassing the NMDA receptor dysfunction. As an adjunctive treatment, sildenafil combined with risperidone significantly improved the negative symptoms of the illness (Akhondzadeh *et al.* 2011).

An alternative approach to improve NMDA receptor functioning by increasing NO production has recently been investigated in a randomized clinical trial in schizophrenia. In this study, an intravenous infusion of SNP in patients already on antipsychotics and in the early stages of schizophrenia, produced rapid (within 4 hours of a single infusion) improvements of positive, negative, anxiety and depressive symptoms of the illness as compared to those patients receiving a placebo infusion (Hallak *et al.* 2013). Following a single infusion of SNP at a dosage of 0.5μg/kg/min, significant effects were found on the total scores and on each of the 4 subscale scores (thinking disorder, withdrawal-retardation, anxiety-depression, and activation subscales) of the 18-item Brief Psychiatric Rating Scale (BPRS) and also on the PANSS negative subscale scores, with no significant cardiovascular side-effects, SNP cyanide toxicity, or exacerbation of symptoms (Hallak *et al.* 2013). Symptom improvement also persisted for 4 weeks after the infusion (although adjustment of the doses of the antipsychotics was allowed 7 days after the infusion). Rapid-acting therapies that can control acute psychotic episodes within emergency and acute care settings are needed as existing antipsychotic medications often require significant time to produce an effect that will adequately reduce psychotic symptoms. A single infusion of SNP has also been used in 2 cases of treatment-refractory schizophrenia, with improvements in all symptoms achieved within a few hours of receiving the adjuvant treatment (Maia-de-Oliveira *et al.* 2014). Control of both acute and treatment-resistant psychosis

by the intravenous administration of NO donors may be a promising option for efficient symptom relief within a reasonable time frame. Future development of SNP analogs or alternative drug delivery formulations to improve compliance and suitability for long-term use may soon be an effective treatment option for schizophrenia.

1.9 REGULATORY MECHANISMS OF NO IN THE BRAIN

1.9.1 NO mediates neuronal development

Neuronal proliferation, differentiation, migration, and synaptogenesis are cellular processes that are critical during early brain development. NO-mediated signal transduction is involved in all of these events, and the adequate production of NO is essential for the eventual establishment of functional neuronal circuits. Neurons that produce NO are among the earliest differentiating cells in the cerebral cortex. They have been found as early as 15 gestational weeks within the subplate and basal ganglia of developing human embryos, and are present at 17 gestational weeks in the cortical plate. By 24 weeks gestation, NO-expressing neurons are present throughout the full thickness of the cortex (Judas *et al.* 1999). The presence of NO-producing cells during critical neuronal developmental periods suggests that NO may be essential for the proper formation and subsequent migration of neurons in the developing brain, and disruption of NO synthesis could lead to impairment, particular to neuronal connectivity as is seen in schizophrenia. In pre-clinical studies NO has been shown to inhibit proliferation of neurons during both embryonic and neonatal periods (Wingrove *et al.* 1999; Tanaka *et al.* 1994). In cultured slices of neonatal rat cerebellum, cells that were exposed to an NO inhibitor caused an increase in granule-cell proliferation and prevention of glial cell differentiation, suggesting that NO influences termination of proliferation of cells and initiation of differentiating of cells in the developing cortex (Tanaka *et al.* 1994).

1.9.1.1 *Relevance of regulated neuronal development in schizophrenia*

Subplate formation, neuronal migration towards the developing cortex, and programmed cell death of overabundant neurons and synaptic connections may also be disrupted in schizophrenia. The subplate neurons are important in establishing normal patterns of cortical wiring (Anderson 2003). Disordered placement of subplate neurons have been reported in both DLPFC as well as the temporal lobe in patients with schizophrenia (Akbarian *et al.* 1993a; Akbarian *et al.* 1993b). These neurons contain NADPHd, which is co-expressed with nNOS, and may indicate reduced or disrupted NO signaling in the illness. Disturbances of the subplate neurons may be responsible for frontal lobe function deficits that emerge as schizophrenia develops into adolescence and adulthood. These deficits may be a direct result of altered migration of neurons in these brain areas and the disorganized arborization of these early cortical connections (Akbarian *et al.* 1993a; Akbarian *et al.* 1993b). Striatal NOS-positive interneurons have also been found to be malformed in schizophrenia (Lauer *et al.* 2005). Neurons that have large pear shaped somas with asymmetrical intermediate axon branching and curled dendrites have been observed in the putamen in particular, a focal brain region that has been identified as abnormal in schizophrenia (Lauer and Beckmann 1997; Shenton *et al.* 2001; Shenton *et al.* 2010).

1.9.2 NO regulates synaptic structure and plasticity

Nitric oxide is also an important regulator of synaptic structure and plasticity. Dendritic remodeling of hippocampal neurons is known to be the cellular basis of learning and memory and NO has been a well-known modulator of LTP and LTD (for review see Garthwaite and Boulton 1995). Nitric oxide signaling has also been shown to regulate synaptic structure and the formation of dendritic spines on post-synaptic neurons via specific interactions between the nNOS and the PSD-95 protein (Nikonenko *et al.*

2008). Neuronal NOS is bound to the plasma membrane by the PDZ2 domain of the PSD-95 protein in the post-synaptic neuron.

In PSD-95-overexpressed hippocampal CA1 cells, it was demonstrated that the overexpression of PSD-95 protein induces multiple axons to form synapses on a given spine. Induced mutations at the PDZ2 region of the PSD-95 protein prevented multi-innervated spine (MIS) formation, and both knockdown and pharmacological inhibition of nNOS on PSD-95-overexpressed cells also prevented MIS formation. The use of the NO donors diethylenetriamine (DETA) NONOate and the cGMP analogue 8-bromo-cGMP sodium salt (8-Br-cGMP) resulted in an increase in MIS formation in PSD-95 transfected cells and PDZ2 mutant cells, indicating that nNOS and NO signaling are key regulators of synapse formation and essential for the creation of sufficient amounts of functional synapses formed on the neuron (Nikonenko *et al.* 2008). These findings may have important implications during periods critical for synaptic formation and stabilization in schizophrenia. In a normal developing brain, many newly formed synapses are normally retracted leaving an optimal number to go on to form functional neuronal circuits (Konradi and Heckers 2003). A disruption in NO production and signaling would have significant impact on the quality and quantity of functional synapses formed.

1.9.2.1 *Relevance of synaptic plasticity in schizophrenia*

Numerous studies over the years have identified structural abnormalities in schizophrenia, including increases in ventricular volumes and loss of cortical grey matter (Andreasen *et al.* 1982; Delisi *et al.* 1995; Keshevan *et al.* 1998a; Thompson *et al.* 2001; Shenton *et al.* 2001; Shenton *et al.* 2010). Morphometric studies suggest that there is no change in the total number of neurons in the cortex, but there is evidence of neuronal disorganization, decrease in neuronal size, and reduced neuropil (axons, dendrites, and synaptic terminals) that have been identified (Selemon *et al.* 1995; Selemon and Goldman-Rakic 1999). A loss of dendritic spines and

total dendritic length of cortical pyramidal neurons may be a result of hypoglutamatergic transmission in the illness (Garey *et al.* 1998; Glantz and Lewis 2000; Black *et al.* 2004). Pyramidal cells in cortical layer III and V are glutamatergic (and contain NMDA receptors located on their dendrites and dendritic spines) and are involved in various projections between many cortical areas in the brain. Nitric oxide produced in the brain by a functional NMDA receptor is an important signaling molecule involved in many neuronal processes, including early developmental synaptogenesis (Gally *et al.* 1990; Contestabile 2000). Growth cones of axons are induced to branch and develop synapses depending on the temporal correlated increase in intracellular levels of NO and Ca^{2+} in both pre-and postsynaptic sites (Gally *et al.* 1990). An alteration of NO synthesis and metabolism could impact functional synaptic connections in the brain. Nitric oxide may be one of many important molecular players, particularly during critical periods of development that may be disrupted in schizophrenia.

1.10 THESIS OVERVIEW

The development of effective pharmacological treatments for schizophrenia continues to be a research priority. Cellular systems involved in processes that are critical to the etiology of schizophrenia may be important molecular targets to consider for future therapeutics. Nitric oxide-based therapies may be a promising target for the development of novel antipsychotic drugs. The studies described in this thesis will contribute to our understanding of the possible therapeutic benefit of pharmacological-mediated NO neurotransmission in schizophrenia. Specifically, this thesis addresses the following questions:

1. Does the administration of the amino acid Arg (the substrate for the nNOS enzyme which catalyzes the formation of NO) have therapeutic benefit as an augmenting treatment to antipsychotic therapy? (Chapter 2)
2. Is Arg able to improve specific antipsychotic treatment-refractory symptoms? (Chapter 2)

3. Is Arg safe to use as an augmenting treatment to antipsychotic medications and does it exacerbate any existing medication side-effects or generate new side-effects? (Chapter 3)

4. Do plasma levels of Arg increase following long-term supplementation in humans? (Chapter 4)

5. Does long-term supplementation of Arg affect levels of other plasma amino acid measurements? (Chapter 4)

6. Does Arg cross the blood-brain barrier after administration? (Chapter 4)

7. What can ornithine (Orn) and Cit whole brain levels tell us about how arginase and NOS compete for Arg binding? (Chapter 4)

8. Do clozapine or risperidone influence whole brain levels of Arg, Orn, or Cit and are there any differences in these amino acid levels that occur between these two drugs? (Chapter 4)

9. What are the geneotype frequencies of Neuronal Period-Arylhydrocarbon Nuclear Translocator (ARNT)-Single-minded (PAS) domain containing 3(*NPAS3*) and *COMT* genes (and their variants specific to schizophrenia) in this population of patients? (Chapter 5)

10. Does Arg have any effect on PCP-induced hyperactivity? (Chapter 6)

11. Is Arg able to enhance the locomotor-reducing effect of clozapine or risperidone in a PCP model of psychosis? (Chapter 6)

1.11 RATIONALE AND RESEARCH HYPOTHESES

The studies described in this thesis tested the ability of the amino acid Arg (given at a dose of 6g per day) to further improve antipsychotic-resistant symptoms of schizophrenia. I expected that Arg would be a safe augmenting treatment to antipsychotic medications and that it would not exacerbate any existing medication side-effects (such as EPS, tardive dyskinesia and metabolic or cardiac side-effects). I measured plasma Arg levels both pre- and post-Arg treatment to determine if plasma Arg increases significantly following long-term supplementation in humans. I also measured a number of other plasma amino acids that are known to be directly related to NMDA

receptor functioning to determine if Arg had any effect on levels of these amino acids. I expected that Arg would cross the blood-brain barrier after administration, and confirmed this by determination of rat whole brain Arg levels. I also measured rat whole brain Orn and Cit levels as an indirect examination of the arginase and NOS enzyme activity in the brain and their ability to compete for Arg substrate binding. I also examined the influence of the antipsychotic drugs clozapine and risperidone on whole brain levels of Arg, Orn, or Cit and examined the difference in these amino acid levels between these two drugs. I expected that clozapine would have a greater influence on these amino acids as compared to risperidone. The majority of patients who responded to Arg in the clinical trial (discussed in Chapter 2) were taking clozapine. In Chapter 5, I determined the genotype frequencies of *NPAS3* and *COMT* genes (and their variants specific to schizophrenia) to see if these gene variants (expressed in combination) would predict worse treatment outcomes (as measured by the PANSS scale) in this clinical sample of patients with schizophrenia. In Chapter 6, I also examined the effect of Arg on PCP-induced hyperactivity and predicted that it would further enhance the locomotor-reducing effect of clozapine in a PCP model of psychosis.

1.12 ANALYTIC METHODS RELEVANT TO THIS THESIS

1.12.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a type of chromatography that is used for separating, analyzing, and measuring relative amounts of different components in a mixture or sample (Odontiadis and Rauw 2007). In HPLC, a sample is introduced into a liquid (mobile phase), which flows at high pressure through an analytical column that is packed with solid stationary phase. The mobile phase carries different components of the sample along with it at different rates. How fast a component moves is dependent on its affinity for the mobile or stationary phases. The solvent that emerges from the column carries the separated compound and passes into a detector. Fluorescence detection was combined

with HPLC in this thesis to measure levels of amino acids (Chapter 4). The detector was set at an ultraviolet (UV) light wavelength that was absorbed by all of the derivatized amino acids that were separated in the sample. The amount of light emitted by each amino acid is directly proportional to its concentration, and the time it takes for each amino acid to come off the column (retention time) was used to identify the specific amino acid measured. The samples were derivatized with the fluorescent agent *o*-phthaldialdehyde to produce highly fluorescent derivatives of the amino acids (Odontiadis and Rauw 2007).

1.12.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique that is used to selectively amplify specific deoxyribonucleic acid (DNA) sequences across several orders of magnitude within a very short time period. The PCR method is a repeated thermal cycling of three steps. First the DNA is isolated and denatured by heating it to 95°C. This process is essential for disrupting the hydrogen bonds that hold the DNA in a helix formation and allows the strands to separate into single-stranded DNA. The sample is then cooled to between 50-65°C to allow two primers to anneal to the complementary 3' (three prime) ends of each of the sense (non ribonucleic acid [RNA] coding) and antisense (RNA coding) strand of the DNA target. Primers used in this thesis were for two Neuronal Period-Arylhydrocarbon Nuclear Translocator (ARNT)-Single-minded (PAS) domain containing 3(*NPAS3*) variants (within exon 6 and 12 of the gene), and one variant for the *COMT* gene. Primers specific to the *COMT* variant were combined with the *NPAS3* variants for DNA amplification (Chapter 5). The reaction is again heated to 72°C, an optimum temperature for *Taq* polymerase (an enzyme isolated from the bacterium Thermus aquaticus) to act. The DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, again using the target DNA as a template. These steps are repeated 20-40 times, resulting in an exponential amplification of the DNA of interest. There is a

final elongation step held at 70-74°C for 5-15 min after the last PCR cycle to ensure any remaining single-strand DNA is fully extended (Ishmael and Stellato 2008).

1.12.3 Multiplex single-base extension PCR

The SNaPshot® Multiplex Kit is a single-base extension (SBE) primer extension assay that uses fluorescently labelled dideoxynucleotide triphosphates (ddNTPs) that are encorporated at the single nucleotide polymorphism (SNP) site. (Podini and Vallone 2009). The encorporation of ddNPTs allows the investigation of each of the 4 nitrogen bases at the mutation site. Thermal cycling allows the SBE primer to anneal to the amplicons generated in the initial PCR step and permits the amplification of the fluorescent ddNTPs. Fragments are then loaded onto a capillary electrophoresis instrument where electropherograms are generated and the data is analysed by a commercially available software program (in this case GeneMapper Software version 4) to generate an image of vertical coloured bins of data containing peaks that define and assign the corresponding alleles of interest (Podini and Vallone 2009).

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CHAPTER 2.

THE EFFICACY OF ADJUNCTIVE L-ARGININE IN TREATMENT-RESISTANT SCHIZOPHRENIA

2.1 INTRODUCTION

Schizophrenia is an illness that begins very early in brain development. It is mediated by both genetic and environmental factors and it follows a neuropathological course that originates long before the emergence of symptoms. Schizophrenia is characterized by distinct phases (related to progressive brain pathology) which are organized into premorbid, prodromal, progressive, and residual stages of the disease trajectory (Lieberman *et al*. 2001). Unfortunately, because of the subtle signs and symptoms that occur during the earliest phases of the illness, treatment is often started long after these initial symptoms emerge. Attenuated psychotic symptoms and other associated symptoms such as anxiety, dysphoria and social withdrawal that develop during the prodromal stage occur an average of 3 years before diagnosis and antipsychotic medications are initiated (McGlashan 1996). It is the frank psychotic symptoms that occur at the end of the prodromal period which usually drive a formal assessment and initiation of treatment for those with schizophrenia. Long periods of untreated active psychotic symptoms are associated with worse global functioning and more periods of re-hospitalization (Wyatt 1991); more severe negative symptoms and cognitive impairment (Waddington *et al.* 1995; Scully *et al.* 1997); and less improvement of the positive symptoms of the illness (Syzmansky *et al.* 1996). There is also evidence that the duration of untreated psychosis has direct physiological consequences to the brain and leads to a substantial reduction of cortical grey matter volume (Lieberman *et al.* 2001).

Most patients experiencing first-episode schizophrenia are often quite responsive to treatments. They are able to recover symptomatically within 3-4 months and can reach a stable period of remission within one year (Lieberman *et al.* 1996). A careful balance of the lowest possible dose of antipsychotic medication that is able to control symptoms and produce the least amount of side-effects contributes to patient compliance to treatment. Reaching a therapeutic threshold that can balance an effective dose of

medication with a minimal hypokinesia-rigidity extrapyramidal side-effect (EPS) (and no other EPSs) has been a guideline used by clinicians to signify a neurologically optimum dose (Haase 1961; McEvoy *et al.* 1986).

Unfortunately, as schizophrenia progresses, the majority of patients will have episodes of exacerbation and remission of psychosis. Relapses are common, and it has been estimated that greater than 80% of subjects relapse within the first 5 years of diagnosis (Robinson *et al.* 1999a). Multiple relapses due to antipsychotic non-adherence, substance abuse, or even natural relapses are associated with poorer long-term outcomes (Andreasen *et al.* 2013). Many patients will also experience residual symptoms such as depression, anxiety, negative symptoms or persistent psychotic symptoms despite compliance to adequate doses of antipsychotic medications.

For those patients who do not achieve adequate symptom control or sustained response to dopamine receptor-2 (D2)-blocking agents, clozapine is currently the drug of choice for treatment-resistant illness (Kane *et al.* 1988; Lieberman *et al.* 1994; Meltzer 1997). Clozapine is recommended when patients have had minimal response to two chemically unrelated antipsychotic drugs at adequate therapeutic doses for at least a 6 week duration of treatment (CPA 2005; APA 2004; WFSBP 2012; NICE 2014). Even with the use of clozapine, over 40% of patients will not have an optimal response (Kane *et al.* 1988) or fail to respond. This group have an ultra-resistant subtype of the illness (Mouaffak *et al*. 2006). Also, many patients are not able to tolerate clozapine because of the associated cardiac side-effects or related blood dyscrasias that can develop while taking this drug. It is also very common for this subpopulation of chronically ill patients to be non-compliant with the blood monitoring requirements that are mandatory with clozapine use.

Augmentation strategies are needed for this treatment-refractory population of patients to improve the residual psychopathology that has been non-responsive to antipsychotic drugs (including clozapine) and also non-responsive to other adjuvant drugs including antidepressants, mood

stabilizing agents and a variety of other medications used for their anxiolytic properties.

Drugs acting on glutamatergic pathways have been of interest as a non-dopaminergic approach to improve antipsychotic treatment in schizophrenia. Strategies designed to improve glutamate (Glu) *N*-methyl-D-aspartate (NMDA) receptor functioning have traditionally targeted the extracellular domain of the receptor. The glycine modulatory site has been of interest as a target to counteract NMDA receptor hypofunction in schizophrenia and agonists or partial agonists of this binding site on the receptor have been examined for their effect in several clinical trials. Glycine, a naturally occurring amino acid, is a co-agonist of the NMDA receptor and it is required along with Glu to activate the NMDA ion channel (Johnson and Ascher 1987; Kleckner and Dingledine 1988). The glycine binding site (specifically on the NR1 subunit) of the NMDA receptor was originally discovered by Johnson and Ascher (1987). In preclinical electrophysiological studies utilizing the outside-out patch clamp technique, NMDA response was discovered to be directly potentiated by glycine. This binding site was distinct from the strychnine-sensitive glycine inhibitory receptor as potentiation by glycine was not blocked by strychnine (Johnson and Ascher 1987).

The use of glycine as an augmenting treatment in schizophrenia was initially examined in a few small open-label clinical trials. Glycine was used at doses between 5 and 25g per day (Waziri 1988; Rosse *et al.* 1989; Costa *et al.* 1990), with benefits reported in patients at the higher doses. In more recent controlled clinical trials, adequate doses of 60g of glycine augmented with first-generation antipsychotic (FGA) and second-generation antipsychotic (SGA) medication was reported to improve negative symptoms (Javitt *et al.* 1994; Javitt *et al.* 2001; Heresco-Levy *et al.* 1996; Heresco-Levy *et al.* 1999; Heresco-Levy and Javitt 2004), cognitive symptoms (Heresco-Levy *et al.* 1996; Heresco-Levy *et al.* 2004; Javitt *et al.* 2001) and the

depressive symptoms of the illness (Heresco-Levy *et al.* 1996). The use of D-serine, an allosteric modulator at the glycine co-agonist site, has also been of interest as an augmenting strategy for improving the deficit symptoms of schizophrenia and may be more effective than glycine due to a greater affinity for the glycine/serine binding site and its increased ability to cross the blood-brain barrier (Nunes *et al.* 2012; Matsui *et al.* 1995; Bauer *et al.* 2005). Of interest, D-serine preferentially binds to synaptic NMDA receptors and may have more of a neuroprotective effect as compared to glycine which binds to extrasynpatic NMDA receptors (Vizi *et al*. 2013). Therapeutic effects of D-serine have been demonstrated when added to standard antipsychotic therapy in patients with acute (Lane *et al.* 2005), chronic (Lane *et al.* 2010), and refractory illness (Tsai *et al.* 1998), and D-serine has been reported to be safe and effective when used at dosages up to 120mg/kg per day (Kantrowitz *et al.* 2010). The anti-tuberculosis drug D-cycloserine, an analog of D-serine and also active at the glycine site, has also been reported to be beneficial for the negative symptoms of schizophrenia (Heresco-Levy *et al.* 1998; Heresco-Levy *et al.* 2002; Goff *et al.* 1999). Unfortunately, glycine, D-serine, and D-cycloserine have all been reported to be less effective at improving the negative and cognitive symptoms in treatment-refractory patients who are taking clozapine (Tsai *et al.* 1999; Diaz *et al*. 2005; Goff *et al.* 1999; Buchannan *et al.* 2007).

Another approach to improve NMDA receptor functioning has been to block the reuptake of synaptic glycine. The amino acid sarcosine, a glycine transporter-1 (GlyT1) inhibitor, has been shown to improve the negative, cognitive, and also depressive symptoms of schizophrenia (Tsai *et al.* 2004; Lane *et al.* 2005). Unfortunately, sarcosine is also an ineffective augmenting strategy when added to clozapine (Lane *et al.* 2006), a drug that already has known GlyT1 antagonist properties (Williams *et al.* 2004). Recently, bitopertin, another GlyT1 inhibiting drug, was investigated as an adjunct to antipsychotics to improve the negative symptoms of the illness (Umbricht *et al.* 2014). In subsequent phase III trials, bitopertin failed to improve the

primary outcome measure of Positive and Negative Syndrome Scale (PANSS) negative symptom scores over placebo.

Drugs that can reduce presynaptic Glu release have also been explored in patients with schizophrenia. Lamotrigine, a drug that inhibits presynaptic Glu release by the blockade of voltage-sensitive sodium channels has been demonstrated to improve clinical outcomes as an augmenting strategy to clozapine treatment (Dursun *et al.* 1999; Dursun and Deakin 2001; Tiihonen *et al.* 2003; Kremer *et al.* 2004; Zoccali *et al.* 2007). These effects may be related to clozapine's low D2-receptor occupancy and interaction with the glutamate system (as compared to other antipsychotic drugs) which may be further enhanced by lamotrigine (Dursun and Deakin 2001). More recent studies have examined the efficacy between the metabotropic glutamate 2/3 (mGlu2/3) receptor agonist pomaglumetad methionil (also known as LY2140023) and atypical antipsychotics (Patil *et al.* 2007; Kinon *et al.* 2011).

An alternative approach to improve NMDA receptor functioning in schizophrenia is to specifically target the glutamate-NO-cGMP signaling cascade. A series of preclinical experiments have explored different components of the pathway in a phencyclidine (PCP) animal model of psychosis (Bujas-Bobanovic *et al.* 2000; Bird *et al.* 2001) and the results have subsequently stimulated the investigation of the therapeutic benefit of NO in schizophrenia (Hallak *et al.* 2013; Maia-de-Oliveira *et al.* 2014). The PCP-treated rat is one of the best pharmacological models of schizophrenia because it reproduces many of the features of psychosis. Phencyclidine is a non-competitive antagonist of the NMDA receptor and it binds to a specific site within the pore of the receptor (Zukin and Zukin 1979; Zukin and Javitt 1989). The NMDA receptor blockade caused by PCP is thought to be consistent with the dysregulated Glu mechanisms that contribute to psychosis.

Rodents that have been administered PCP exhibit hyperlocomotion, fast movements, stereotyped behaviours, and ataxia (due to cerebellar drug toxicity). A dose of 5mg/kg of PCP in rats was found to be optimal for the effects mentioned above without inducing ataxia. Also, the neuroanatomical sites of PCP drug action (identified with c-Fos) were consistent with brain regions that are also associated with schizophrenia (Bird *et al.* 2001). Neuronal populations that have been metabolically activated (by a variety of stimuli) can be directly identified by the expression of the c-Fos protein (a product of the immediate-early gene *c-fos*) (Herrera and Robertson 1996; Dragunow and Robertson 1987). Further investigations of the neuronal nitric oxide synthase (nNOS) enzyme (by the use of knock-down and knock-out methods) suggested that an intact NO-producing pathway is essential for inducing the psychotic-like effects from PCP (Bird *et al.* 2001). Pharmacological manipulation of the NO system by the use of the drug sodium nitroprusside (SNP), a NO donor, blocked PCP-induced behaviour and reduced c-Fos activation, indicating that NO was able to reverse all of the PCP effects (Bujas-Bobanovic *et al.* 2000). The use of NG-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, was also investigated and L-NAME potentiated PCP-induced behaviour and PCP neuronal activation, generating worse effects in the animals (Bujas-Bobanovic *et al.* 2000).

These preclinical observations led to the development of the research hypothesis that the NO generated from SNP may improve the symptoms of schizophrenia. It has been demonstrated that in acute psychosis, intravenous infusion of SNP in patients already on antipsychotics and in the early stages of schizophrenia produced a rapid (within 4 hours of a single infusion) improvement of the positive, negative, anxiety and depressive symptoms of the illness as compared to those patients receiving a placebo infusion (Hallak *et al.* 2013). A single infusion of SNP has also been reported to be of benefit in treatment-refractory schizophrenia (Maia-de-Oliveira *et al.* 2014). These findings are important as existing antipsychotic medications require a long period of time to produce an effect that will adequately reduce psychotic

symptoms. Rapid-acting therapies that can control acute psychotic episodes and improve treatment resistance within a reasonable timeframe are needed.

To add to the clinical experience of the effects of NO in schizophrenia, the primary goal of the investigations in this chapter was to examine the therapeutic effects of the amino acid L-Arginine (Arg) (the substrate of the nNOS enzyme that produces NO in the brain) in patients with schizophrenia. As an augmenting strategy, the use of Arg was examined to see if it could improve the therapeutic efficacy of antipsychotic therapy. The use of Arg as an augmenting strategy in schizophrenia is a progression from previous work. Intravenous Arg has been shown to evoke patterns of regional brain activation and deactivation in the frontal and temporal cortex, cingulate, parahippocampal gyrus, caudate, and thalamus, with partial overlap with areas of the brain that were also affected by ketamine in healthy volunteers (Lees 2007; Deakin *et al.* 2008). This indicates that sufficient Arg is able to cross the blood-brain barrier to modulate central nervous system (CNS) function in discrete brain areas that have been linked to schizophrenia.

2.2 METHODS

2.2.1 Trial design and setting

This was a randomised, double-blind, cross-over, placebo-controlled clinical trial that was conducted at the Centre for Psychiatric Assessment and Therapeutics (CPAT) at Alberta Hospital Edmonton, Edmonton, Alberta, Canada (http://clinicaltrials.gov/ct2/show/NCT00718510). Approval was received from the University of Alberta Research Ethics Board (REB approval No. MS3_Pro00002053).

2.2.2 Participants

Patients diagnosed with schizophrenia using the Diagnostic and Statistical Manual of Mental Disorders (*DSM-IV TR*) (APA 2000) criteria who were clinically stable and had no change in the dosage of the antipsychotic medication they were taking for 8 weeks prior to enrollment were chosen to participate. All of the *DSM-IV TR* diagnostic investigations were carried out

by the Principal Investigator who is a psychiatrist. Thirteen subjects (12 males/1 female) of mean age 36±11.5 years with a mean duration of illness of 15.2±10.9 years were recruited from the Adult Psychiatry Rehabilitation Program at Alberta Hospital Edmonton and from the Edmonton Addictions and Mental Health Clinic (see Figure 2-1). Psychiatrists at both of these centres referred appropriate patients to the clinical trial based on the inclusion criteria. Nine subjects were receiving clozapine (range of 100-900mg/day), 2 were receiving oral risperidone (4mg/day), 1 patient was on risperidone Consta® (50mg every 2 weeks) and 1 patient was receiving olanzapine (25mg/day). Seven subjects (53.8%) were also receiving concomitant antidepressant medication and 2 (15.4%) were receiving concomitant mood stabilizers (see Table 2-1).

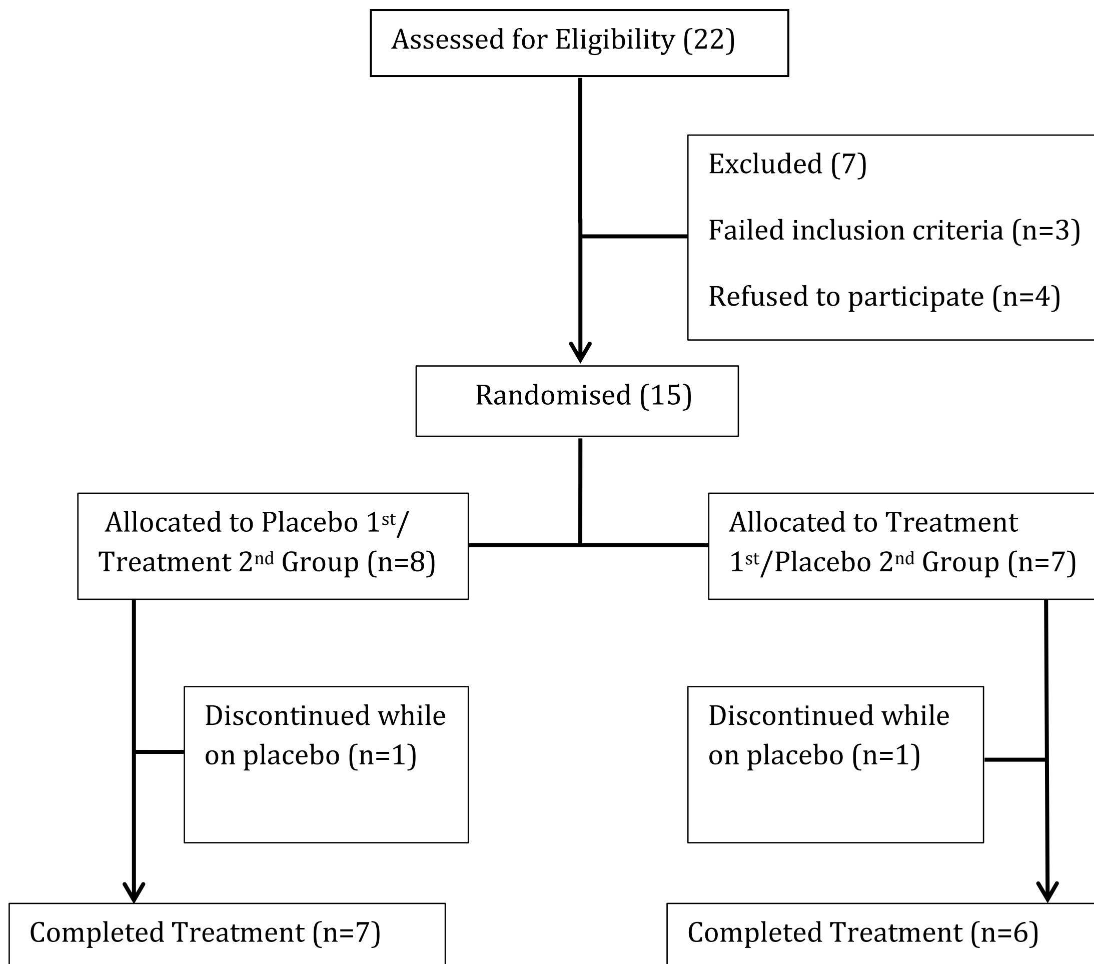

Figure 2-1. Flow diagram of the Arg clinical trial.

Table 2-1. Baseline demographic data

Age	Gender	DSM IV TR Diagnosis	Duration of Illness (years)	Number of Admissions	Antipsychotic Medication	Total Dosage
24	Male	Schizophrenia (Catatonic)	3	3	Clozapine	650mg
28	Male	Schizophrenia (Undifferentiated)	8	4	Clozapine	450mg
20	Male	Schizophrenia (Undifferentiated)	2	1	Risperidone	4mg
30	Male	Schizophrenia (Paranoid)	7	5	Clozapine	450mg
24	Male	Schizophrenia (Residual)	9	3	Clozapine	200mg
43	Male	Schizophenia (Paranoid)	20	5	Clozapine Perphenazine	900mg 14mg
42	Male	Schizophrenia Disorganized)	24	6	Clozapine	900mg
47	Male	Schizophrenia (Paranoid)	27	5	Clozapine	100mg
39	Male	Schizophrenia (Paranoid)	16	3	Clozapine	425mg
58	Male	Schizophrenia (Paranoid)	41	7	Olanzapine	25mg
26	Female	Schizophrenia (Undifferentiated)	10	1	Clozapine Quetiapine	100mg 200mg
48	Male	Schizophrenia (Paranoid)	18	2	Risperidone Consta Risperidone	50mg q2wks 1mg
39	Male	Schizophrenia (Paranoid)	13	2	Risperidone Quetiapine	4mg 350mg

Mean age (36, SD 11.5)
Mean duration of illness years (15.2, SD 10.9)
Mean number of hospitalizations (3.6, SD 1.9)

2.2.3 Randomisation

The clinical trial participants were randomised to receive 6 grams (3 grams twice per day at 0800h and 2000h) of Arg or placebo capsules (cross-over design) in addition to their antipsychotic treatment as usual. Randomisation (on a balanced 1:1 ratio) and blinding was coordinated by the Alberta Hospital Edmonton research statistician and the hospital pharmacist was responsible for dispensing the Arg and placebo capsules. All other clinical research investigators, primary psychiatrists, care providers, and patients were blind to the allocation of the treatment sequence. The capsules were dispensed on a 3 week basis to the nursing staff on the hospital units or to the patients directly if they were accessing the clinical trial on an outpatient basis. Compliance to treatment was assessed based on self-report and collateral history from the hospital staff as applicable.

2.2.4 Intervention

L-Arginine (500 mg) and matching placebo capsules were obtained from Douglas Laboratories, London, ON (Health Canada NHPD-approved, NPN 80000048). The active treatment period was 21 days with a wash-out period of 5 days followed by re-commencement on the alternative arm of the randomization. Clinical assessments were administered on days 0, 21, 26, 49, and 56 to determine clinical efficacy of Arg therapy. Blood samples (20ml) were collected at days 0, 21, 26, 49 and 56 and used to prepare plasma samples which were monitored for levels of Arg and other amino acids. The blood samples that were collected were also monitored for: overall liver function by measuring alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) levels; thyroid stimulating hormone (TSH) function; complete blood count (CBC) and differential including platelets; and renal function by measuring urea and creatinine. On day 0, day 21, and day 56, an electrocardiogram (ECG) was performed for cardiac monitoring.

2.2.5 Primary and secondary outcome measures

The PANSS is a 30-item clinical instrument that is often used to measure symptom improvement following pharmacological intervention in clinical trials. The PANSS was used to evaluate the positive, negative and general psychopathology symptoms of schizophrenia (Kay *et al.* 1987) and to determine the primary efficacy of Arg therapy. Secondary outcome measures included: The Clinical Global Impression (CGI) scale, a 3-item scale that rates treatment response and monitors the clinical course of all psychiatric illnesses (Guy *et al.* 1970) and the Calgary Depression Rating Scale for Schizophrenia (CDRS), a 9-item scale that is used to rate the depressive symptoms in patients with schizophrenia (Addington *et al.* 1990). This latter scale is a well-validated instrument that allows the rater to differentiate between the associated depressive symptoms versus the negative features of schizophrenia. All of the research data was collected and stored securely inside a locked cabinet in a secure storage facility on site at CPAT.

2.2.6 Determination of sample size and statistical analysis

The statistical power analysis was calculated using the data from the previous augmentation studies in schizophrenia. We calculated that 14 patients per treatment arm was able to detect a group difference on the primary outcome measure (PANSS), giving a 90% chance of detecting a minimum difference between treatment groups of 4 points. This included a drop-out rate of about 10% during this trial. To assess treatment response to Arg therapy relative to placebo, a two-factor repeated measures analyses of variance (ANOVA) was used across all subjects with the within-subject factor being the treatment phase (Arg/placebo or placebo/Arg) and the between-subject factor being the day of treatment (time).

2.3 RESULTS

2.3.1 Effect of Arg augmentation on PANSS scores

The mean PANSS score at baseline prior to Arg treatment was 72±5.8. The majority of the clinical sample n=12 (92.3%) (mean score 73±6.0) were moderately ill (according to the PANSS rating) with schizophrenia (Kay *et al.* 1987). There was one participant who would be described as mildly ill (with a PANSS total score of 57) as the PANSS recommended cut-off score for mild illness is 58 (Kay *et al.* 1987). The distribution of PANSS responder rates indicated that the majority of patients recruited n=7 (53.8%) had a reduction in total PANSS scores of <10 % while receiving either Arg treatment or placebo, with n=2 (15.4%) having a reduction in total PANSS scores of 10-15% while on either Arg or placebo, and n=4 (30.8%) with no reduction in PANSS total scores while receiving either treatments (see Table 2-2).

The primary effect observed with Arg treatment was a significant between-treatment difference in the general psychopathology symptoms in those patients while augmented with Arg versus placebo [$F(1,11)=5.03$, $p<0.05$] (see Figure 2-2), with no effect for time [$F(1,11)=0.19$, $p=0.66$] or interaction [$F(1,11)=0.22$, $p=0.64$]. There was a reduction in the PANSS negative symptoms scores, however the Arg/placebo comparison was not statistically significant for treatment [$F(1,11)=0.18$, $p=0.67$] or time [$F(1,11)=0.47$, $p=0.5$]; however there was an interaction effect [$F(1,11)=4.69$, $p<0.05$]. There were no significant changes in positive symptom scores at the end of 3 weeks of Arg for treatment [$F(1,10)=4.15$, $p=0.07$], time [$F(1,10)=3.54$, $p=0.08$] or interaction [$F(1,10)=0.78$, $p=0.39$]; however, two patients receiving placebo did report a placebo effect (see Table 2-3).

Table 2-2. Percentage of PANSS Responder Rates

	Total ***n***	**<10%** PANSS reduction *n* (%)	**10-15%** PANSS reduction *n* (%)	**15-50%** PANSS reduction *n* (%)	**50-100%** PANSS reduction *n* (%)
Arg	13	7 (53.8)	2 (15.4)	0	0
Placebo	13	7 (53.8)	2 (15.4)	0	0

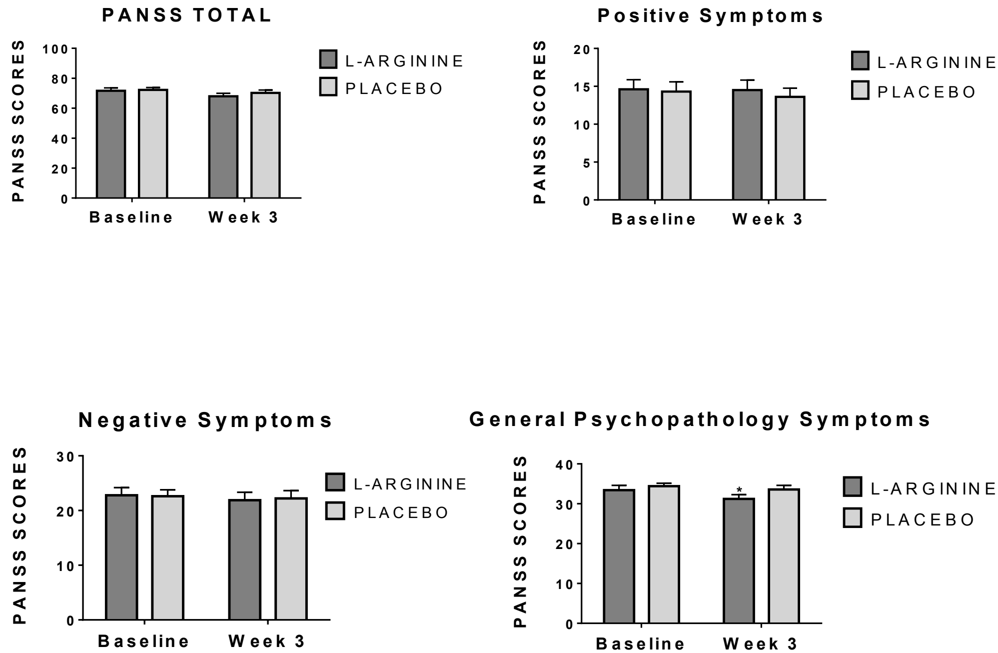

Figure 2-2. Mean±SEM PANSS total score, positive symptom score, negative symptom score, and general psychopathology symptom score values of patients on Arg vs. patients on placebo measured at baseline (day 0) and week 3 (day 21) of treatment. n=13 per group. * denotes a significant difference (p<0.05) between treatments as assessed by a two-factor repeated measures ANOVA.

Table 2-3. Three factor PANSS, CDSS and CGI scores of augmenting antipsychotic treatment with Arg and placebo

	Treatment Assignment	Day of Treatment Day 0	Day 21	ANOVA Treatment x time (d.f.=1,11)
PANSS Positive symptoms	Arg	14.6 (4.4)	14.5 (4.6)	*F*=4.15
	Placebo	14.3 (4.5)	13.6 (4.0)	*p*=0.07
PANSS Negative symptoms	Arg	22.8(5.0)	21.9(5.2)	*F*=0.19
	Placebo	22.6(4.3)	22.2(5.2)	*p*=0.67
PANSS General psychopathology symptoms	Arg	33.4(4.4)	31.2(3.9)	*F*=5.03
	Placebo	34.4(2.7)	33.6(3.7)	*p*<0.05*
PANSS Total symptoms	Arg	71.6(7.2)	68.0(7.2)	*F*=0.69
	Placebo	72.2(5.7)	70.2(7.0)	*p*=0.42
CDSS	Arg	4.5 (3.4)	3.4 (2.7)	*F*=0.37
	Placebo	4.5 (2.8)	3.8 (2.1)	*p*=0.55
CGI	Arg	4.0 (.0)	3.6 (0.5)	*F*=0.59
	Placebo	4.0 (.0)	3.7 (0.4)	*p*=0.46

2.3.2 Effect of Arg augmentation on CGI and CDSS Scores

All of the patients were given a rating of 4 (moderately ill) on the CGI-Severity Score while receiving both Arg treatment and placebo. The CGI-Improvement score did not change in n=7 (53.8%) of patients while on Arg therapy; however n=6 (46.1%) did score "minimally better." On placebo treatment, n=10 (76.9%) remained "unchanged" and n=3 (23.1%) scored "minimally better." Because the patients in the study had been stabilized on antidepressant medication and had minimal mood symptoms at the time of enrollment, the antidepressant effect of Arg could not be evaluated with the CDSS (see Table 2-4).

Table 2-4. CGI Responder Rates

CGI-improvement score	**Total *n (%)***	**Very much worse**	**Much worse**	**Minimally worse**	**Unchanged**	**Minimally better**	**Much better**	**Very much better**
Score		**7**	**6**	**5**	**4**	**3**	**2**	**1**
Arg	13	0	0	0	7(53.8)	6(46.1)	0	0
Placebo	13	0	0	0	10(76.9)	3(23.1)	0	0
CGI-severity score	**Total *n***	**Extremely ill**	**Severely ill**	**Markedly ill**	**Moderately ill**	**Mildly ill**	**Borderline mentally ill**	**Normal, not at all ill**
Score		7	6	5	4	3	2	1
Arg		0	0	0	13(100)	0	0	0
Placebo		0	0	0	13(100)	0	0	0

2.4 DISCUSSION

2.4.1 Arg augmentation has an anxiolytic effect

This clinical trial demonstrated the effect of Arg treatment on intrinsic anxiety symptoms in patients with treatment-resistant schizophrenia. Those patients who responded to Arg therapy did have a reduction specifically in the anxiety (item number 2) and tension (item number 3) symptom scores that reduced the general psychopathology subscale of the PANSS as compared to those patients on placebo. This effect was difficult to demonstrate within a cluster analysis of the data as anxiety and tension are often combined with a number of depressive symptom items when using the 5 factor analysis of the PANSS (Lindenmayer *et al*. 1994).

The mechanisms involved in the anxiolytic effect of Arg are currently unknown, but may be directly related to an increase in endogenous central NO production that was generated by increasing Arg (substrate) availability to the nNOS enzyme. Nitric oxide has been investigated for its direct role in modulating neurotransmission. Preclinical studies that have investigated the effect of a variety of NO-producing drugs in microdialysis experiments (within a variety of brain regions) as well as in synaptosome preparations have confirmed that the release and also the reuptake of several neurotransmitters, including catecholamines, acetylcholine, and excitatory and inhibitory amino acids, may be regulated by NO (Pogun and Kuhar 1994; Lonart *et al.* 1992; Guevera-Guzman *et al.* 1994; Prast and Philippu 2001).

The NO-mediated increase in the release of Glu from presynaptic glutamatergic neurons may have enhanced the effect of post-synaptic NMDA receptors on activation of gamma-aminobutyric acid (GABA)-producing neurons. The activation of these neurons may have contributed to the anxiolytic effect of Arg. In animal models, Arg (at doses of 2000mg/kg) has been shown to increase brain GABA concentrations within 10 minutes of administration and also to reduce the activity of GABA transaminase (GABA-T), the enzyme that degrades GABA to succinic semialdehyde (Jayakumar *et al.* 1999; Paul and Jayakumar 2000). The NO donor SNP has also been shown

to increase the amount of GABA (by two-fold) that is released in the hypothalamus in animal models when exposed to this drug (Seilicovich *et al.* 1995). This finding is interesting as the anxiety symptoms measured on the Brief Psychiatric Rating Scale (BPRS-18) subscale in the SNP clinical trial in early-stage schizophrenia were some of the first symptoms to respond to the treatment (Hallak *et al.* 2013), suggesting this particular effect of SNP on GABA may occur in humans as well.

The anxiolytic effect of Arg may also have been related to the modulation of the tonic concentrations of serotonin and norepinephrine in the brain. In animal models, it has been reported that increased levels of NO can block the reuptake of monoamines (Pogun and Kuhar 1994; Lonart *et al.* 1992; Kiss and Vizi 2001). The inhibition of monoamine reuptake coupled with increased NO production may be responsible for the anxiolytic effects of some antidepressant drugs. In clinical studies, the administration of paroxetine, a selective serotonin reuptake inhibitor (SSRI) drug that is prescribed primarily for depression but is often used for its anxiolytic effects has been reported to increase plasma levels of the NO metabolites nitrate and nitrite (NO_x) in healthy male controls (Lara *et al.* 2003a). Paroxetine has also been reported to increase plasma NO_x in patients diagnosed with major depression within two weeks of initiating treatment and even before the antidepressant effects are observed (Chrapko *et al.* 2006). This mechanism may be directly related to serotonin transporter (SERT) functioning as there are direct linkages between the nNOS enzyme and the SERT via the postsynaptic density 95kDa (PSD-95) protein that directly links the nNOS to the NMDA receptor (Chanrion *et al.* 2007).

Those with schizophrenia that experience persistent psychic anxiety that is integral to their disease have poorer prognostic outcomes and limited choices for treatment (Sim *et al.* 2006; Cunill *et al.* 2009). When anxiety persists beyond initial treatment and stabilization, recommendations include the short-term use of benzodiazepines (for short-term use only during acute phases due to risk of dependency and abuse) (Wolkowitz and Pickar 1991;

Bandelow *et al.* 2008; Dold *et al.* 2012), and the addition of an SSRI for longer-term augmentation of antipsychotic medications. In general, there are no specific guidelines for clinicians to follow to treat the integral anxiety symptoms of schizophrenia, even though it is estimated that up to 60% of patients with a chronic psychotic disorder have persistent anxiety symptoms (CPA 2005; APA 2004; WFSBP 2012; NICE 2014; Braga *et al.* 2013). A distinct symptom separate from psychosis, anxiety infiltrates through all phases of the illness. Anxiety (social anxiety in particular), has been identified as an important factor in children who later convert to schizophrenia (Johnstone *et al.* 2005); it has shown to be an important predictor of a worse initial clinical outcome following a first psychotic break (Craig *et al.* 2002; Strakowski *et al.* 1993); and it is also a prominent predictor of relapse that is associated with much more severe clinical features and poorer outcomes (Docherty *et al.* 1978; Sim *et al.* 2006; Cunill *et al.* 2009).

There are currently few drugs that are being investigated to treat intrinsic anxiety symptoms in schizophrenia. Pregabalin is an anticonvulsant medication similar to gabapentin that is indicated for neuropathic pain. It has also been used for treating generalized anxiety disorder (Baldwin *et al.* 2013) and social anxiety disorder (Kawalec *et al.* 2015). Pregabalin's anxiolytic, anticonvulsant, and analgesic effects are mediated through its actions on the α2-delta subunit of voltage-gated calcium channels and it is able to reduce presynaptic release of Glu in hyper-excited neurons (Baldwin *et al.* 2013; Kawalec *et al.* 2015) while having no affinity to any of the GABA receptors (Bandelow *et al.* 2007). Pregabalin at dosages of over 300mg per day has been used in patients with schizophrenia who have treatment-resistant anxiety and it has been reported to be able to reduce anxiety and mood symptoms, psychotic symptoms, and general psychopathology symptoms in patients on stable doses of SGAs. Augmentation with pregabalin was also reported to promote the dose reduction of antipsychotic medications (Englisch *et al.* 2010). Unfortunately, following 7 weeks of

treatment, there was substantial weight gain associated with its use, an additional unwanted side-effect that would significantly influence treatment compliance in patients with schizophrenia (Englisch *et al*. 2010). Pregabalin is currently being investigated in a phase IV randomised controlled trial in anxiety-comorbidity in schizophrenia at flexed doses between 75mg per day up to 600 mg per day (with doses above 150 mg given twice per day) (NCT01496690). This study in Denmark is currently recruiting patients.

An extended-release formulation of quetiapine is also currently being investigated for comorbid anxiety in schizophrenia in an open label trial (NCT01672554). A previous clinical trial found this form of quetiapine particularly helpful for persistent anxiety in the illness (Bui *et al.* 2013). Other SGAs are also currently being investigated for their ability to reduce comorbid anxiety in schizophrenia and include aripiprazole (NCT00177008). Addition of aripiprazole to clozapine has also been reported to be useful in decreasing anxiety in treatment-resistant schizophrenia (Chanachev *et al.* 2011). Prior investigations of compounds including the neurosteroids dehydroepiandrosterone and pregnenolone (Ritsner *et al.* 2011), and the neurohormones oxytocin and vasopressin (Feifel *et al.* 2012; Newman and Landgraf 2012) and other therapeutics that have been used for comorbid anxiety have suggested that these compounds are ideal because they are not likely to increase the side-effect burden to patients on antipsychotic drugs (for review see Garay *et al.* 2015). Drug targets specific to the modulation of NO could also be of therapeutic benefit to those that have developed persistent comorbid anxiety that has become resistant to current available treatments without the added risk of additional side-effects.

2.4.2 Arg augmentation with clozapine

The majority of responders to Arg therapy (80%) were taking clozapine, which may suggest an enhanced effect of both of these substances on NMDA receptor functioning. Clozapine, on its own, has known agonist properties at NMDA receptors. In animal models, it has been shown to be the

most effective antipsychotic for blocking NMDA antagonist neurotoxicity produced by PCP, suggesting agonist properties at the NMDA receptor that prevented the neuronal damage (Olney and Farber 1994). Clozapine has also been shown to have GlyT1 antagonist properties (Williams et al., 2004), which may offer an explanation for the lack of response that patients taking clozapine have had to augmented glycine, D-serine, and D-cycloserine therapy as previously discussed (Tsai *et al.* 1999; Potkin *et al.* 1999; Evins *et al.* 2000; Goff *et al.* 1999). In human studies, clozapine has also been associated with significant blunting of ketamine-induced psychosis in patients with schizophrenia, reducing the positive symptoms of the illness (Malhotra *et al.* 1997). Clozapine has also been reported to increase Glu binding by over 35% in the thalamus as compared to drug-free patients and those on typical antipsychotic drugs (Bressan *et al.* 2003). The modulation of NMDA receptor function by clozapine's effect on these extra-cellular mechanisms coupled with the effect of increased Arg substrate availability to the nNOS and intra-cellular NO signaling cascades may have provided enhanced NMDA receptor functioning in the participants in this clinical trial. A safe and effective supplement like Arg that improves NMDA receptor signaling coupled with added anxiolytic benefits, may be a justified alternative to target intrinsic anxiety in treatment-refractory schizophrenia.

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CHAPTER 3.

SAFETY AND TOLERABILITY OF ADJUVANT L-ARGININE IN SCHIZOPHRENIA

3.1 INTRODUCTION

Health risks associated with the use of antipsychotic medications to treat the symptoms of schizophrenia are of concern to both patients and clinicians. Most of the side-effects that are experienced with antipsychotic drugs are a result of their direct action on a variety of neurotransmitter receptors, both within and outside of the central nervous system (CNS). Adverse effects that are of most concern are those that affect neurological function (including acute and chronic extrapyramidal side-effects [EPS], neuroleptic malignant syndrome, and tardive dyskinesia); metabolic side-effects (contributing to weight gain, blood glucose abnormalities and dyslipidemia); cardiovascular side-effects (tachycardia and hypotension); and a variety of other very distressing side-effects such as seizures, blood dyscrasias, opthalmological abnormalities, and dermatological side-effects (Allison *et al.* 1999; Correll 2011; De Hert *et al.* 2011).

Distressing side-effects are an important predictor of treatment adherence, which is essential for the sustained recovery of first-episode psychosis as well as long-term treatment outcomes (Perkins 2002; Perkins 2006). The search for a safe and effective augmenting compound that is not likely to increase these side-effects and that can also improve the efficacy of antipsychotic therapy is important for the treatment of schizophrenia.

L-Arginine (2-amino-5-guanidinopentanoic acid) is classified as a semi-essential or conditionally essential amino acid, depending on the developmental stage and health status of the individual. L-Arginine is one of three amino acids with basic side-chains, and it is capped by a guanidino group at the distal end of a 3-carbon aliphatic straight chain (see Figure 3-1). Dietary Arg is absorbed into the mucosa within the small intestine and

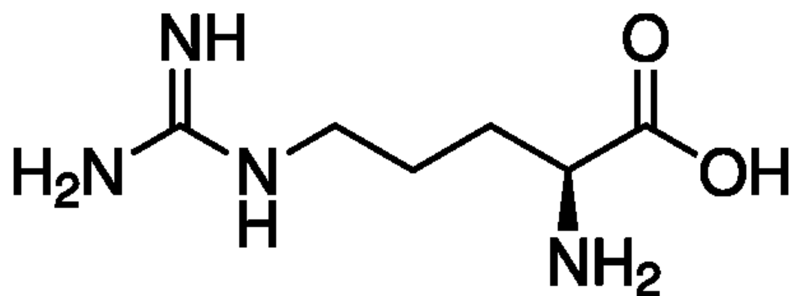


Figure 3-1. Chemical structure of Arg, capped by a guanidino group at the distal end of a 3-carbon aliphatic straight chain, a basic side-chain amino acid.

transported to the liver where the majority of it is metabolized by the enzyme arginase (to produce L-ornithine [Orn] and urea via the urea cycle). Other metabolic enzymes that use Arg as a substrate include arginine decarboxylase (ADC; to produce agmatine) and arginine:glycine amidinotransferase (AGAT; to produce creatine). The enzyme NOS also uses Arg as its substrate to produce NO and citrulline (Cit) (Wu and Morris 1998; Morris 2004). It is the NO pathway that is of potential interest as a therapeutic target in schizophrenia (Chapter 2).

As a precursor for NO, oral supplementation with Arg has been investigated the most in studies where the therapeutic benefit of vasodilation is required, such as in congestive heart failure, angina pectoris, hypertension, coronary artery disease, as well as erectile dysfunction in men (Watanabe *et al.* 2001; Ceremuzynski *et al.* 1997; Huynh and Tayek 2002; Boger 2007; Klotz *et al.* 1999; Chen *et al.* 1999). Vasodilation occurs when Arg is utilized by the endothelial nitric oxide synthase (eNOS) enzyme to produce NO that diffuses across the cell membrane into underlying smooth muscle. The NO generated binds to its receptor guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP) that initiates signaling cascades that promote the vascular smooth muscles to relax and dilate (Watanabe *et al.* 2001).

In clinical studies, the use of Arg has been reported to be well tolerated when administered in doses up to 30g per day (Bode-Boger *et al.* 1998). Reported side-effects of oral Arg include stomach discomfort, nausea, stomach cramps and an increase in number of stools directly related to local NO production within the intestinal tract. Increased intestinal NO production disrupts water and electrolyte transport as well as vasomotor functioning which can result in the generation of osmolytic diarrhea (Izzo *et al.* 1998; Stark and Szurszewski 1992).

Other potential side-effects of Arg include changes in levels of numerous chemicals and electrolytes in the blood. Examples include high potassium, high chloride, low sodium, low phosphate, high blood urea nitrogen and high creatine levels related to the urea cycle (L-Arginine

Monograph 2005). L-Arginine has also been reported to modulate blood glucose levels (Morris 2004). All participants who were in the study and on the drug metformin to manage the metabolic side-effects of antipsychotic medications were monitored closely for any blood glucose changes.

The objectives of this section were to examine the clinical safety and tolerability of Arg as an augmenting treatment to antipsychotic medication in schizophrenia and to evaluate whether Arg would exacerbate any existing antipsychotic medication side-effects or generate additional side-effects in the participants. I expected that Arg would be a safe augmenting strategy to antipsychotic drugs. Although Arg supplementation has never been studied in schizophrenia, Arg has safely been used in many other clinical trials (Watanabe *et al.* 2001; Ceremuzynski *et al.* 1997; Huynh and Tayek 2002; Boger 2007; Klotz *et al.* 1999; Chen *et al.* 1999). To ensure the clinical safety of Arg use in patients with schizophrenia, I excluded participants with any relevant medical illnesses, especially related to impaired cardiac functioning, as Arg may be detrimental to those with a history of myocardial infarct (MI) (Shulman *et al*. 2006). I also excluded patients who were taking any medications that would increase the known side-effects of Arg. I examined neurological side-effects (EPS and tardive dyskinesia); metabolic side-effects (weight gain and changes to serum urea and serum creatinine levels); and hemodynamic side-effects (changes to electrocardiogram [ECG] recordings, blood pressure and heart rate) throughout treatment to ensure the safety and tolerability of Arg treatment.

3.2 METHODS

3.2.1 Comorbid medical exclusions

Patients were excluded from the clinical trial if they had a relevant medical illness such as serious renal, diabetes, hepatic, or cardiac illnesses including low or high blood pressure. In particular, a history of past or recent cardiac illness, MI, abnormal ECG or receiving current treatments for cardiac illness were all considered to be contraindications for Arg therapy. A

previous study that investigated the effect of Arg on vascular stiffness following an MI resulted in 6 patient deaths in the Arg group compared to placebo. Most deaths were in patients over 60 years of age and could not be directly attributed to the Arg therapy; however, there is a concern that Arg may worsen outcomes in patients with a history of MI (Schulman *et al.* 2006).

L-Arginine may also increase the risk of bleeding due to the ability of NO to inhibit platelet aggregation as a direct result of vasodilation (Tsao *et al.* 1994). Because of this risk, patients using anticoagulants or antiplatelet drugs or those who had underlying bleeding disorders were also excluded from the study. Patients with any history of genital herpes infections or receiving active treatment for the herpes virus were also excluded. L-Arginine competes with the same amino acid transporter system as lysine, which is commonly used to treat the herpes virus (Dall'Asta *et al.* 2000; Banos *et al.* 1974). Patients with asthma have been shown to have increased arginase expression in alveolar and bronchial cells in the lungs (Vercelli 2003). It has also been discovered that those with asthma have enhanced arginase activity in plasma (Morris 2004). Arginase is the enzyme that catalyses the conversion of Arg to Orn. Ornithine is the precursor of proline and the polyamines, which both play a role in collagen synthesis and cell proliferation, mechanisms that could lead to cell proliferation and airway wall thickening in those with asthma (Maarsingh *et al.* 2006). Related to these risks, as well as the risk of airway inflammation from Arg substrate binding to the inducible nitric oxide synthase (iNOS) enzyme in the lungs (Redington 2006), all of the patients with a pre-existing diagnosis of asthma were excluded from the clinical trial.

Participants with gout, elevated blood-urea-nitrogen (BUN), or elevated serum creatine and creatinine were also excluded due to Arg's direct involvement in the urea cycle and creatine synthesis. Patients who were pregnant or planned to become pregnant, or were breastfeeding, were also excluded from the study. Any prior history of intolerance to Arg or allergies to any of the ingredients in the Arg or placebo capsules excluded

those participants from the study. Finally, participants with any diagnosis of substance abuse (except nicotine or caffeine) or dependence within the last three months according to the Diagnostic and Statistical Manual of Mental Disorders (*DSM-IV TR*) (APA 2000) criteria were also excluded from the study.

3.2.2 Concomitant drug exclusions

Patients who were currently receiving nonsteroidal anti-inflammatory drugs (NSAIDs) or other drugs that can cause stomach and gastrointestinal side-effects were asked to discontinue these drugs while taking Arg. A washout period of one-week was utilised. Also, a one-week washout period was utilized for the use of medications such as angiotensin-converting enzyme (ACE) inhibitors (drugs that potentiate vasodilation) and potassium-sparing diuretics (drugs that can alter potassium levels in the body) since Arg may also increase serum potassium levels, especially in those patients with severe liver disease.

3.2.3 Drug safety monitoring

3.2.3.1 Udvalg for Kliniske Undersøgelser (UKU) Side-Effect Rating Scale

To assess for drug safety, the Udvalg for Kliniske Undersøgelser (UKU) Side-Effect Rating Scale was used to monitor the systemic side-effects of antipsychotic medications (Lingjaerde *et al.* 1987). The UKU is a comprehensive 64-item scale that divides side-effects into 4 broad categories: Psychic side-effects (such as concentration difficulties, sedation, and sleep disturbances); neurologic side-effects (includes those symptoms associated with extrapyramidal effects such as dystonia, akathesia, and tremor); autonomic side-effects (such as salivation, constipation, and orthostatic hypotension); and also includes an "other" category which lists many other common side-effects such as pruritis and photosensitivity reported with the use of antipsychotic drugs (Lingjaerde *et al.* 1987). This particular scale was developed by a task force from the Scandinavian College of Neuropsychopharmacology who identified the need for a comprehensive

side-effect rating scale that also incorporated methodological issues in relation to psychopharmacological research (http://scnp.org/home.html). The UKU is currently the only prospective, clinician administered (not self-rated) scale that assesses the full scope of side-effects across a number of physiological systems in the body that are sensitive to and affected by antipsychotic medication. Patients can also access a self-rated version of UKU as well.

3.2.3.2 Abnormal Involuntary Movement Scale (AIMS)

The Abnormal Involuntary Movement Scale (AIMS) is frequently used by clinicians to screen for and systematically rate any abnormal involuntary movements which would aid the clinician in formulating a diagnosis of tardive dyskinesia associated with antipsychotic therapy if it is present (Guy *et al.* 1970). The AIMS is a 12-item scale and can be quickly administered within 10 minutes and so it is helpful to track the development and severity of TD on each treatment visit. This scale is often used in clinical trials because of these features.

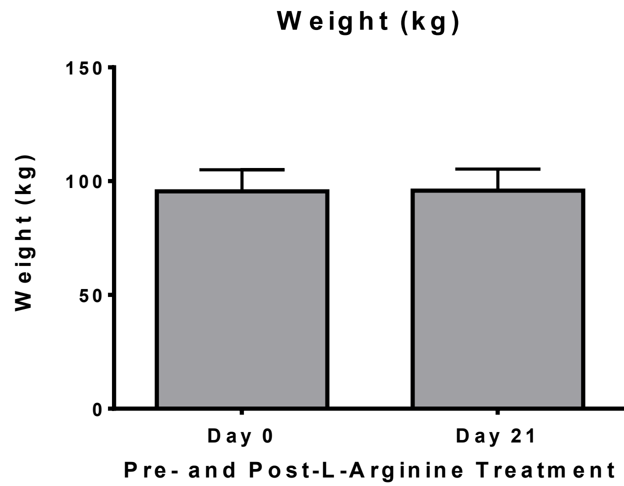
3.3 RESULTS

3.3.1 Neurologic side-effects

There were n=4 (31%) participants in this clinical trial that had been exposed to both first-generation antipsychotic (FGA) and second-generation antipsychotic (SGA) drugs and n=9 (69%) participants that had only been exposed to SGAs throughout their illness. Pre-existing extrapyramidal effects (in particular akinesia and tremor) that were experienced by the participants in this study reached a point prevalence rate of 54% and 38% respectively, prior to receiving Arg therapy, with no other exacerbation of EPS reported. Tardive dyskinesia was not detected in any of the 13 patients recruited for the study and there was no new emergence of EPS or TD symptoms during either Arg or placebo treatment.

3.3.2 Metabolic side-effects

At baseline, metabolic parameters that were collected in the patients included weight and body mass index (BMI) measurements. Fasting plasma glucose and fasting lipid values were not specifically measured for the clinical trial, however, regular monitoring of plasma glucose and fasting lipid values were routinely collected by the treatment team as part of antipsychotic drug metabolic monitoring protocols. Patients enrolled in the clinical trial who were also taking metformin were within normal limits for fasting plasma glucose measured both pre- and post-Arg treatment. There was no significant weight gain as a result of receiving Arg therapy. Mean pre-treatment weight was (95.5 kg [210.5 lbs]±31.4) and post-treatment weight was (95.8 kg [211.2 lbs]±31.1) with no significant change in weight from Arg therapy (p=0.43). Mean BMI measured pre-treatment was (30.8 ± 8.0 kg/m^2) and post-treatment was (30.9 ± 7.9 kg/m^2). There was also no significant change in BMI pre- or post-treatment with Arg therapy (p=0.57). These results are shown in Figure 3-2.



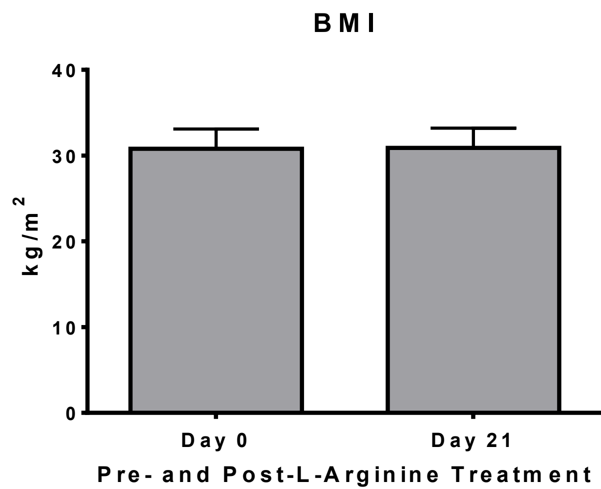
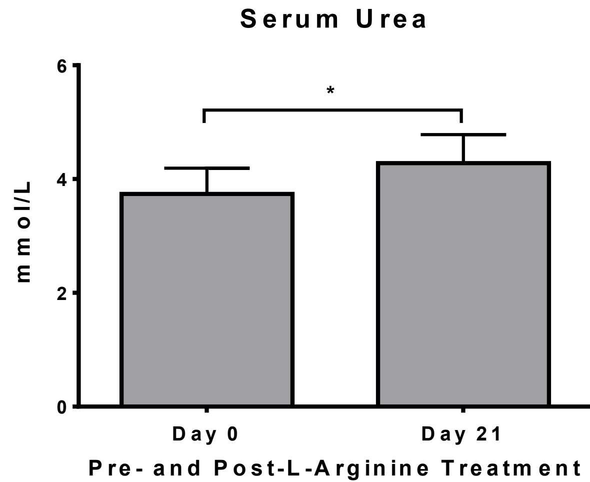


Figure 3-2. Mean±SEM Weight (kg) and Body Mass Index (BMI; kg/m^2) measurements pre-and post-Arg treatment, n=13 per group, as assessed by a paired samples two-tailed *t*-test.

L-Arginine at 6 g per day did significantly increase mean serum urea levels. Pre-treatment mean serum urea concentrations (3.7±1.4 mmol/L) were significantly lower than the mean serum urea concentrations measured at the end of 3 weeks of Arg treatment (4.3±1.5 mmol/L)[t=-2.45, df11, $p < 0.05$]. L-Arginine is metabolized by the enzyme arginase in the liver to produce Orn and urea as by-products of the urea cycle, so increased production of urea was expected. Serum creatinine levels (which are influenced by the catabolism of Arg by the enzyme AGAT) did not change significantly following 3 weeks of Arg therapy [t=0.53, df11, p=0.60] (these results are shown in Figure 3-3). Monitoring of laboratory blood chemistry, endocrine, liver, and kidney functioning measures did not reveal any abnormal values.

3.3.3 Cardiovascular side-effects

There were no significant hemodynamic effects of Arg on systolic blood pressure measurements between treatment [$F(1,11)$=2.02 p=0.18] or time [$F(5,55)$=1.42 p=0.23]; diastolic blood pressure measurements between treatment [$F(1,11)$=3.30 p=0.10] or time [$F(5,55)$=1.01 p=0.42]; and no significant effect on heart rate between treatment [$F(1,11)$=0.25 p=0.63] or time [$F(5,55)$=1.29 p=0.28]. These results are shown in Figures 3-4 through 3-6. All of the ECG recordings that were taken were within normal limits for all of the participants in the clinical trial.



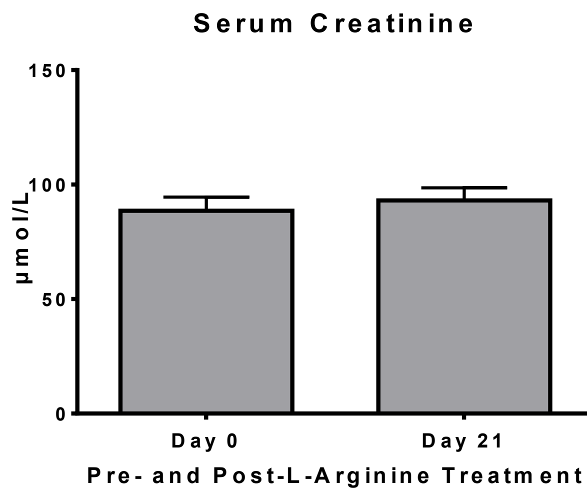


Figure 3-3. Mean±SEM serum urea (mmol/L) and serum creatinine (µmol/L) levels pre- and post-Arg treatment. n=13 per group. * denotes a significant difference ($p<0.05$) following Arg treatment as assessed by a paired samples two-tailed *t*-test.

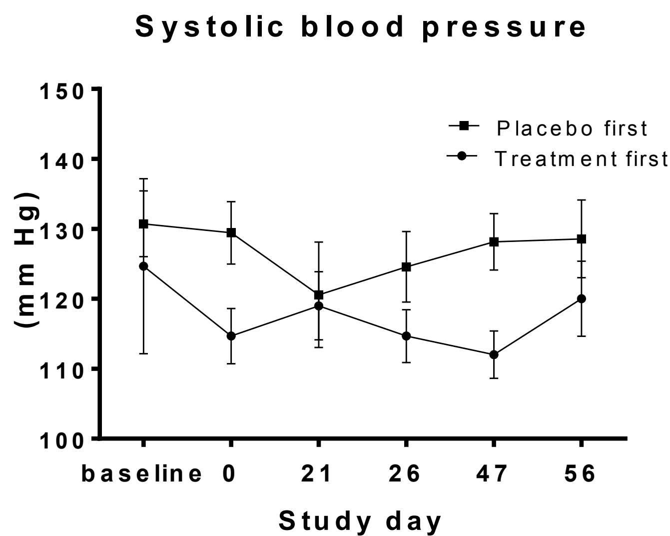


Figure 3-4. Mean±SEM systolic blood pressure values (mmHg) taken at baseline and days 0,21,26,47, and 56 of placebo first and Arg treatment first clinical groups. n=13 per group, as assessed by a two-way repeated measures ANOVA.

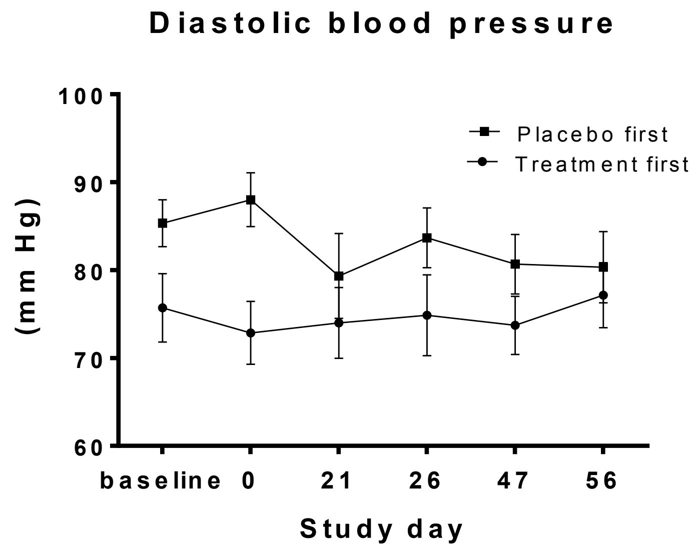


Figure 3-5. Mean±SEM diastolic blood pressure values (mmHg) taken at baseline and days 0,21,26,47, and 56 of placebo first and Arg treatment first clinical groups. n=13 per group, as assessed by a two-way repeated measures ANOVA.

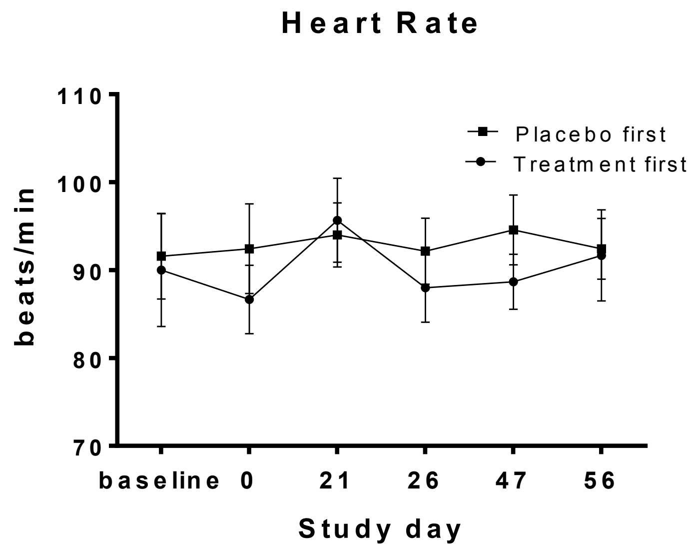


Figure 3-6. Mean±SEM heart rate values (beats/min) taken at baseline and days 0,21,26,47, and 56 of placebo first and Arg treatment first clinical groups. n=13 per group, as assessed by a two-way repeated measures ANOVA.

3.4 DISCUSSION

The augmentation of antipsychotic medication with the amino acid Arg at the dose used in this study (6g per day) proved to be a safe strategy in the treatment of schizophrenia. The addition of Arg to antipsychotic drugs did not exacerbate the neurological side-effects (akinesia and tremor) that were present as a result of ongoing antipsychotic therapy. The majority of the participants in this study n=9(69%) have only been prescribed SGAs to treat their illness. The yearly incidence rates of neurological side-effects such as EPS and TD have been estimated in adult patients that have only been exposed to SGAs throughout their illness at 0.7% for TD and 3.7% for EPS (Tenback *et al.* 2010). The point prevalence of EPS in this clinical sample of patients only exposed to SGAs was n=5(56%) for akinesia and n=4(44%) for tremor. Although the incidence of EPS and TD is low in patients exposed only to SGAs, the persistence of these symptoms can be substantial if they do develop. In the 4 patients who have also had lifetime exposure to FGAs, akathisia was experienced by n=3 (75%) and n=2 (50%) also had a visible tremor. These persistent side-effects are also consistent with incidence and prevalence of EPS found in other patient populations treated with FGAs (Tenback *et al.* 2010).

L-Arginine also did not affect the metabolic side-effects of antipsychotic therapy. There was no significant weight gain or change to BMI when compared to pre-treatment measurements. The BMI is a measure of body mass divided by the square of the body height and is expressed in units of kg/m^2 (WHO 2006). The mean baseline BMI values for the participants in this study (30.8 kg/m^2) indicated an obese rating (BMI≥30 kg/m^2) (WHO 2006). Also, these parameters indicate that only n=2 (15%) of the participants were of normal weight (BMI 18.5-24.9 kg/m^2); n=6 (46%) were overweight (BMI 25-29.9 kg/m^2); and n=5 (38%) were classified as obese (BMI≥30 kg/m^2). Of the obese participants, n=2 (40%) reached the very severely obese category, both having a BMI ≥ 40 kg/m^2 (WHO 2006). The mean duration of illness in the participants was 15.2±10.9 years, and so the

majority of the participants have been exposed to antipsychotic medications for many years, which would substantially put them at risk for weight gain (Parsons *et al.* 2009).

Antipsychotic drug-induced weight gain involves multiple mechanisms thought to be related to serotonergic (5-HT_{2C} and 5-HT_{2A}), histaminic (H_1), and adrenergic (α-1) receptor blockade (Casey and Zorn 2001; Wirshing *et al.* 1998). The weight gain can also be related to endocrine abnormalities (plasma leptin increases as a result of increased adiposity) (Kraus *et al.* 1999) that together can lead to an increased appetite and reduced satiety (for reviews see Stanton 1995; Nasrallah 2003). Also, n=9 (69%) of the participants were taking clozapine. Olanzapine and clozapine are known to cause the most significant weight gain of all of the antipsychotic drugs (Allison *et al.* 1999; Wetterling and Mussigbrodt 1999; Wirshing *et al.* 1998; Newcomer 2005; Newcomer and Hennekens 2007). The negative symptoms of the illness (such as avolition and amotivation) that often persist despite adequate treatment also indirectly contribute to a significant amount of weight gain. It is very difficult for patients who are experiencing these symptoms to engage in any kind of regular exercise regime. Considering these challenges, it is crucial to find augmenting agents to antipsychotic drugs that will not exacerbate weight gain, a side-effect that is linked to an increased risk for diabetes and cardiovascular disease that is particular to this population of patients (Allison *et al.* 1999).

L-Arginine has been investigated for its specific effect on adiposity in both animal and human studies, and the production of NO does play an important role in fat metabolism. In an obese animal model of type 2 diabetes mellitus, supplemental Arg resulted in a 16% loss of body weight that was independent of calorie or nutrient content in the diet (Fu *et al.* 2005). In the Arg-treated animals, the adipose tissue examined had increased messenger ribonucleic acid (mRNA) levels of key genes responsible for the oxygenation of the energy substrates glucose and fatty acids as compared to alanine-treated animals (used as an isonitrogenous

control). L-Arginine supplementation also led to decreased abdominal adipose tissue mass (by 34%) and reduced serum glucose, triglycerides, and leptin, with increased released glycerol, a marker within adipocytes that indicates lipolysis, findings which were all mediated by NO production (Fu *et al.* 2005). In human studies, when given at a doses of approx. 8.3g per day (80mg/kg of body weight), Arg was able to further reduce weight, fat mass, waist circumference, and blood glucose levels in diet-controlled patients with type 2 diabetes mellitus compared to controls (Lucotti *et al.* 2006). The improvement of these metabolic features by Arg supplementation is encouraging, as it has been estimated that over 32% of patients with treated schizophrenia have metabolic syndrome, with their illness duration being the strongest factor that influences its development. In clozapine-treated patients, the prevalence of metabolic syndrome is over 51% (Mitchell *et al.* 2013), and approximately 25% of all patients treated with clozapine go on to develop type 2 diabetes (Lamberti *et al.* 2005; Zhang *et al.* 2011). High rates of metabolic syndrome and type 2 diabetes are directly associated with clozapine's high affinity to H_1 receptors and its propensity to cause a significant amount of weight gain (Allison *et al.* 1999; Wetterling and Mussigbrodt 1999; Wirshing *et al.* 1998; Newcomer 2005; Newcomer and Hennekens 2007).

Other mechanisms involved in the weight loss effect of Arg are related to the generation of creatine. As mentioned previously, Arg is utilized by a number of metabolic enzymes. The enzyme AGAT catalyses the transfer of an amidino group from Arg to glycine to produce creatine. Creatine is a nonessential amino acid that can be phosphorylated by creatine kinase to form phosphocreatine (PCr), which is used as an energy reserve in tissues such as skeletal muscle and brain. Phosphocreatine increases the ability of cells to resynthesize adenosine triphosphate (ATP) from adenosine diphosphate (ADP) to meet the high energy demands of the brain and muscles (Walliman *et al.* 2011). The increase in PCr stores leads to increases in lean body mass. Weight lifters and body builders use Arg and creatine

supplements to increase muscular endurance and to increase calorie burn as the increase in lean muscle tissue is much more metabolically active (Mendes and Tirapequi 2002; Paddon-Jones *et al.* 2004).

Weight gain is one of the major factors for non-compliance to antipsychotic treatment. Although we did not get a weight-loss effect in this clinical trial, the use of an augmenting compound in schizophrenia with even a modest weight gain potential would be of concern. The long-term use of this amino acid that can further improve residual anxiety symptoms (Chapter 2), promote abdominal weight loss and prevent the development of metabolic syndrome and type 2 diabetes, with no exacerbation of neuroleptic side-effects, should be an ideal augmentation strategy to antipsychotic therapy.

The sexual side-effects of antipsychotic medications are also very distressing to patients. Sexual dysfunction is rated as one of the top 3 side-effects, these are not only most troubling to patients but also have the most influence on non-adherence (Lambert *et al.* 2004). In patients, prevalence rates of sexual dysfunction are high at (49-59%) for males and (25-49%) for females (Bobes *et al.* 2003; Khawaja 2005). Erectile dysfunction occurs at rates of between 30-52% in males on antipsychotic drugs (Bobes *et al.* 2003; Macdonald *et al.* 2003). Again, the mechanisms for sexual dysfunction related to antipsychotic drug use are complex, but prolactin elevation caused by dopamine receptor-2 (D2) antagonism as well as the antiadrenergic and anticholinergic effects of these drugs have all been implicated (Haddad and Wieck 2004).

L-Arginine therapy has been used in clinical trials to improve erectile dysfunction in men (Klotz *et al.* 1999; Chen *et al.* 1999). The increase in the endogenous generation of NO from the nNOS enzymes within the cavernous nerves and of endothelial nitric oxide synthase (eNOS) enzymes within the endothelial cells of the blood vessels of the penis activate cGMP-dependent protein kinases and stimulate the uptake of calcium by the endoplasmic reticulum in adjacent muscle cells (Hurt *et al.* 2002). Reduced levels of

cytoplasmic calcium cause the myocin light chains in the muscle cells to relax and promote vasodilation (Watanabe et al. 2001; Burnett 2006). We had one clinical trial participant who experienced an increase in sexual desire, as well as an increase in the number and quality of his erections while on Arg therapy. Perhaps the use of Arg could be trialled as an initial treatment in patients who are experiencing erectile dysfunction before medications such as phosphodiesterase 5 (PDE-5) inhibitor drugs like sildenafil (Viagra®) and tadalafil (Cialis®) are prescribed.

In summary, the use of L-arginine at 6g to stimulate the glutamate-NO-cGMP signaling pathway in schizophrenia has proven to be a safe augmenting strategy. L-Arginine did not exacerbate any neurologic, metabolic, or cardiovascular side-effects that are common to antipsychotic therapy.

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CHAPTER 4.

ARGININE METABOLISM

4.1 INTRODUCTION

The amino acid arginine (Arg) is classified as a "semi-essential" amino acid. It is required for protein synthesis during periods of growth in infants and children and it is also required during periods of increased protein catabolism (such as during infection or serious illness). In healthy adults, dietary Arg is not usually required as it can be synthesized *in vivo* from citrulline (Cit) and converted back to Arg within the proximal tubular cells of the kidney in a pathway known as the intestinal-renal axis (Morris 1992; 2002). Arginine plays a role in many biosynthetic pathways in the body, and it is an important link between the urea cycle and the Krebs cycle (also known as the citric acid cycle or tricarboxylic acid [TCA] cycle) shown in Figure 4-1. Metabolic enzymes that are linked to these pathways and use Arg as a substrate include arginine decarboxylase (ADC; EC 4.1.1.19) to produce agmatine; arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1) to produce creatine; arginase (EC 3.5.3.1) to produce ornithine (Orn) and urea; and nitric oxide synthase (NOS; EC 1.14.13.39) to produce nitric oxide (NO) and Cit. Ornithine produced via arginase is further metabolized to glutamate (Glu) via ornithine transaminase (ORN-T; EC 2.6.1.13) and to the polyamines putrescine, spermidine, and spermine via ornithine decarboxylase (ODC; EC 4.1.1.17) (Morris 2004).

Of all of the enzymes that utilize Arg as a substrate, arginase is probably the most critical to control the regulation of intracellular Arg concentrations due to its role in the urea cycle. There are two types of arginase enzymes in the body: those found within hepatic cells and those found within a variety of extra-hepatic cells. Type I arginase (Arg1) is a cytosolic enzyme widely distributed throughout the hepatocytes in the liver, and it is an important enzyme that hydrolyses Arg to produce Orn and urea. Type II arginase (Arg2) or extra-hepatic arginase, is found in the endothelial cells of blood vessels, enterocytes of the small intestine, kidney, mammary glands, lungs and also in both neurons and glia in the brain (Vockley *et al.* 1996; Carraway *et al.* 1998; Jenkinson and Grigor 1994). In the brain, Arg2 is

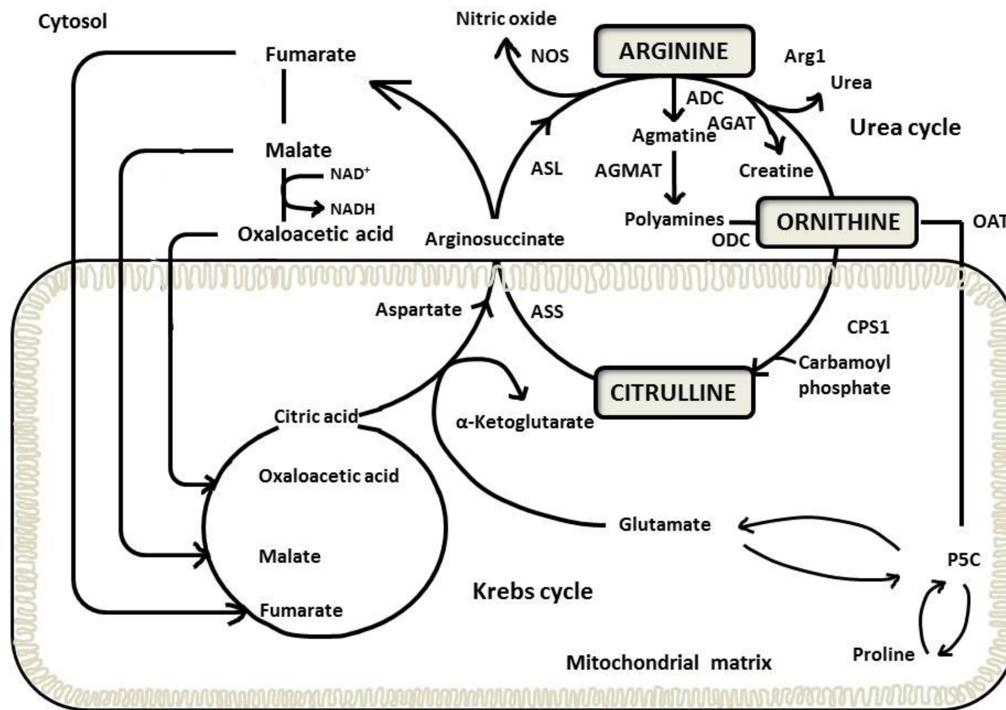


Figure 4-1. Schematic diagram of the biosynthetic pathways of Arg metabolism. ADC: arginine decarboxylase; AGAT: agmatinase; Arg1: arginase type 1; ASL: arginosuccinate lyase; ASS: arginosuccinate synthetase; CPS1: carbamoyl phosphate synthetase 1; NAD^+: nicotinamide adenine dinucleotide; NADH: nicotinamide adenine dinucleotide plus hydrogen; NOS: nitric oxide synthase; OAT: ornithine aminotransferase; ODC: ornithine decarboxylase; P5C: l-Δ^1-pyrroline-5-carboxylate. (Adapted from Lehninger *et al.* 2008).

a mitochondrial isoform of the enzyme, and it is thought to play a more important role in the regulation of intracellular Arg concentrations (Que *et al.* 2002).

Dietary Arg is absorbed by active transport across the brush-border membrane into the bloodstream via the splanchnic region of the small intestine which is the primary site for regulation of whole body amino acid metabolism. The majority of orally ingested Arg remains in the peripheral circulation. Bioavailability of absorbed oral Arg at 6g per day has been calculated to be approximately 68% (Bode-Borger *et al.* 1998). Additional kinetic studies examining plasma Arg flux from orally ingested whole protein feedings in healthy humans have been studied by the use of isotopically labelled tracers for Arg. Losses relating to first-pass metabolism through urine and feces have been estimated at approximately 38%, with approximately 62% of dietary Arg remaining in the peripheral circulation (Castillo *et al.* 1993).

Intracellular pools of Arg within neurons and glia cells in the brain are dependent on either local protein breakdown within the cells or by active transport mechanisms that can carry circulating plasma Arg across the blood-brain barrier (BBB) and across plasma cell membranes. Arginine can also be produced by the recycling of Cit to Arg within the cell itself in the brain via argininosuccinate synthetase (ASS; EC 6.3.4.5) and argininosuccinate lyase (ASL; EC 4.3.2.1), the same enzymes that recycle Cit to Arg within other cells in the body (e.g. the kidney). There are a number of different transporter systems responsible for amino acid transport into the cells in the body. These systems are organized based on substrate specificity to the carrier and on their dependence on the sodium gradient. Amino acids with cationic side chains (Arg, lysine [Lys], and Orn) utilize system y^+ carriers, a family of cationic amino acid transporters (CATS), to facilitate their transport across the inside (luminal surface) and outside (abluminal surface) of capillary membranes throughout the body including the cerebral vessels of the BBB (Oldendorf and Szabo 1976; Closs and Mann 1999;

Malandro and Kilberg 1996). The system y^+ CATS have been further differentiated by their cell-specific expression. There are 4 isoforms of CATS that have been cloned and labelled for their expression in various tissue types: Cationic amino acid transporter type 1 (CAT1) is expressed in several tissue types excluding hepatocytes; cationic amino acid transporter type 2B (CAT2B) is expressed in a variety of tissues including the brain and excluding the liver; cationic amino acid transporter type 2A (CAT2A), an alternatively spliced variant of CAT2B, is restricted to hepatocytes; and cationic amino acid transporter type 3 (CAT3) is found in rodent brain only (Braissant *et al.* 1999).

Studies examining central nervous system (CNS) and neural cell cultures have identified system y^+ carrier proteins within various neurons, glia, and at various sites within the BBB membranes and cerebral capillaries throughout the cortex and cerebellum (Westergaard *et al.* 1993; Aldridge *et al.* 1996; Schmidlin and Wiesinger 1994; Lopes *et al.* 1994; Tan and Ng 1995). All of the metabolic enzymes that use Arg as a substrate have also been localized within neurons and glia, including cells that also contain neuronal nitric oxide synthase (nNOS), suggesting that Arg biosynthesis and catabolism are indeed important mechanisms required not only for normal brain metabolism but also to facilitate NO production (Nakamura *et al.* 1991; Braissant *et al.* 1999). In a preclinical study that examined the distribution of cells expressing CAT1, CAT3, ASS, ASL, nNOS and the arginases, it was discovered that cells expressing nNOS do appear to express all of the cellular components of the entire Arg-Cit-NO-cycle (Braissant *et al.* 1999).

In the experiments described in this Chapter, I measured human plasma concentrations of Arg following 21 days of Arg treatment p.o. at 6 g per day. I also measured concentrations of a number of other plasma amino acids that are known to be directly related to NMDA receptor functioning to determine if Arg had any effect on these amino acid concentrations in human plasma. I examined Arg's ability to cross the BBB after administration, and investigated rat whole brain Arg levels following i.p. administration in dose-

and time-dependent studies. I included rat whole brain Orn and Cit levels as an indirect measure of arginase and NOS enzyme activity in the brain and their ability to compete for Arg substrate binding. I also examined the influence of the antipsychotic drugs clozapine and risperidone on whole brain levels of Arg, Orn, or Cit in a phencyclidine (PCP) animal model of schizophrenia and I then examined the differences in these amino acid levels between these two drugs.

4.2 METHODS

4.2.1 Participants

Thirteen subjects (12 males/1 female) of mean age 36±11.5 years with a Diagnostic and Statistical Manual of Mental Disorders (*DSM-IV TR*) (APA 2000) diagnosis of schizophrenia with a mean duration of illness of 15.2±10.9 years were recruited from the Adult Psychiatry Rehabilitation Program at Alberta Hospital Edmonton and also from the Edmonton Addictions and Mental Health Clinic (see Chapter 2). The *DSM-IV TR* diagnosis was confirmed by the primary treating psychiatrist and also by the Principal Investigator of the clinical trial who is also a psychiatrist. Approval was received from the University of Alberta Research Ethics Board (REB approval No. MS3_Pro00002053).

4.2.2 Medication administration

The participants received an oral dose of 6 g p.o. (3 g twice per day at 0800h and 2000h) of Arg for a 21 day period in addition to their prescribed antipsychotic medication. L-Arginine (500 mg) capsules were obtained from Douglas Laboratories, London, ON (Health Canada NHPD-approved, NPN 80000048) (see Chapter 2).

4.2.3 Human plasma collection

Between the hours of 0800h and 0900h (before the morning administration of Arg), 20 ml of blood was collected into two 10ml ethylenediaminetetraacetic acid (EDTA) lavender-topped vacutainers. The

samples were put on ice immediately after collection and spun in a 4°C centrifuge at 2000 x g for 10 minutes. Plasma was then divided into approximately 1.5 ml aliquots in screw capped polypropylene tubes and stored in a -20°C freezer. Samples were then transported to the Neurochemical Research Unit, University of Alberta and stored at -80°C until the time of analysis.

4.2.4 Determination of human plasma amino acid levels

Plasma levels of the amino acids aspartate (Asp), glutamate (Glu), L- and D- serine (Ser), glutamine (Gln), glycine (Gly), Arg, taurine (Taur), alanine (Ala), and gamma-aminobutyric acid (GABA) were determined by high performance liquid chromatography (HPLC) with fluorimetric detection after derivatization with N-isobutyryl-L-cysteine (IBLC) and *o*-phthaldialdehyde (OPA) to produce fluorescent derivatives, using a modification of a procedure previously used by Grant et al. (2006). A 100µl portion of plasma was added to 200µl of ice-cold methanol (MEOH) and kept on ice for 10 minutes. The sample was centrifuged at 4°C for 5 minutes at 12,000 x g, and the supernatant was then transferred to a HPLC vial. A sample volume of 5µl was reacted with a mixture of OPA/IBLC (5µl) reagent in the injection loop of a Waters Alliance 2690XE system for 5 minutes before injection onto the analytical column. The derivatizing mixture was prepared by dissolving 2mg OPA and 3mg IBLC in 150µl MEOH and 1350µl 0.1M of sodium borate. Chromatographic separation was achieved by using the Waters Alliance 2695XE Separations Module with a Waters Symmetry C_{18} 3.5µm(4.6 x 150mm) column and a Waters 474 Scanning Fluorescence Detector set at an excitation wavelength of 344nm and an emission wavelength of 433nm. Mobile phase "A" consisted of 85% 0.04M sodium phosphate buffer and 15% MEOH, with the pH adjusted to 6.2. Mobile phase "B" consisted of 53.4% 0.04M sodium phosphate buffer and 44.2% MEOH also with a pH of 6.2. Initially there was 83% mobile phase "A" and 17% mobile phase "B" with a flow rate of 0.5mg/min, gradually increasing to

100% “B” and then returning to initial conditions, with a total run time of 60 minutes.

4.2.5 Statistical analysis of human data

Human plasma data were analysed by paired samples two-tailed *t*-tests for the pre-and post-treatment analysis as all of the 13 participants did receive both placebo and Arg treatment (cross-over design). Statistical significance was established using a probability value of $p<0.05$.

4.2.6 Animal drug administration

For the dose-dependent study, adult male Sprague-Dawley rats were injected with 20% polyethylene glycol (PEG) 300 in 0.9% saline or Arg monohydrochloride (HCl) dissolved in 20% PEG 300 in 0.9% saline (at concentrations of 500mg, 1000mg and 1500mg/kg). Clozapine HCl was dissolved in 20% PEG 300 in 0.9% saline (at a concentration of 10mg/kg); and risperidone HCl was dissolved in 20% PEG 300 in 0.9% saline (at a concentration of 1mg/kg). The three doses of Arg were administered alone and also in combination with clozapine (at 10mg/kg) and risperidone (at 1mg/kg). All of the drugs were administered intra-peritoneally (i.p.) in a volume of 2ml/kg of body weight for the studies described. All of the animals received an i.p. injection of PCP HCl dissolved in 0.9% saline (at a concentration of 5mg/kg) 30 minutes following the initial treatment injection; n=6 animals for each 12 treatment conditions. Animals were euthanized by decapitation under light isoflurane anesthesia at 90 minutes after the initial treatment injection and whole brain samples were flash frozen in isopentane on dry ice, and stored at -80˚C until the time of analysis. For the Arg time-dependent study, adult male Sprague-Dawley rats were injected with vehicle (20% PEG 300 in 0.9% saline) or Arg HCl at 1000mg/kg (dissolved in 20% PEG 300 in 0.9% saline). There were n=5 for each treatment condition with the exception of the vehicle treated groups (n=3). In this experiment, the animals were also euthanized by decapitation under light isoflurane anesthesia at 15 minutes, 30 minutes, 1 hour, or 2 hours after

the injection and whole brain samples were prepared and stored as described above. All of the animal procedures were conducted in accordance with the Alberta Innovates Technology Futures (AITF) Health Non-Clinical Service Standard Operating Procedures and also approved by the University of Alberta Biosciences Animal Care and Use Committee and in accordance with the Canadian Council on Animal Care guidelines.

4.2.7 *Ex vivo* determination of whole brain amino acid levels

For both of the dose- and time-dependent studies, brain levels of the amino acids Asp, Glu, Ser, Gln, Arg, Cit, Gly, Taur, Ala, GABA and Orn were measured. Partially-thawed brains were dissected along the medial longitudinal fissure, and cerebral hemispheres were homogenized in 5 volumes of ice-cold 100% MEOH. A small amount of homogenate (100µl) was removed to a microfuge tube and was centrifuged (12 000 x g for 5min at 4°C). Following centrifugation, the supernatant was added to 900µl of ice-cold Millipore (MP) filtered H_2O, vortexed, and 100µl was transferred to HPLC inserts inside HPLC vials. For the Orn analysis, 100 µl of the supernatant was directly transferred to HPLC inserts. A portion of the supernatant (5µl) was reacted with OPA (5µl) in the injection loop of a Waters Alliance 2690XE system for 1.5 minutes before injection onto the analytical column (Waters Symmetry C_{18} 3.5µm (4.6 x 150 mm). A Waters 474 fluorescence detector was set to an excitation wavelength of 344 nm and an emission wavelength of 433 nm. Mobile phases "A" and "B" were prepared as described above for the determination of the human amino acid levels. Initially there was 60% mobile phase "A" and 40% mobile phase "B" with a flow rate of 0.5ml/min, gradually increasing to 100% "B" and then returning to initial conditions, with a total run time of 60 minutes. Data were collected and analysed using the Empower Pro software package (Waters).

4.2.8 Statistical analysis of animal data

Animal data were analysed by a one-way analysis of variance (ANOVA) followed by the Neuman-Keuls multiple comparison test. Statistical significance was established using a probability value of $p<0.05$.

4.3 RESULTS

4.3.1 Arginine human plasma levels

Oral supplementation of Arg at 6g per day did not significantly increase plasma levels of Arg. Pre-treatment mean Arg levels (16.5± 2.3 µg/ml) were not significantly different from mean Arg plasma levels at the end of 3 weeks of oral Arg treatment (19.2± 5.2 µg/ml) [t =-1.55, df11, p=0.15]. Participants had blood sampling done before taking their initial morning dose of Arg, at trough conditions. These results are shown in Figure 4-2.

4.3.2 Glutamine human plasma levels

The administration of Arg (6g per day) resulted in a significant decrease in plasma Gln levels following 3 weeks of Arg therapy. Pre-treatment mean Gln concentrations (76.5± 14.8 µg/ml) were significantly higher than the mean Gln plasma concentrations measured at the end of 3 weeks of oral Arg treatment (63.8± 15.1 µg/ml)[t =3.38, df11, $p<0.006$]. These results are shown in Figure 4-3

4.3.3 *Ex vivo* amino acid levels

4.3.3.1 Dose-dependent study

The *ex vivo* dose-dependent study results in rats indicate that brain Arg levels were significantly increased following 500mg/kg, 1000mg/kg, and 1500mg/kg of Arg measured at 90 minutes post-injection [$F(3,20)$ =4.46 $p<0.02$], reaching 168±11%, 144±20%, and 194±30% of the % Arg in the vehicle + PCP treatment group respectively. Brain Arg levels in the 1500mg/kg Arg treatment group were significantly increased in comparison to brain Arg levels in the vehicle + PCP treatment group

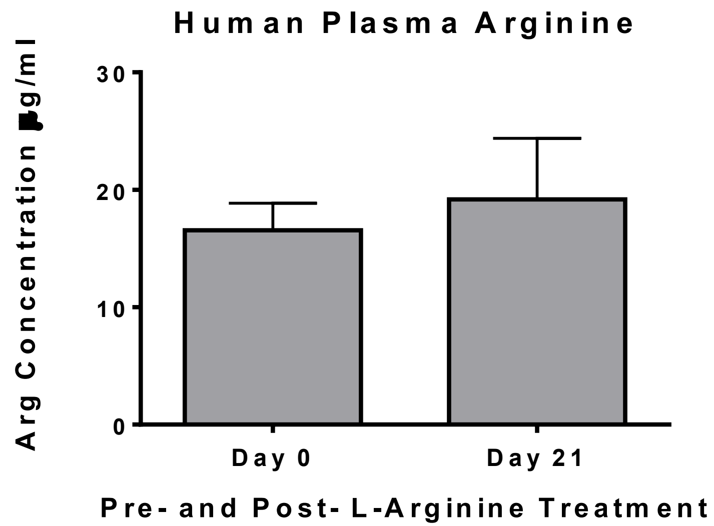


Figure 4-2. Mean±SEM plasma levels of Arg (μg/ml) measured pre- and post-Arg treatment given at 6 grams per day. n=13 for each group, as assessed by a paired samples two-tailed *t*-test.

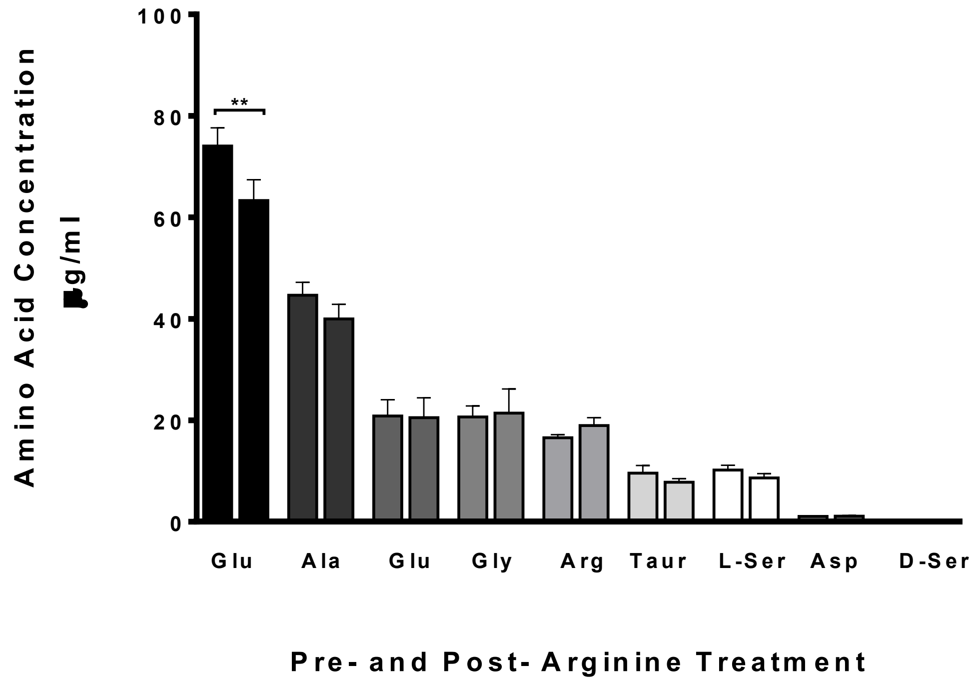


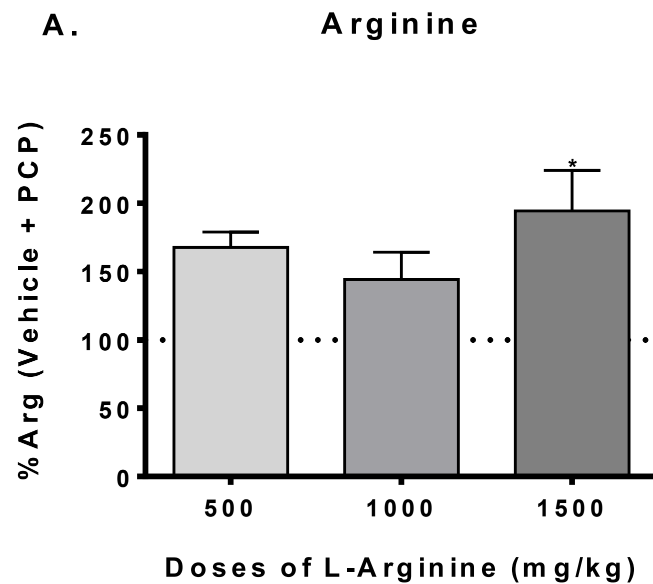
Figure 4-3. Plasma levels of Gln, Ala, Glu, Gly, Arg, Taur, L and D-Ser, and Asp measured pre- and post-Arg treatment. n=13 for each group. Results are expressed as mean±SEM amino acid value (μg/ml). **denotes a significant difference (p<0.01) between pre-and post-Arg treatment, as assessed by a paired samples two-tailed t-test.

($p<0.05$) as assessed by the Neuman-Keuls multiple comparison test. Brain Orn levels were also significantly elevated at each of the Arg doses [$F(3,20)=7.55$ $p<0.002$], reaching 208±21%, 160±25%, and 290±45% of the % Orn values in the vehicle + PCP group respectively. Brain Orn levels in the 500mg/kg and 1500mg/kg Arg treatment groups were significantly increased in comparison to brain Orn levels in the vehicle + PCP treatment group ($p<0.05$, $p<0.001$) and significantly increased between the 1000mg/kg and 1500mg/kg Arg treatment groups ($p<0.05$). Brain Cit levels in the dose-dependent experiments were not significantly elevated at each of the doses of Arg that were administered [$F(3,20)=1.71$ $p=0.19$] (110±5%, 105±11%, and 126±10%) of the % Cit values in the vehicle + PCP group.

4.3.3.2 Time-dependent study

In the time-dependent study, the administration of Arg at 1000mg/kg increased brain Arg concentrations at each of the time intervals tested. Arginine was able to cross the BBB quickly and levels were significantly elevated at the 15 min [$t=3.36$, df6, $p<0.02$], 60 min [$t=3.45$, df6, $p<0.02$], and 120 min [$t=3.53$, df6, $p<0.02$] time points tested, reaching 182±41% of vehicle control values at the 15 minute time point and remaining elevated for up to 120 min post-injection, with final values reaching 163±56% of vehicle controls. Arginine concentrations were not significant at the 30 min time point [$t=0.72$, df6, $p<0.49$]. Brain Orn levels that were generated from the catabolism of Arg by the enzyme arginase were increased from vehicle controls within all of the time points tested, but reached a significant increase only at the 60 min time point [$t=2.55$ df6, $p<0.04$] 140±26% following the Arg injection. The effect of a single Arg injection on whole brain Cit levels was fast-acting. Citrulline levels that were generated from NOS activity increased significantly [$F(3,16)=1.99$ $p<0.001$] and reached 256±103% of control values within 15 minutes of the Arg injection, then returned to control values at the 30 min (115±39%), 60 min (86±8%), and 120 min (135±29%) time intervals tested. Brain Cit levels at the 15 min time interval

were significantly increased as compared to the vehicle only values at 15 min and also significantly increased compared to the 30 min, 60 min, and 120 min time intervals tested ($p<0.001$), as assessed by the Neuman-Keuls multiple comparison test. Arginine had no dose- or time-effect on Gln whole brain levels or any influence on the other amino acids that were measured. The influence of supplemental Arg on reducing Gln levels in human plasma seems to be directly related to long- term dosing only. Data from the dose-and time-dependent studies are shown in Figures 4-4 through 4-7.



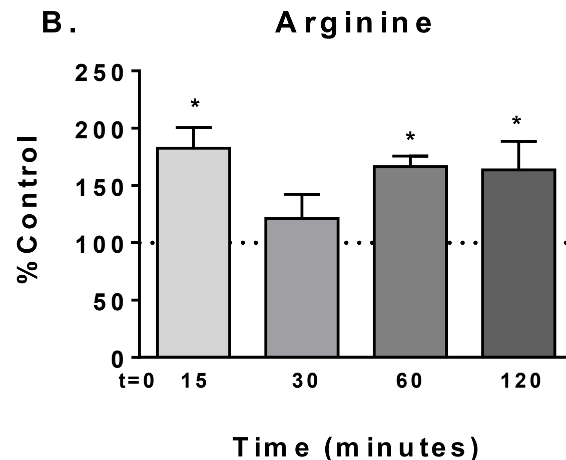
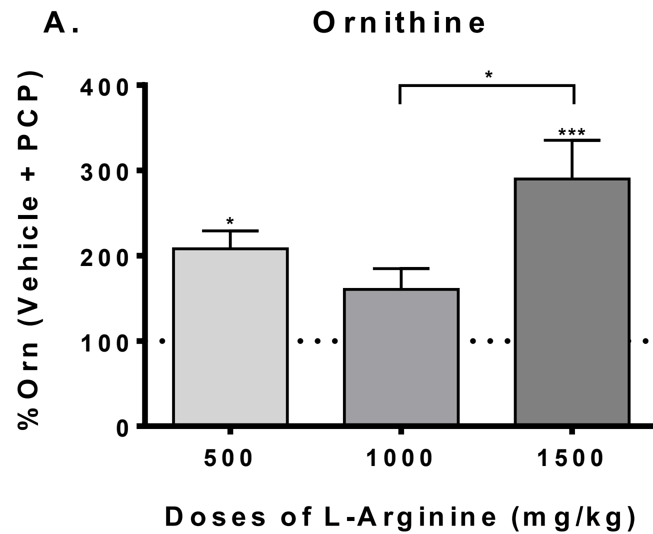


Figure 4-4. A) Dose-dependent (500, 1000, 1500mg/kg at 90 minutes) and B) time-dependent (1000mg/kg) effects of Arg on whole brain Arg levels. n=3-6 per group. Results are expressed as mean ±SEM % Arg in the vehicle +PCP treatment group for dose-dependent data and mean±SEM % Arg in the control (vehicle only) treatment group for the time-dependent data. *denotes p<0.05, compared to vehicle + PCP values (A) and compared to vehicle only (B), as assessed by the Neuman-Keuls multiple comparison test (dose-dependent results) and one-way ANOVA followed by unpaired two-tailed t-tests (time-dependent results).



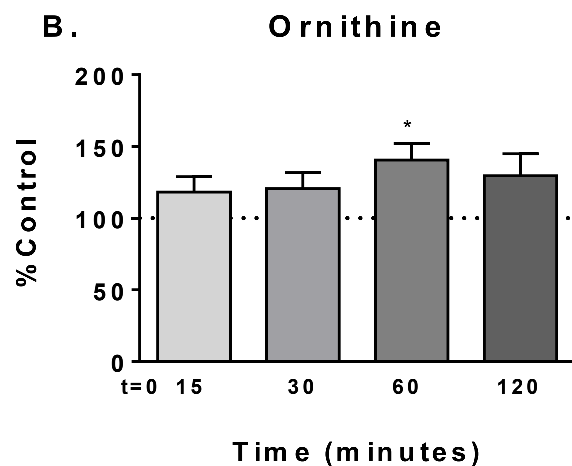
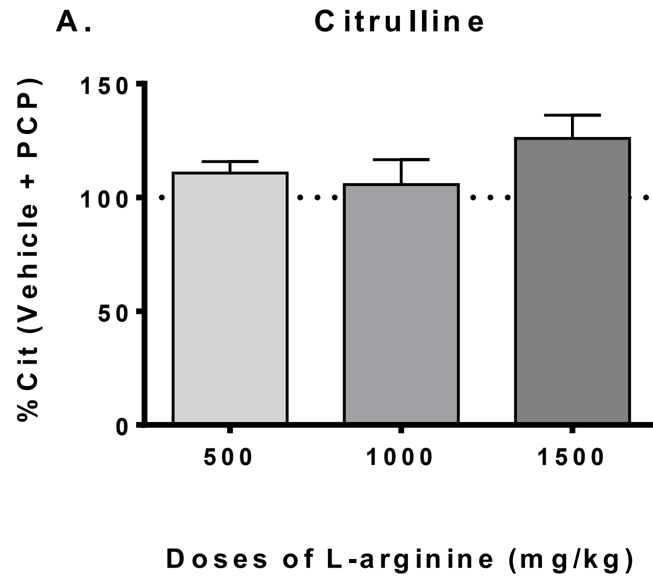


Figure 4-5. A) Dose-dependent (500, 1000, 1500mg/kg at 90 minutes) and B) time-dependent (1000mg/kg) effects of Arg on whole brain Orn levels. n=3-6 per group. Results are expressed as mean±SEM % Orn in vehicle +PCP treatment group for dose-dependent data and mean±SEM % Orn in control (vehicle only) treatment group for the time-dependent data. * denotes $p<0.05$, compared to vehicle + PCP values and between 1000mg/kg and 1500mg/kg values (A) and compared to vehicle only values (B). *** denotes $p<0.001$, compared to vehicle + PCP values (A), as assessed by the Neuman-Keuls multiple comparison test (dose-dependent results) and one-way ANOVA followed by unpaired two-tailed *t*-tests (time-dependent results).



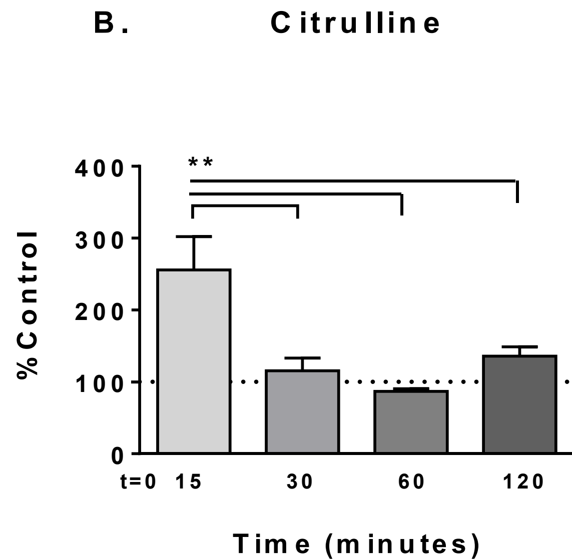
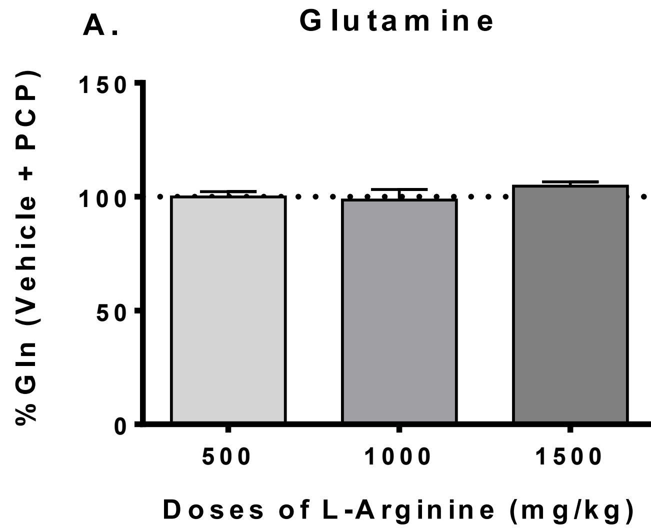


Figure 4-6. A) Dose-dependent (500, 1000, 1500mg/kg at 90 minutes) and B) time-dependent (1000mg/kg) effects of Arg on whole brain Cit levels. n=3-6 per group. Results are expressed as mean±SEM % Cit in vehicle +PCP treatment group for dose-dependent data and mean±SEM % Cit in control (vehicle only) treatment group for the time-dependent data. ** denotes $p<0.01$, compared to vehicle only values (B) and between 30, 60, and 120 min values (B), as assessed by the Neuman-Keuls multiple comparison test.



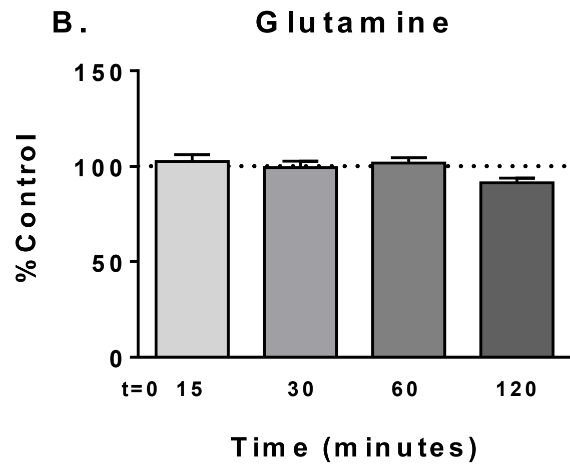
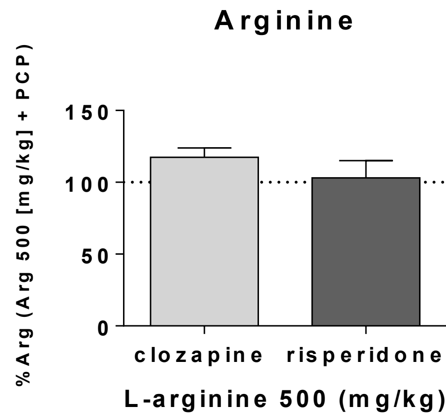
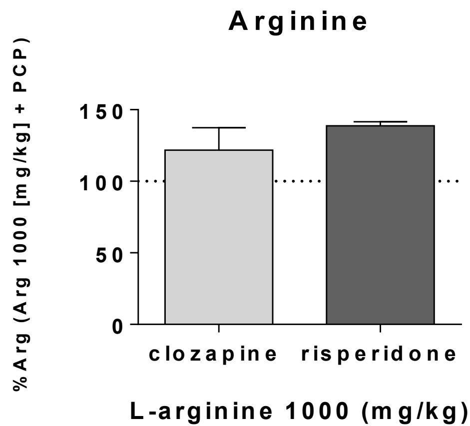


Figure 4-7. A) Dose-dependent (500, 1000, 1500mg/kg at 90 minutes) and B) time-dependent (1000mg/kg) effects of Arg on whole brain Gln levels. n=3-6 per group. Results are expressed as mean±SEM % Gln in vehicle +PCP treatment group for dose-dependent data and mean±SEM % Gln in control (vehicle only) treatment group for the time-dependent data.

4.3.3.3 Antipsychotic drug effects

Combining Arg (at doses of 500, 1000, and 1500mg/kg) with clozapine (10mg/kg) or risperidone (1mg/kg) in PCP-treated animals did not significantly change brain Arg levels at Arg 500mg/kg [t=1.06, df10, p=0.32], Arg 1000mg/kg [t=1.06, df10, p=0.32], or Arg 1500mg/kg [t=0.53, df10, p=0.61]; brain Orn levels at Arg 500mg/kg [t=2.01, df10, p=0.07], Arg 1000mg/kg [t=0.99, df10, p=0.34] or Arg 1500mg/kg [t=0.99, df10, p=0.34]; or brain Cit levels at Arg 500mg/kg [t=0.08, df10, p=0.93], Arg 1000mg/kg [t=0.76, df10, p=0.46], or Arg 1500mg/kg [t=0.22, df10, p=0.83] over those animals who had Arg administered alone (also PCP-treated) at the equivalent doses (% Arg, Orn, and Cit used as comparison). There were no significant differences between these two drugs on any of the amino acids measured. These results are shown in Figures 4-8 through 4-10.





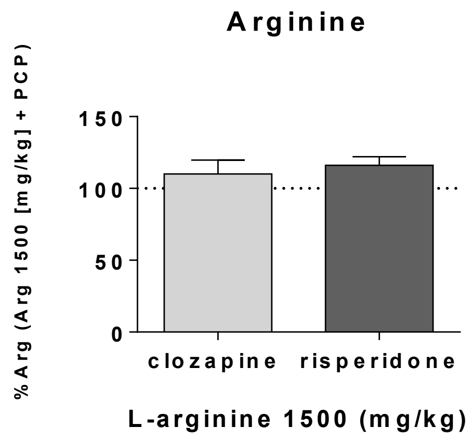
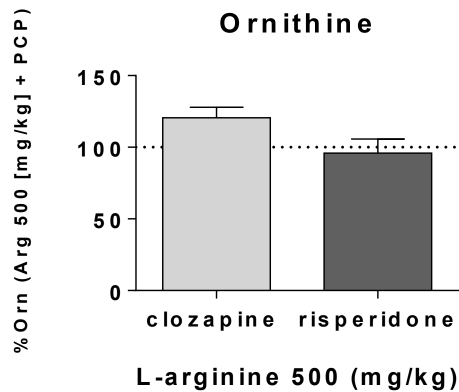
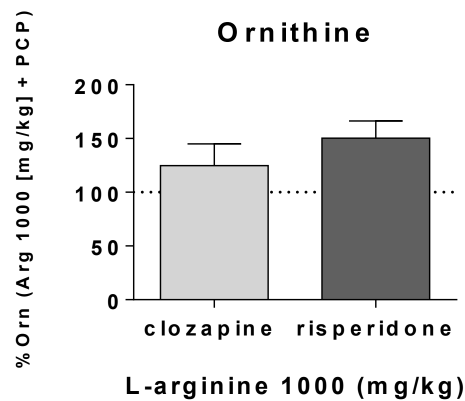


Figure 4-8. Dose-(500, 1000, 1500mg/kg) dependent effects of Arg combined with clozapine (10mg/kg) and risperidone (1mg/kg) measured at 90 minutes on whole brain Arg levels. n=6 per group. Results are expressed as mean ±SEM % Arg in Arg (500, 1000, 1500 mg/kg) + PCP (5mg/kg) treatment groups respectively.





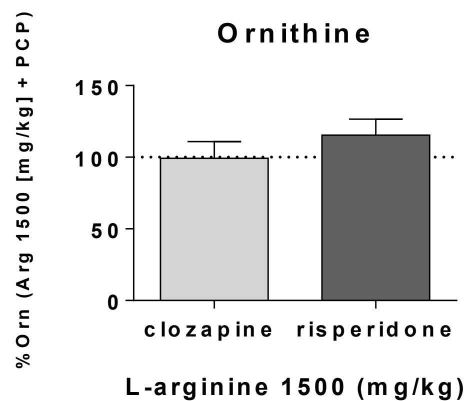
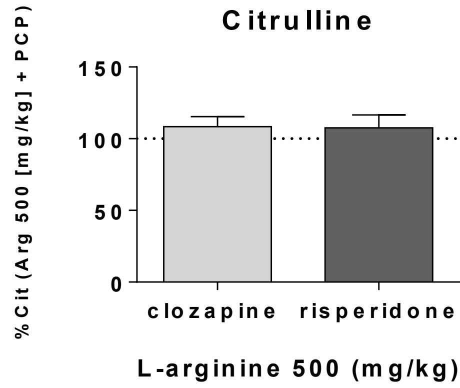
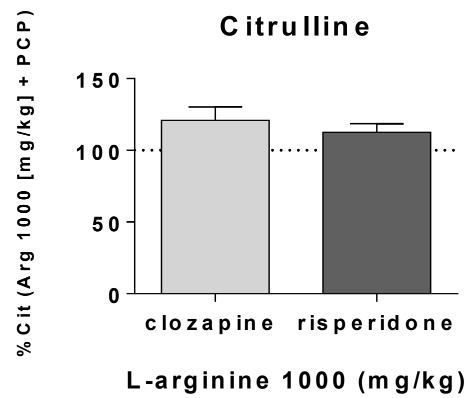


Figure 4-9. Dose-(500mg, 1000mg, 1500mg/kg) dependent effects of Arg combined with clozapine (10mg/kg) and risperidone (1mg/kg) measured at 90 minutes on whole brain Orn levels. n=6 per group. Results are expressed as mean±SEM % Orn in Arg (500, 1000, 1500 mg/kg) + PCP (5mg/kg) treatment groups respectively.





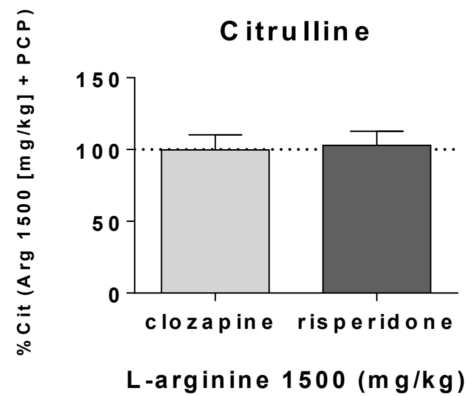


Figure 4-10. Dose-(500mg, 1000mg, 1500mg/kg) dependent effects of Arg combined with clozapine (10mg/kg) and risperidone (1mg/kg) measured at 90 minutes on whole brain Cit levels. n=6 per group. Results are expressed as mean±SEM % Cit in Arg (500, 1000, 1500 mg/kg) + PCP (5mg/kg) treatment groups respectively.

4.4 DISCUSSION

4.4.1 Human arginine plasma levels

Plasma Arg concentrations following 6 g of oral Arg per day that were determined at trough conditions were accurate. This study confirms findings from several other studies that in the post-absorptive state, plasma Arg concentrations are physiologically maintained at approximately 15.1±2.6 μg/ml in adults (Armstrong and Stave 1973; Tangphao *et al.* 1999; Cynober 2002). Maximum concentration of Arg (C_{max}) following oral doses of 6 grams per day is usually reached within about 90 minutes of administration at values of 310±152μmol/l (53.9±26.4μg/ml) (Bode-Boger *et al.* 1998). Similar C_{max} concentrations have been reported following oral doses of 10g of Arg per day (50.0±2.6μg/ml) (Tangphao *et al.* 1999). The half-life of oral Arg has been measured at approximately 90min and the bioavailability is approximately between 63 and 68% (Castillo *et al.* 1993; Bode-Boger *et al.* 1998). Participants in this study had their blood taken following an overnight 12 hour fast, and were *non per os* (NPO) at the time of the blood collection. No differences have been detected in Arg plasma concentration even 5 hours after administration under conditions of long-term oral dosing due to the rapid clearance of Arg from the plasma (Bode-Boger *et al.* 1998).

4.4.2 Human glutamine plasma levels

The mechanisms responsible for decreasing plasma Gln levels following Arg supplementation in this study are most likely related to what is known as the metabolic zonation of function of the hepatocytes (Jungermann and Kietzmann, 1996; Gebhardt 1992) that was influenced by the increased intake of Arg. Although most liver tissue appears to be quite uniform histologically, there is a large degree of heterogeneity in terms of specific hepatocyte function depending on the region of the liver in which the cells are located (zonation of function). The smallest functional unit in the liver is called the acinus, and its boundaries are based on its individual blood supply,

each one representing a microcirculatory unit (for reviews see Jungermann and Kietzmann, 1996; Gebhardt 1992).

Dietary amino acids (including Arg) are absorbed via the intestine and are carried to the portal vein of the liver via the mesenteric veins. Arginine-rich blood from the intestines enters the portal vein and mixes with oxygen-rich blood from the hepatic artery and then flows through the blood vessels (called sinusoids) towards the central vein of the liver. Hepatocytes along this path express differences in their function (specific to enzymatic activity) that influences hepatic metabolism. Two pathways of ammonia detoxification are localized differently in the acinus. Cells that surround the portal vein are categorized as belonging to the periportal zone, and those cells found close to the central vein are part of the perivenous zone (Jungermann and Kietzmann 1996; Gebhardt 1992).

Periportal cells and proximal perivenous cells contain a high number of urea cycle enzymes and favour urea synthesis, particularly at high ammonia concentrations. Perivenous cells favour Gln synthesis. This has been confirmed by functional studies that have used metabolically and structurally intact perfused liver with ammonia ion gradients along the liver acinus during both antegrade and retrograde perfusions (Haussinger 1983). Immunohistochemical localization has also confirmed several urea cycle enzymes in the periportal area including carbamoyl phosphate synthetase 1 (CPS1; the initial rate-limiting enzyme of the urea cycle) (Gaasbeek *et al.* 1984), and several Arg metabolizing enzymes (Miyanaka *et al.* 1998). This is in comparison to the Gln synthesizing enzyme (glutamine synthetase) which is restricted to the hepatocytes in the perivenous acinus (Gebhardt and Mecke 1983). Further characterization of the perivenous glutamine synthetase-containing hepatocytes has revealed that more than 60% of total hepatic Glu uptake occurs in these cells (Haussinger and Gerok 1983).

Normal dietary intake of Arg is approximately 4-6 g per day (Bode-Boger *et al.* 1998). Arginine intake that exceeds the normal daily requirement increases the rate of Arg catabolism, induces urea synthesis, and

activates enzymes within the periportal area of the acinus. In this study, we increased dietary Arg intake by 6g per day. A direct relationship has been demonstrated between the amount and activity of many liver enzymes and the amount of protein in the diet (Schimke and Doyle 1970). Adaptive responses of hepatic glutaminase activity to high protein diets in rats have shown greater glutaminase activity and increased levels of hepatic glutaminase mRNA, indicating increased rates of transcription of the glutaminase gene and subsequent decreased Gln synthesis (Watford *et al.* 1985; Watford 1993). Supplemental diets specifically rich in Arg in animal models also have decreased serum Gln levels (Wu and Morris 1998; Boza *et al.* 2000) and have been shown to reduce glutamine synthetase activity in the rat liver (Boza *et al.* 2000).

To our knowledge, Gln levels have never been directly measured in human plasma in studies of long-term Arg supplementation. The mechanisms responsible for decreased Gln levels in human plasma that are shown here appear to be consistent with the normal physiological action of liver enzymes that catabolize excess nitrogen from an amino acid-rich diet and are also consistent with data from several animal experiments that have investigated these particular metabolic pathways.

4.4.3 Whole brain amino acid levels in rats

The results of the dose- and time-dependent studies confirm that Arg administered i.p. at all of the doses tested does cross the BBB (within 15 minutes of administration) and concentrations remained elevated up to 2 hours post-injection. Arginine concentrations that were measured at the 30 minute time point were lower than those Arg concentrations measured at the other time intervals. This is attributed to lower Arg levels measured in 3 of the 5 animals in the treatment group at this time point as compared to the other time intervals measured. The most substantial effect of Arg in the time-dependent studies was the potentiation of whole brain Cit that reached 256% of control values within 15 minutes of the Arg injection, returning to control

values at all of the subsequent time points tested. The generation of Cit as an indicator of endogenous NO production is often used as an indirect measure of NO biosynthesis. Increases in whole brain Orn concentrations were much more pronounced in the dose-response studies (measured at 90 minutes post-injection) which may indicate sustained arginase activity influenced by increased intracellular Arg that was made available to the enzyme. The competing actions of NOS and arginase for Arg substrate binding may help explain these findings. A lower concentration of Arg is required to achieve half-saturation of all of the isoforms of the NOS enzymes (K_m estimated to be between 2-20μM) than the concentration of Arg that is required for the arginase enzymes (K_m estimated between 2-20mM) (Griffith and Stuehr 1995). This indicates that NOS competes well initially for Arg binding, and that the enzyme is able to reach maximum velocity much quicker than that of arginase, producing Cit immediately. The significant elevation of Cit at the 15 minute time point measured would be consistent with these findings. However, the concurrent activity of the arginase enzymes seems to also be essential for the direct regulation of NO production, having a maximum velocity (V_{max}) of more than 1000 times than that of the NOS enzymes (1400μmol/min per mg for arginase; 1 μmol/min per mg for NOS), indicating that arginase is able to utilize and convert a large amount of intracellular Arg to Orn and urea over time even when the arginase enzymes become saturated (Griffith and Stuehr 1995; Vockley *et al.* 1996; Gotoh and Mori 1999; Corraliza *et al.* 1995; Wang *et al.* 1995; Daghigh *et al.* 1994).

The competing influence of arginase on NO production has been examined in several macrophage models that were stimulated with polysaccharides to induce cytokine stimulated inducible nitric oxide synthase (iNOS) NO production. The activity of both isoforms of arginase does appear to be effective in limiting NO production by iNOS (Buga *et al.* 1996; Chang *et al.* 1998). The inhibitory effects of the arginases on NO production has also been examined in a cell line expressing nNOS, with greater Arg utilization of the cytosolic Arg1 than the mitochondrial Arg2 isoform, indicating the Arg2

isoform found predominantly in neurons and glia may not play as an important role in the regulation of NO production by nNOS as the Arg1 isoform does (Que *et al.* 2002).

Whole brain Cit concentrations remained relatively unchanged in the dose-dependent study. This may be directly related to PCP's ability to bind to an inhibitory site within the ion channel pore of the NMDA receptor and reduce Ca^{2+} entry into the cell (Zukin and Zukin 1979). The nNOS enzyme is dependent on Ca^{2+} binding to calmodulin for activation exclusively when the neuron depolarizes and Ca^{2+} enters the cell. Calmodulin binding enhances the electron transfer in the reductase domain and increases nNOS activity and NO production (Zhou and Zhu 2009). At the 5mg/kg dose of PCP used in this study, PCP may have reduced nNOS activity. Neuronal NOS activity has been investigated in animals dosed with PCP, and nNOS activity was found to be increased in only those animals that received the lowest dose of PCP (2mg/kg). In that study, PCP did reduce Arg levels measured specifically in the prefrontal cortex (PFC) but it had no influence on Cit or Orn levels (Knox *et al.* 2014). All of the animals used in the dose-dependent studies reported in this thesis received PCP and so the specific effect of PCP on whole brain amino acids could not be determined.

The effect of high Orn levels generated by the catabolism of Arg by arginase may have contributed to the anxiolytic effects of Arg supplementation in schizophrenia (discussed in Chapter 2). Although the competing metabolic pathways driven by nNOS and arginase activity have been the focus of this Chapter, the other metabolic pathways of Arg that generate agmatine and the polyamines (putrescine, spermidine, and spermine) should also be considered. Agmatine is the intermediate produced by the decarboxylation of Arg via ADC and it is the precursor for the polyamines. Polyamines are produced either through the catabolism of agmatine via agmatinase (AGMAT) or downstream metabolism of Orn via ODC (Morris 2004). There has been considerable interest in agmatine in particular as a potential pharmacological target for the treatment of

depression and anxiety. Agmatine is an endogenous ligand for alpha(2)-adrenoceptors as well as the type 1 imidazoline receptor (IR-1) which both play an important role in the central control of blood pressure. It also may have antidepressant and anxiolytic properties (Li *et al.* 1994). The discovery that agmatine is synthesized in neurons (Reis and Regunathan 1998; Reis and Regunathan 2000; Zhu *et al.* 2004; Iyo *et al.* 2006), it is released presynaptically by Ca^{2+} dependent depolarization (Goracke-Postle 2006) and it is inactivated specifically by AGMAT (Moldering *et al.* 2003) all support the theory that this guanidino-amine may also act in the brain as a neurotransmitter. Agmatine has been shown to have both antidepressant (Zomkowski *et al.* 2002) and anxiolytic properties (Aricioglu and Altunbas 2003; Lavinsky *et al.* 2003; Gong *et al.* 2006) in animal models. In human studies, plasma levels of agmatine have been reported to be significantly higher in untreated vs treated patients with depression (Halaris *et al.* 1999). Immunohistochemical evidence in human hippocampal tissue also supports increased brain agmatine levels in depression as increased expression of AGMAT was found in this area of the brain in post-mortem tissue from subjects with affective disorders (Bernstein 2012). The compensatory upregulation of AGMAT may explain the increase of agmatine (thought to be an endogenous antidepressant) in the illness (Bernstein 2012).

It is hoped that this analysis of the biosynthetic pathways involved in Arg metabolism and their influence on Arg and related amino acid concentrations in human plasma and also in rodent brain discussed in this Chapter will help to better understand the therapeutic role of Arg as an adjuvant treatment strategy in schizophrenia.

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CHAPTER 5.

NPAS3 AND *COMT* GENOTYPING IN SCHIZOPHRENIA

5.1 INTRODUCTION

5.1.1 *NPAS3* gene

Neuronal Period-Arylhydrocarbon Nuclear Translocator (ARNT)-Single-minded (PAS) domain containing 3(*NPAS3*) is a gene located on chromosome 14 that spans approximately 862kb (Pickard *et al.* 2005). The NPAS3 protein belongs to the larger family of basic helix-loop-helix (bHLH)/PAS proteins that function as transcription factors. Transcription factors assemble and bind to promoter regions of specific deoxyribonucleic acid (DNA) sequences and control the transcription of genetic information from DNA to messenger ribonucleic acid (mRNA). The NPAS3 transcription factor has 3 regions that are vital to regulating the expression levels of over 3000 target genes (Sha *et al*. 2012). The protein contains a bHLH domain that is involved in DNA binding in the promotor region of target genes. A paired PAS domain alters the affinity for partner binding in response to cellular signalling, and a C-terminal region contains a transcriptional activation domain that is responsible for modulating the expression of target genes (Brunskill *et al.* 1999). In the brain, any functional change in the activity of transcription factors would impact the expression of target genes and have the potential to alter a variety of proteins involved in neuronal development. In the case of NPAS3, this includes various mechanisms involved in neurogenesis, including cell differentiation, neuronal migration and cell functioning (Pickard *et al.* 2005).

The *NPAS3* gene was originally identified as a possible schizophrenia candidate gene in a Scottish mother and daughter who were both diagnosed with schizophrenia. Both were carriers of a reciprocal balanced translocation between chromosomes 9 and 14 t(9;14)(q34;q13) with a breakpoint junction that spanned a 683 kb interval on chromosome 14q13 containing the *NPAS3* gene (Kamnasaran *et al.* 2003; Pickard *et al.* 2005). A breakpoint within the *NPAS3* gene has the potential to produce a truncated protein that contains the bHLH domain (necessary to bind to DNA) without the functional PAS or transcriptional activation domains. The PAS proteins

must form homo- or heterodimeric complexes to regulate DNA transcription. A truncated protein might sequester dimerization partners and result in a variety of non-functional protein complexes (Kamnasaran *et al.* 2003; Pickard *et al.* 2005).

The *NPAS3* gene consists of 12 exons, and genetic variation within these exons (1 variant within exon 6, and 6 variants within exon 12) has been previously associated with schizophrenia (Macintyre *et al.* 2010). In *NPAS3* knockout mice, various behavioural abnormalities including locomotor hyperactivity, impaired prepulse inhibition of acoustic startle, increased anxiety, decreased nesting behaviour and decreased social recognition have been observed (Erbel-Sieler *et al.* 2004; Brunskill *et al.* 2005). These are behaviours that have also been found in a variety of animal models of schizophrenia (for review see Powell and Miyakawa 2006). Structural abnormalities in *NPAS3* deficient mice include reduced hippocampal volumes, and enlargement of the posterior lateral, third, and forth ventricles which are also well-described deficits that have been observed in structural imaging studies of patients with schizophrenia (Honea *et al.* 2005; Shenton *et al.* 2001; Shenton *et al.* 2010).

Glutamate (Glu) *N*-methyl-D-aspartate (NMDA) receptors are embedded within the postsynaptic membrane along with a variety of cytoskeletal and signaling proteins collectively referred to as the postsynaptic density (PSD) (Sheng and Pak 2000). The four members of the PSD-95 family of PSD/Disc-large/Z0-1 homologous (PDZ) scaffolding proteins are encoded by four genes *PSD-95/ SAP-90* (synapse-associated protein 90), *PSD-93/chapsin-110*, and *SAP-102* and *SAP-97* (Kim and Sheng 2004). PSD-95 directly links the NMDA receptor to the neuronal nitric oxide synthase (nNOS) enzyme, providing the functional connectivity of the glutamate-nitric oxide (NO) pathway (Brenman *et al.* 1996). In *NPAS3* deficient mice, reduced expression of the PSD-95 has been found in the frontal-parietal cortex of the animals compared to wild-type mice (Brunskill

et al. 2005). This reduced expression of PSD-95 may influence differential effectiveness of the antipsychotic drug iloperidone in patients with *NPAS3* genetic variation (Lavedan *et al.* 2009).

5.1.2 *COMT*, a schizophrenia-associated gene

The gene for the enzyme catechol-O-methyltransferase (*COMT*), is located at the 22q11 region of chromosome 22. This region is associated with velocardiofacial syndrome, also known as DiGeorge syndrome or 22q11 deletion syndrome (Bassett *et al.* 2008; Prasad *et al.* 2008). It is estimated that up to 25% of people with this syndrome develop psychosis as they are missing several genes within the 22q11 region of the chromosome, including *COMT.* This gene encodes the catabolic enzyme COMT (EC 2.1.1.6) that is involved in the termination of catecholamine signaling by enzymatic degradation in the synaptic cleft. In relationship to schizophrenia, alterations in COMT function may result in abnormal dopamine (DA) neurotransmission. There are two forms of the COMT protein: a soluble form found in the cell cytoplasm (S-COMT) and a membrane-bound form (MB-COMT) which is the more prevalent form found in the brain (Tenhunen *et al.* 1994). The *COMT* single nucleotide polymorphism (SNP) p.V158M has been of interest as it influences COMT enzymatic function. The SNP encodes a valine (*Val*) to methionine (*Met*) substitution at codon 158 of MB-COMT, with the *Val* allele encoding a protein with significantly greater enzymatic activity than the *Met* allele (Chen *et al.* 2004).

In the present study, I examined the amount and activity of the *NPAS3* gene in this clinical sample of patients with schizophrenia since *NPAS3* plays an important role in brain development and NMDA receptor functioning. I examined variants previously associated with *NPAS3* and schizophrenia to elucidate the integrity of the PSD-95 protein and to assess if these variants could predict the outcome of the effects of adjuvant L-arginine (Arg) therapy. The *COMT* SNP p.V158M was also examined to explore the relationship between this genetic marker and clinical outcomes of Arg treatment.

5.2 METHODS

5.2.1 Participants

Thirteen subjects (12 males/1 female) of mean age 36±11.5 years with a Diagnostic and Statistical Manual of Mental Disorders (*DSM-IV TR*) (APA 2000) diagnosis of schizophrenia with a mean duration of illness of 15.2±10.9 years were recruited from the Adult Psychiatry Rehabilitation Program at Alberta Hospital Edmonton and also from the Edmonton Addictions and Mental Health Clinic. The *DSM-IV TR* diagnosis was confirmed by the primary treating psychiatrist and also by the Principal Investigator of the clinical trial who is also a psychiatrist. Approval for genetic investigations was received from the University of Alberta Research Ethics Board (REB approval No. MS3_Pro00002053) (see Chapter 2).

5.2.2 DNA extraction

For DNA collection the Genotek OraGene OG-250 disc-format vials for saliva collection were used. The supplied receptacle was filled with fresh saliva (4 ml) and sealed with a buffer-filled cap. Upon cap-closure, the buffer was mixed with the saliva to preserve the sample. Samples were then taken to the Cox laboratory, Department of Medical Genetics, University of Alberta for processing. Genomic DNA was extracted using the manufacturer's protocol. The OG-250 vials were mixed by inversion and then incubated for 2 hours at 50°C to ensure complete cell lysis and inactivation of cellular enzymes. After incubation, 500μl of each sample of lysed saliva were transferred to a 1.5mL DNA/RNAse-free microfuge tube. OraGene purifier solution (20μl) was added to each microfuge tube and the samples were mixed by vortexing. Samples were then incubated on ice for 10 minutes prior to centrifugation at 13,000 x g for 15 minutes. The supernatant was removed to clean 1.5mL tubes and 500μl of 100% ethanol were added. To precipitate the DNA, the samples were mixed by inversion and left to stand for 10 minutes at room temperature. The precipitated DNA was then pelleted by centrifugation at 13,000 x g for 2 minutes. The supernatant was

then discarded, and 250µl of freshly prepared 70% ethanol was added and the mixture was left to stand for 1 minute at room temperature. Lastly, the ethanol was removed from the sample and the DNA pellet was resuspended in l ml of FlexiGene (FG3) hydration buffer (Qiagen Whole Blood DNA Isolation kit). The genomic DNA was left overnight at room temperature to allow complete resuspension of the DNA prior to long-term storage at 4˚C.

5.2.3 Genotyping

In this study, a modified SNaPshot method for genotyping was used. The SNaPshot consists of the three major steps: polymerase chain reaction (PCR) amplification; visualization and quantification on PCR fragments; and multiplex single-base extension (SBE) PCR. The PCR step was modified by using a multiplex PCR over the single-plex PCR.   For the PCR primer design the MPprimer program (http://biocompute.bmi.ac.cn/MPprimer/) was used. This program enables a design of primers specific for multiplexing, and in this study the *COMT* variant was combined with 3 *NPAS3* gene variants, see Table 5-1.

5-1. Multiplex PCR Primers

GENE	SNP	FORWARD PRIMER	REVERSE PRIMER	PRODUCT LENGTH(bp)
COMT	rs 4680	5´- TCGTGGACGCCGTGATTCAGGA- 3´	5´- TGGTGTGAACACCTGGTGGGGA- 3´	285
NPAS3	c. 726C>T	5´- AGGATTGTTTCTGGGACCTG- 3´	5´- TTCACATGGGTAACTTGAGCAT- 3´	377
NPAS3	c. 1500G>C	5´- GGAACCAGTCCGAGAACAGCGA- 3´	5´- TCTCCACGTAGCGCTCCACCTT- 3´	228
	c. 1654G>C			

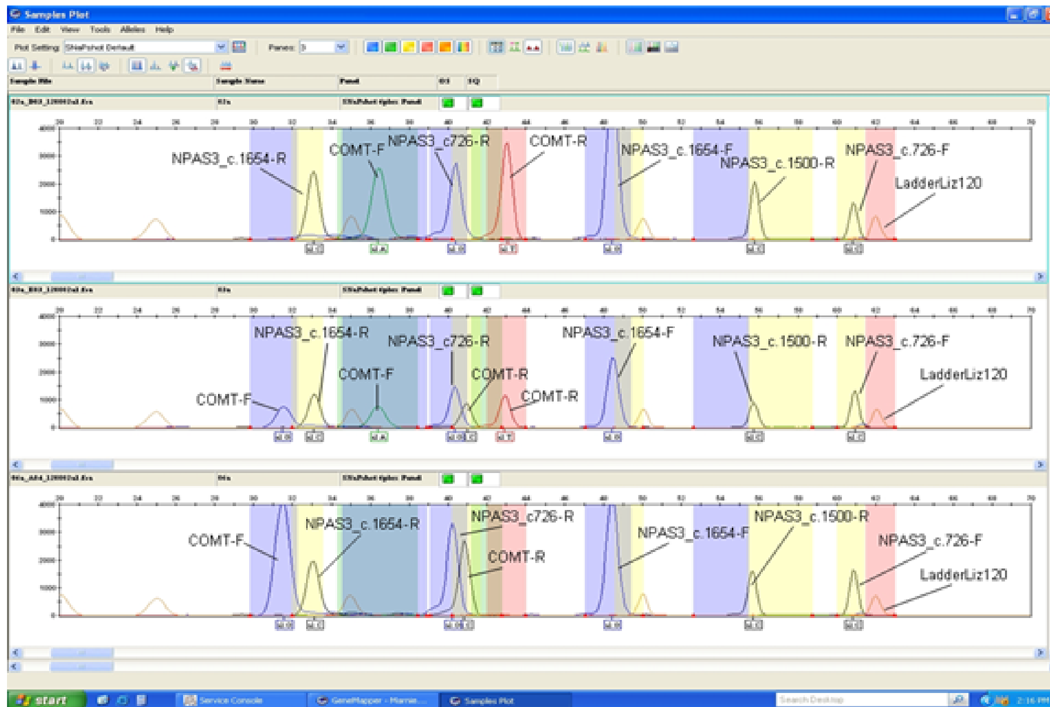
The PCR cycling conditions denatured the samples at 95˚C for 5 minutes. There were 32 cycles of denature at 95˚C for 30 seconds, annealing at 65˚C for 90 seconds, and extension at 72˚C for 30 seconds. The final extension was at 68˚C for 10 minutes and then held at 4˚C to ensure any remaining single-strand DNA was fully extended.

With the Qiagen Type-IT mutation detection kit multiple amplicons were amplified in a single tube. After amplification, amplicons were visualized and quantified by using QIAxcel (Qiagen, gel electrophoresis). In the third step, the SBE PCR method, primers designed upstream from the SNP were extended by a single base that is complementary to the SNP of interest. In the amplification reaction
dideoxynucleoside triphosphates (ddNTPs) from the mixture were incorporated at the end of the primer and the reaction was stopped by the terminator on the deoxynucleoside triphosphates (dNTPs). The SBE primers for each SNP had been designed by a BatchPrimer3 program (http://probes.pw.usda.gov/batchprimer3/index.html) and multiplexed with the previous multiplexed amplicons in a single tube (see Table 5-2). In the final step, ladder Liz120 was added to each sample, which were denatured at 98˚C for 3 minutes, chilled on ice for addition 3 minutes and visualized by a 4 capillary Sequencer 3130. Analysis was done by GeneMapper v.4 as shown in Figure 5-1.

Table 5-2. SNaPshot Primers-Single-Base Extension (SBE) PCR

Gene	Count	Primer_type	Orientation	Start	Length	Tm	GC%	Any compl	3' compl	Q Score	SNP	Pos	Primer Seq
COMT_rs4680	1	SBE	FORWARD	161	16	58.58	62.5	2	2	72.41	G/A	177	GGTGGATTTCGCTGGC
NPAS3_Exon12_c1645	2	SBE	REVERSE	214	28	60.79	52.63	4	2	85.94	G/C	195	agactgactTCCACCTTGATCTGCATCG
NPAS3_Exon6 c.726	2	SBE	REVERSE	310	34	61.6	61.11	4	1	74.17	C/T	292	gactgactgactgactGCAATTCCCACCTGGCTC
COMT_rs4680	2	SBE	REVERSE	197	34	60.72	50	4	2	88.61	G/A	177	acgactgactgactCATGCACACCTTGTCCTTCA
NPAS3_Exon12_c1645	1	SBE	FORWARD	179	42	58.93	68.75	3	3	78.16	G/C	195	gagactgactgactgactgactgactCTTCGGTGCTCTGGGC
NPAS3_Exon12_c1500	1	SBE	REVERSE	58	50	59.93	58.82	2	1	86.25	G/C	41	agactgactgactgactgactgactgactgactGCGTTGCCCGACTTCTT
NPAS3_Exon6 c.726	1	SBE	FORWARD	274	58	58.22	66.67	7	3	77.55	C/T	292	gactgactgactgactgactgactgactgactgactgactCTCTCAGTCGGAGACCCC

SBEprimers design with BatchPrimer3 program. Tails added to 5'-end of primers "GACT". Amplification used SNaPshot kit protocol (Applied Biosystems)



Genotyping data from Sequener 3130 (Applied Biosystems). Analysed with GeneMapper version 4.

Figure 5-1. SNP visualization of *NPAS3* and *COMT* genotyping data

5.2.4 Statistical analysis

Standard multiple regression was used to assess the ability of the *NPAS3* c.1654G>C variant and the *COMT* SNP p.V158M variant homozygous for the *Val* allele to predict the reduction of the Positive and Negative Syndrome Scale (PANSS) total score (dependent variable) as treatment response to Arg therapy. Statistical significance was established using a probability value of $p<0.05$.

5.3 RESULTS

5.3.1 *NPAS3* genotyping

This set of experiments was completed in collaboration with Dr. Georgina Mcintyre, Department of Medical Genetics, University of Alberta. Results are presented in Table 5-3. It has been previously observed that the *NPAS3* variant c.726C>T in exon 6 had an allele frequency of 1.2% in a patient group with schizophrenia as compared to healthy controls (Macintyre et al., 2010). This particular exon variant was not observed in this clinical sample of patients with schizophrenia. All of the 13 patients genotyped in this study were homozygous for the C allele of the c.726C>T variant. Within exon 12, the allele frequency of the c.1654G>C variant was 8.0% in this clinical sample, a lower allele frequency but not significantly different than the 13.8% reported frequency expected based on the HapMap data set for the CEU (CEPH, Utah residents with northern and western European ancestry) neurologically normal population (http://hapmap.ncbi.nlm.nih.gov). The clinical significance of the c.1654G>C variant results in an alanine (Ala) to proline (Pro) change which may encode a defective NPAS3 protein or affect proper ribonucleic acid (RNA) splicing and may alter transcriptional processing of *NPAS3* RNA (Pickard *et al.* 2005). One of the patients who did have the c.1654G>C variant did have the highest PANSS (Kay *et al.* 1987) positive symptom scores and the highest PANSS total scores of the clinical cohort. The other patient with the c.1654G>C variant had similar PANSS scores to the rest of the clinical sample.

Table 5-3. *NPAS3* and *COMT* variants in clinical trial participants

Participant ID	NPAS3 Variant c.726C>T	NPAS3 Variant c.1654G>C	COMT Variant p.V158M	PANSS Total	PANSS Positive	PANSS Negative	PANSS General
	Genotype	***Genotype***	***Genotype***				
1A	C/C	G/G	*Met/Met*	52	13	15	24
2A	C/C	G/G	*Met/Met*	74	8	27	39
3A	C/C	G/G	*Val/Met*	70	8	32	30
4A	C/C	G/G	*Val/Met*	78	18	25	35
6A	C/C	G/G	***Val/Val***	67	8	27	32
7A	C/C	**G/C**	*Val/Met*	65	15	20	30
8A	C/C	G/G	*Val/Met*	66	16	21	29
9A	C/C	G/G	*Val/Met*	65	14	22	29
11A	C/C	G/G	***Val/Val***	75	16	21	38
12A	C/C	G/G	*Met/Met*	68	19	18	31
13A	C/C	**G/C**	*Val/Met*	81	26	24	31
14A	C/C	G/G	***Val/Val***	67	22	13	32
15A	C/C	G/G	***Val/Val***	69	17	20	32

5.3.2 *COMT* genotyping

COMT genotyping revealed that no one in this clinical sample had 22q11 deletion syndrome. Homozygotes of the *Val* allele (n=4, 31%) encode an enzyme with higher activity, increasing prefrontal cortical (PFC) DA catabolism and this is thought to be detrimental to executive frontal lobe functioning in schizophrenia (Egan *et al.* 2001; Malhotra *et al.* 2002). There were n=6 (46%) of the patients that were heterozygous for the *Val/Met* allele, and n=3 (23%) of the patients were homozygotes for the *Met/Met* allele encoding an enzyme with the lowest activity. The *COMT* genotype frequencies found in this clinical trial did not deviate from Hardy-Weinberg equilibrium. The frequencies were as would be predicted in the absence of non-random mating or selection factors. The *NPAS3* c.1654G>C variant with an added *COMT* dosage effect of the *Val* allele both in combination was an important clinical consideration that may have predicted worse clinical outcomes for the study participants. Standard multiple regression analysis was used to assess the ability of the *NPAS3* c.1654G>C variant and the *COMT* SNP p.V158M *Val* allele variant to predict treatment response to Arg therapy. The coefficients for the *NPAS3* c.1654G>C variant and *COMT* SNP p.V158M *Val* allele were -0.625 and 0.0417 respectively, which yielded a multiple R^2=0.213, $F(2,10)$=1.36, p=0.30.

5.4 DISCUSSION

This study has identified a *NPAS3* variant that has been previously associated with schizophrenia. The *NPAS3* c.1654G>C variant found in exon 12 of the *NPAS3* gene was confirmed in two patients. One of the individuals, a female, had the highest PANSS positive symptom scores and PANSS total scores of the clinical sample. The other male carrier had PANSS scores similar to the other clinical trial participants. Although this variant is also found in neurologically normal populations, its relevance to schizophrenia and processes that are involved in brain development supports a neurodevelopmental origin of the illness.

The most prominent impact of the *NPAS3* c.1654G>C variant in schizophrenia may be related to NPAS3 posttranslational regulation (Wong *et al.* 2013). In humans, *NPAS3* mRNA levels have been found to be highest during the neonatal period and then to decrease significantly throughout infancy and toddlerhood, with lowest levels detected at school age. These levels remain stable into adulthood. NPAS3 protein expression is highest during the first decade of life (Wong *et al.* 2013). In schizophrenia, NPAS3 mRNA levels measured in adulthood are consistent with levels measured in adult healthy controls; however, females with schizophrenia have been found to have significant decreases in NPAS3 protein in comparison to female healthy controls and also in comparison to males with the illness (Wong *et al.* 2013).

Micro-ribonucleic acid (miRNA) expression has also been increased in patients with schizophrenia in comparison to controls (Wong *et al.* 2013). MicroRNAs are cellular RNA fragments that prevent the production of proteins by binding to and deactivating the mRNA that is needed to produce the protein. Since there has been no reported difference in NPAS3 mRNA in patients with schizophrenia as reported by Wong *et al.* (2013), NPAS3 biosynthesis or function may have be altered in early development in the carriers of the *NPAS3* c.1654G>C variant. Decreases in NPAS3 protein in females with schizophrenia may suggest a change in NPAS3 protein expression or a decrease in the translational ability of stable mRNA (biological processes that may have also impacted the female patient in this study).

The reduction of NPAS3 protein function in both of the patients with the *NPAS3* c.1654G>C variant may have impacted the integrity of the Glu NMDA receptor and response to Arg therapy. The PSD-95 protein is the primary protein that determines the functional connection and integrity between the Glu NMDA receptor and the NO system. Therefore, a fully functional PSD-95 protein may have determined the outcome of the effects of Arg therapy in the two *NPAS3* c.1654G>C carriers. In *NPAS3* knockout mice,

reduced expression of PSD-95 protein in the frontal-parietal cortex has been found in comparison to wild-type mice (Brunskill *et al.* 2005). This suggests that any alteration in NPAS3 protein function or activity may lead to an interruption in NO signaling. This may have been one of many factors underlying their disease. Due to the heterogeneity of schizophrenia, patients without the *NPAS3* variant in this clinical trial may have had other genetic or environmental influences that contributed to the development of their illness.

COMT genotyping identified patients that are homozygotes of the *Val* allele. These individuals encode a COMT enzyme with approximately 40% higher activity than those who are homozygotes for the *Met* allele (Chen *et al.* 2004). The MB-COMT form of the enzyme is expressed primarily in neurons and it is found in abundance in the PFC and hippocampus rather than in striatal or brainstem DA neurons (Matsumoto *et al.* 2003; Kastner *et al.* 1994). Abnormal PFC functioning measured by cognitive tests and PFC activation has been found in those patients who are homozygotes of the *Val* allele in relationship to increased DA catabolism in this region of the brain (Egan *et al.* 2001).

The absence of DA transporters (DAT) in the PFC may also allow COMT and variation in its enzymatic activity to have a greater impact on cortical DA signaling (Lewis *et al.* 2001). Decreased PFC DA signaling induced by COMT *Val* allele activity may play a role in the regulation of mesencephalic DA activity and may lead to an increase in DA levels in the striatum. In *COMT* knockout mice DA levels have been reported to be unchanged in the striatum but have been increased in the PFC under certain conditions such as stress (Gogos *et al.* 1998). There was no change found in norepinephrine levels in the animals, suggesting that COMT may have a selective impact on PFC DA signaling. These findings do support the idea that the COMT *Val* allele may lead to both cortical DA deficiency and mesolimbic hyperdopaminergia. This would be in keeping with the DA hypothesis of schizophrenia that states that DA hyperactivity in the mesolimbic area of the

brain leads to the positive symptoms of the illness, and DA hypoactivity within the cortex contributes to the negative symptoms of the illness (Weinberger 1987; Weinberger *et al.* 1988).

In this study, the gene-gene interaction of *NPAS3* and *COMT* and their combined impact on treatment response to Arg therapy was not significant. Genotyping and identification of the *NPAS3* c.1654G>C variant with an added *COMT* dosage effect of the *Val* allele was a clinical consideration that in combination did not predict worse clinical outcomes for patients. None of the study participants had combined *NPAS3* and *COMT* variants of interest. The *NPAS3* c.1654G>C variant found in two of the participants may have impacted the integrity of the PSD-95 protein and contributed to the etiology of their illness. Nitric oxide is generated in the brain by an intact NMDA receptor, PSD-95 scaffolding protein, and nNOS enzyme. Downstream signaling mechanisms that are regulated through a fully functional NMDA receptor, including the production of NO, may be influenced by *NPAS3* and *COMT* functioning. Future studies that include a larger sample size to investigate these pathways further in schizophrenia are warranted.

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CHAPTER 6.

PRECLINICAL STUDIES ON L-ARGININE AND TWO ATYPICAL ANTIPSYCHOTICS

6.1 INTRODUCTION

A comprehensive model of schizophrenia is difficult to reproduce in animals due to the heterogeneity of the illness in humans. To recreate all of the physiological mechanisms underlying schizophrenia goes well beyond a single lesion, a manipulated gene, or an environmental insult in an animal as there are many more biological mechanisms of the disease that are uniquely human and still remain unknown. One of the best pharmacological probes to use as an effective animal model for schizophrenia is the non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist drug phencyclidine (PCP).

Phencyclidine (1-[1-phenycyclohexyl]piperidine) was first developed by the pharmaceutical company Parke-Davis, and was marketed under the trade name Sernyl® as an anesthetic agent in the late 1950s. From early experience in humans who received PCP for anesthesia purposes, it was found that 30% of patients developed an emergence delirium as they were recovering. This phenomenon was characterized by acute disorientation, feelings of dissociation, excitation, unmanageability and paranoia (Greifensten *et al*. 1958). Interest grew in the potential of PCP to be a pharmacological model for psychosis and schizophrenia due to these behavioural effects of the drug. Initial studies that investigated the psychotomimetic effects of PCP in both healthy controls and patients with schizophrenia confirmed that the drug was able to induce in healthy controls a variety of positive, negative, and cognitive symptoms consistent with the illness and to exacerbate psychosis in patients, having long-lasting effects in those patients even after a single dose (Luby *et al.* 1959). Consequences of long-term PCP exposure due to its abuse induced a syndrome that includes psychosis, auditory hallucinations, flattened affect, formal thought disorder, thought blocking, and social withdrawal (Allen and Young 1978; Smith 1980).

The psychotomimetic effects of non-competitive NMDA receptor antagonist drugs continued to gain interest when it was discovered that patients with schizophrenia had lower levels of glutamate (Glu) in their cerebrospinal fluid (CSF) as compared to healthy controls (Kim *et al.* 1980). This is further evidence to support the hypoglutamatergic hypothesis of schizophrenia (see Chapter 1) which is supported by the NMDA receptor blocking property of PCP and other drugs. The drug ketamine (a related arylcyclohexylamine derivative with similar pharmacological action as PCP) (Siegel *et al.* 1978), was used in further clinical trials as it was a shorter-acting and a safer alternative to PCP use in humans (Krystal *et al.* 1994; Lahti *et al.* 1995; Lahti *et al.* 2001; Malhotra *et al.* 1996; Malhotra *et al.* 1997).

In rodent studies, single-dose PCP administration of 5 mg/kg can produce a behavioural response in the animals that is thought to be relevant to the clinical presentation of schizophrenia. Increased locomotor activity, speed of movements, and several stereotyped behaviours (turning, head weaving, circling and back peddling) in animals have been useful behavioural indicators of the ability of PCP to induce psychosis in humans (Fessler *et al.* 1980; Castellini and Adams 1981b; Moghaddam and Adams 1998). Stereotyped behaviour (repetitive head weaving and head bobbing) have also been reported in humans experiencing acute psychosis from PCP (Luby *et al.* 1959), and this may have some relationship to catatonic motor behaviour and disturbances in perseveration of thought, both of which can be features of schizophrenia (Moghaddam and Adams 1998). Acute PCP dosing in animals can also: disrupt prepulse inhibition (Geyer and Braff 1987; Braff *et al.* 1992), a measure consistent with the startle-gating deficits in schizophrenia; decrease social interaction (Sams-Dodd *et al.* 1996; Sams-Dodd *et al.* 1998) which may have a relationship to the persistent social deficits of the illness; and reduce cognition (Egerton *et al.* 2005; Egerton *et al.* 2008)(for additional reviews see Thornberg and Saklad 1996; Steinpreis 1996; Jentsch and Roth 1999).

The primary pharmacological action of PCP involves binding to an inhibitory site within the ion channel pore of the NMDA receptor, acting as a non-competitive antagonist (Zukin and Zukin 1979). Phencyclidine binds to this site with high affinity in a saturable, reversible, and selective fashion. Studies that have investigated [^{3}H]-PCP binding in rat brain membrane report that it is not displaced by the ligands Glu or NMDA (Vincent *et al.* 1983). The NMDA receptor blockade by PCP may be consistent with the dysregulated Glu mechanisms that are thought to contribute to the pathophysiology of schizophrenia. Downstream signaling mechanisms that are regulated through a fully functional NMDA receptor, including the production of nitric oxide (NO), may be disrupted in the illness. Glutamate binding at NMDA receptors is the primary activating signal for the synthesis of NO in the brain (Garthwaite *et al.* 1989). Phencyclidine-induced antagonism of NMDA receptors might lead to a decrease in NO production as release of NO is dependent upon the depolarization of the neuron and influx and binding of Ca^{2+} to calmodulin to activate the neuronal nitric oxide synthase (nNOS) enzyme (Garthwaite *et al.* 1989).

Both preclinical and human studies have been helpful to understand the role of NO in the mechanism of action of PCP and to also learn about its therapeutic effect in schizophrenia. The effects of the NO donor drug sodium nitroprusside (SNP) studied in PCP-treated rats has provided further insight into what contribution NO may have in PCP-induced psychosis. Sodium nitroprusside is classified as a nitrovasodilator drug that has traditionally been used for acute hypertensive crisis. Structurally, SNP is a sodium salt with 5 cyanide ions that are complexed to a central iron molecule (Friederich and Butterworth 1995). The SNP molecule is very unstable and will decompose when it is exposed to light so it must be protected from any light source and be given by an intravenous (human) or intraperitoneal (i.p.) (animal) injection. When administered, SNP reacts with oxyhemoglobin molecules within erythrocytes to form methemoglobin. This causes the SNP

molecule to become very unstable and results in the quick release of NO (Friederich and Butterworth 1995).

Increased production of NO by SNP has been shown to antagonize behavioural and neurobiological effects of PCP-treated rats (Bujas-Bobanovic *et al.* 2000a; 2000b). Sodium nitroprusside administration (0.5μg/kg/min) in patients with schizophrenia (on antipsychotic medication) was also able to improve acute psychotic symptoms within 2 hours of the start of the infusion and these improvements were maintained for up to 4 weeks (Hallak *et al.* 2013). To further distinguish the antipsychotic effect of SNP on its own, a preclinical study designed to administer a pre-treatment (up to one week) of SNP was able to prevent ketamine–induced effects in the animals (Maia-de-Oliveira *et al.* 2015). The ability of SNP to modulate protein kinases, transcription factors, and other gene products through the generation of NO and cyclic guanosine monophosphate (cGMP) may be responsible for these lasting cascading effects of SNP.

Several studies have also shown that a decrease in NO production (by blocking NOS activity with NOS inhibitors) can enhance locomotor effects of PCP (Noda *et al*. 1995; Noda *et al*. 1996; Bujas-Bobanovic *et al*. 2000b). However, an intact nNOS enzyme is required for PCP to induce hyperlocomotor activity. Mice with reduced expression of nNOS (and also nNOS knock-out mice) have reduced psychotic-like reactions to PCP (Bird *et al*. 2001). Taken together, this evidence suggests that the use (and development) of drugs that target the glutamate-NO system may offer a promising new approach for the treatment of PCP- and ketamine-induced psychosis as well as schizophrenia.

In the experiment described in this Chapter, I examined the dose-dependent relationship of L-arginine (Arg) to PCP-induced hyperlocomotor activity in rats. I examined the ability of Arg (at doses of 500, 1000, and 1500mg/kg) given alone to attenuate the locomotor-producing effects of PCP. I also examined Arg (at all 3 doses) combined with clozapine (10mg/kg) and risperidone (1mg/kg) and measured the concomitant effects

of Arg and these two antipsychotic drugs. As clozapine is known to have partial agonist properties at the NMDA receptor (Olney and Farber 1994; Malhotra *et al*. 1997; Williams *et al*. 2004), I expected that the animals in the clozapine + Arg treatment groups would have a greater reduction in PCP-induced hyperlocomotor activity as compared to the risperidone + Arg treatment groups. The majority of the responders to Arg treatment (80%) in the clinical trial (Chapter 2) were also taking clozapine. The doses chosen for Arg, risperidone, clozapine, and PCP were determined by previous animal studies utilizing these drugs (Noda *et al.* 1995; Umathe *et al.* 2009; Anand *et al.* 2011; Kitaichi *et al.* 1994; Williams *et al.* 2006; Bird *et al.* 2001) and were also based on appropriate antipsychotic dosing recommendations for rodents by Kapur *et al.* (2003).

6.2 METHODS

6.2.1 Animals

For this set of experiments, 12 groups of 6 adult male Sprague-Dawley rats (Charles River Canada Inc.) weighing 205-300g and 7-8 weeks of age were used, and we followed the experimental protocol as outlined in Figure 6-1. Animals were individually housed in Shoebox Cages with environmental enrichment (2 autoclaved Ancare Nestlets) with Certified Irradiated Anderson's Corn-O-Cob Bedding and were allowed *ad libitum* access to Certified Lab Diet Pico Rodent Diet and sterilized water. The animals were maintained on a 12 hour light cycle with temperatures kept between 18 and 26˚C. All of the animal procedures were conducted in accordance with the Alberta Innovates Technology Futures (AITF) Health Non-Clinical Service Standard Operating Procedures and also approved by the University of Alberta Biosciences Animal Care and Use Committee and in accordance with the Canadian Council on Animal Care guidelines.

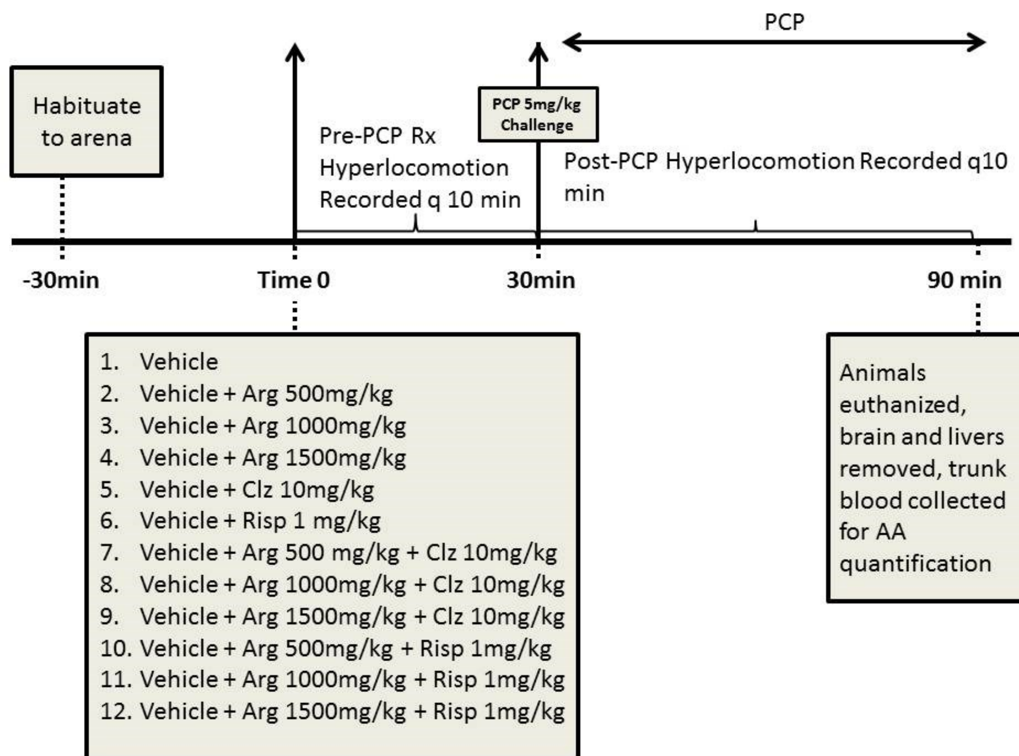

Figure 6-1. Dose-dependent experimental timeline of Arg administered i.p. at doses of 500, 1000, and 1500mg/kg alone and in combination with clozapine (Clz) (10mg/kg) and risperidone (Risp) (1 mg/kg) pre- and post-PCP 5mg/kg challenge.

6.2.2 Pre-treatment procedures

Due to the large number of animals (N=72), the experiment was conducted in 3 separate Cohorts (Cohort A, B, and C) over 4 days, having 2 animals represent each treatment group per Cohort. Animals were observed and body weights were recorded on study days 3, 4, and 6 for Cohorts A, B, and C, respectively during the acclimation and pretest period. An acclimation period of 5, 6, and 8 days for Cohorts A, B, and C was provided. A computer-generated randomization program that used the SAS® PROC PLAN procedure to minimize group differences in body weights was used to randomly assign the animals to the treatment groups.

6.2.3 Drug administration

For the experiments in this study, adult male Sprague-Dawley rats were injected with 20% polyethylene glycol (PEG) 300 in 0.9% saline or Arg monohydrochloride (HCl) dissolved in 20% PEG 300 in 0.9% saline (at concentrations of 500mg, 1000mg and 1500mg/kg). Clozapine HCl was dissolved in 20% PEG 300 in 0.9% saline (at a concentration of 10mg/kg); and risperidone HCl was dissolved in 20% PEG 300 in 0.9% saline (at a concentration of 1mg/kg). The three doses of Arg were administered alone and also in combination with clozapine (at 10mg/kg) and risperidone (at 1mg/kg). All of the drugs were administered i.p. in a volume of 2ml per 1 kg of body weight for the studies described. All of the animals received an i.p. injection of PCP HCl dissolved in 0.9% saline (at a concentration of 5mg/kg) 30 minutes following the initial treatment injection; n=6 animals for each 12 treatment conditions.

6.2.4 Motor activity

During light phase (0800h-1500h), animals were placed in their home cages with bedding and allowed to habituate to the arena for 30 min before testing. Animals were injected i.p. with drug and vehicle at time 0 and hyperlocomotor activity was recorded with the San Diego Instruments Photobeam Activity System-Home Cage (PAS-HC) apparatus (San Diego Instruments, San Diego, CA, USA) with a 4x8 photobeam configuration to record movement by detection of bidirectional photocell beam breaks. Automated locomotor activity was measured in 10 minute intervals for 30 min. Each animal was then removed from the motor activity apparatus and injected i.p. with 5mg/kg of PCP and immediately returned to the cage and motor activity was recorded again at 10 minute intervals for a further 60 min for post-PCP injection analysis. Locomotor activity was recorded for a total of 90 minutes. The animals were then immediately euthanized by decapitation under light isoflurane anesthesia and whole brain samples and livers were dissected out and flash-frozen in dry ice/isopentane (Fisher Scientific, USA) and stored at below -65˚C. Trunk blood was also collected and plasma isolated by centrifugation and stored below -65˚C.

6.2.5 Statistical analysis

Animal data were analysed by a mixed between-within subjects analysis of variance (ANOVA) (Treatment x Arg x Time) with repeated measures on the 3rd factor. Treatment (vehicle, antipsychotic medications, and 3 doses of Arg) were the between factors and time was the within subject factor measured in the analysis. A one-way ANOVA and the Bonferonni *post-hoc* test for multiple comparisons was also used to measure total number of beam break differences between treatment groups at each time point and also the total distance moved between treatment groups post-PCP administration. All of the results are presented as the mean and standard error of the mean (S.E.M) for each experimental group. Statistical significance was established using a probability value of $p<0.05$.

6.3 RESULTS

The measurement of hyperlocomotor activity following a single-dose PCP administration of 5mg/kg in animal models is a reliable method to determine the antipsychotic effects of many drugs. A *post-hoc* one-way ANOVA conducted on the vehicle alone group between the 10 min and 30 min time intervals was used to determine the initial habituation effect over time. There was a significant time effect [F(1.425, 7.123)=33.29 p<0.004] across the first 3 time points measured, and a significant between-subject effect [F(5,10)=3.37 p<0.05] was found. To determine a reference point for a percent of control transformation for the PCP analysis for all of the treatment groups measured between the 30 min to 90 min time intervals, the mean value for the 30 min time interval for the vehicle alone group was subsequently used as the reference point for all further analysis.

A mixed between-within subjects ANOVA was used to assess the effect of 12 different treatment interventions on hyperlocomotor activity across 7 time intervals post-PCP injection (at 30, 40, 50, 60, 70, 80, and 90 min). There was a significant overall interaction effect between the vehicle + PCP group, the clozapine + PCP group, and the risperidone + PCP group over time [F(18,156)=2.34 p<0.003], with no significant interaction effect between the Arg + PCP groups of 500, 1000, and 1500mg/kg over time [F(18, 156)=1.13 p=0.33], and also no significant interaction effect between the clozapine + Arg + PCP treatment groups or the risperidone + Arg + PCP treatment groups over time [F(30, 222)=1.27 p=0.17]. There was a substantial effect for time [F(6, 55) 24.46 p<0.0005], with all groups showing an increase of hyperlocomotor activity at the 40 min and 50 min time intervals tested, then a gradual decline for the remaining time measurements.

The main group effect between the vehicle only group, the clozapine group, and the risperidone group was significant [F(3,60) = 5.15 p<0.003], with separation from the vehicle only group occurring at the first 10 min time interval [F(2,15)=6.36 p<0.01] and also at the 40 min time interval post-PCP injection [F(2,15)=4.47 p<0.05]. There was no significant difference

between the groups at the 50 min time interval [$F(2,15)=3.16$ $p=0.06$]. These results are shown in Figure 6-2. There were no significant between-group differences between the Arg + PCP treatment groups [$F(3,60)=0.34$ $p=0.79$] and no significant differences between the clozapine + Arg + PCP treatment groups and the risperidone + Arg + PCP treatment groups [$F(5,60)=1.13$ $p=0.36$]. However, the total distance moved in the risperidone + Arg treatment groups measured post-PCP administration was significantly reduced [$F(3,20)=3.74$ $p<0.05$]. These results are shown in Figures 6-3 through 6-5.

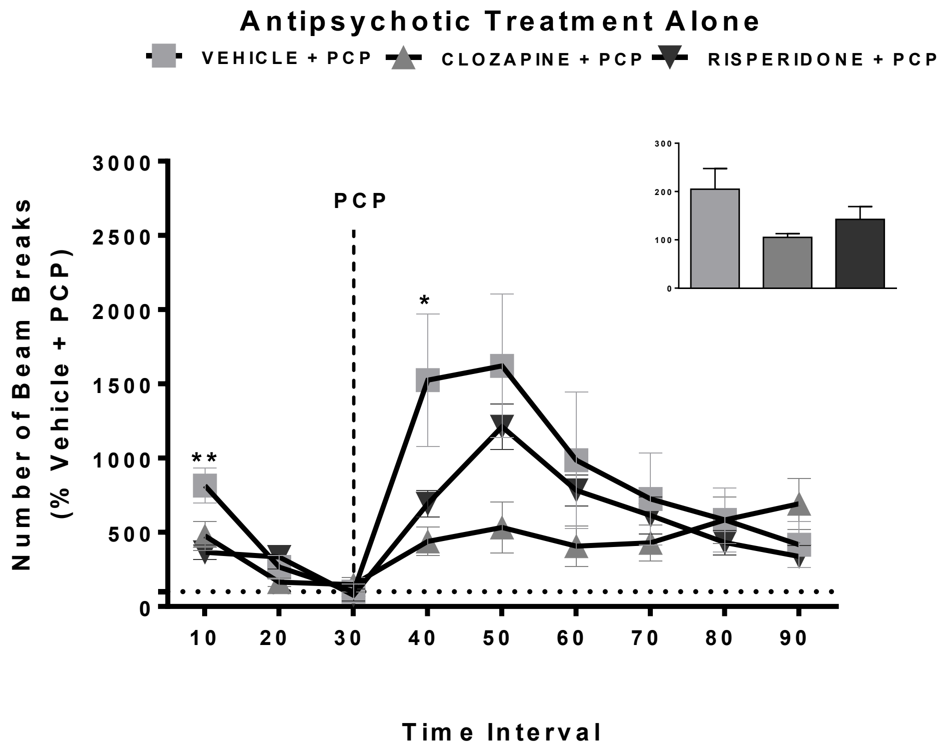

Figure 6-2. Effect of clozapine (10mg/kg) and risperidone (1mg/kg) on PCP (5mg/kg)-induced hyperlocomotion. Results are expressed as mean±SEM % (vehicle + PCP) treatment group of total number of beam breaks at each time interval tested. *denotes significant differences (p<0.05) and (** p<0.01), compared to vehicle + PCP values between treatment groups as assessed by analysis of simple main effects with a Bonferonni correction for multiple comparisons. **Inset:** total distance moved post-PCP administration expressed as mean±SEM. n=6 per treatment group.

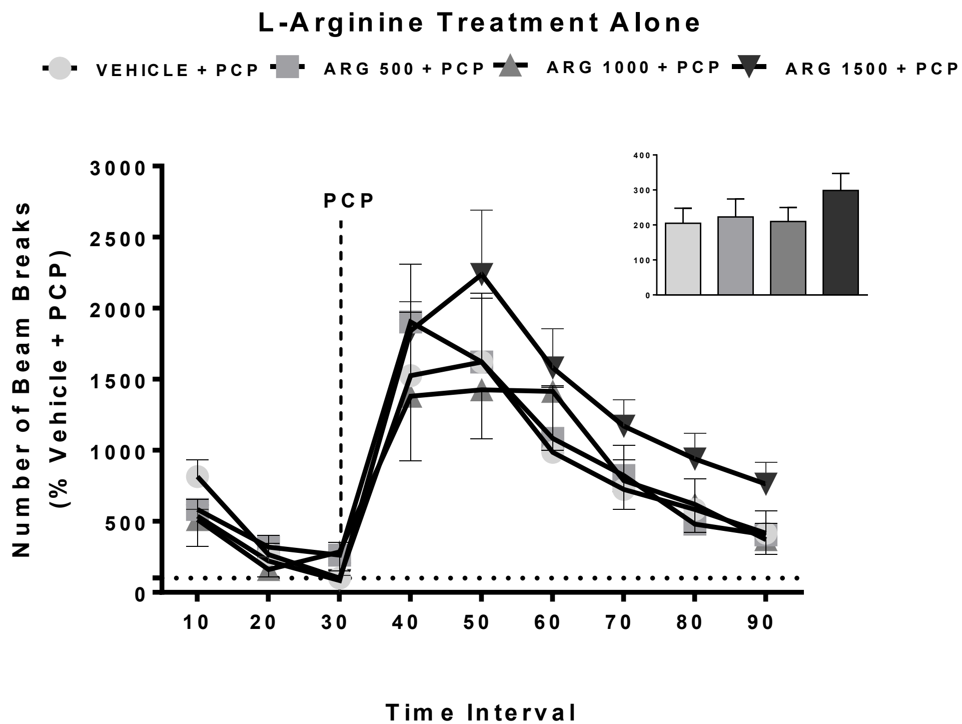

Figure 6-3. Effect of Arg (500, 1000, 1500mg/kg) on PCP (5mg/kg)-induced hyperlocomotion. Results are expressed as mean±SEM % (vehicle + PCP) treatment group of total number of beam breaks at each time interval tested. **Inset:** total distance moved post-PCP administration expressed as mean±SEM. n=6 per treatment group.

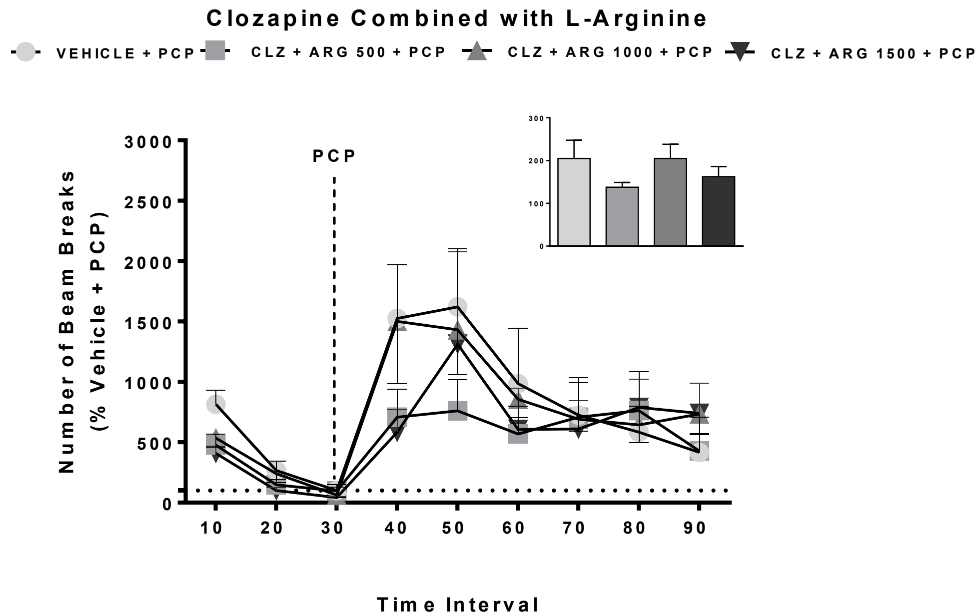

Figure 6-4. Effect of Arg (500, 1000, 1500mg/kg) combined with clozapine (10mg/kg) on PCP (5mg/kg)-induced hyperlocomotion. Results are expressed as mean±SEM % (vehicle + PCP) treatment group of total number of beam breaks at each time interval tested. **Inset:** total distance moved post-PCP administration expressed as mean±SEM. n=6 per treatment group.

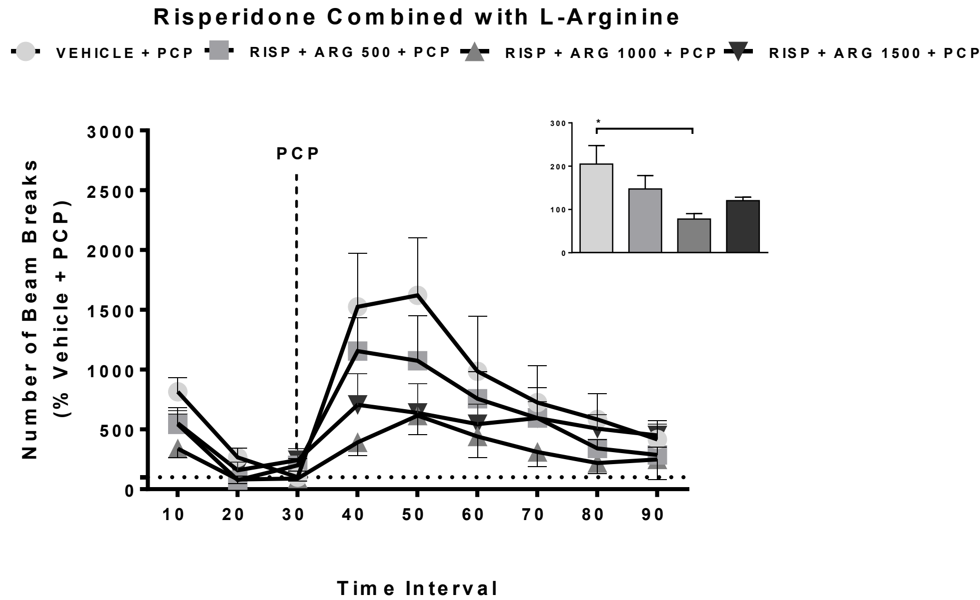

Figure 6-5. Effect of Arg (500, 1000, 1500mg/kg) combined with risperidone (1mg/kg) on PCP (5mg/kg)-induced hyperlocomotion. Results are expressed as mean±SEM % (vehicle + PCP) treatment group of total number of beam breaks at each time interval tested. **Inset:** total distance moved post-PCP administration expressed as mean±SEM. n=6 per treatment group. *denotes significant differences (p<0.05) between vehicle + PCP group and the risperidone + Arg (1000mg/kg) + PCP treatment group as assessed by analysis of simple main effects with a Bonferonni correction for multiple comparisons.

6.4 DISCUSSION

In agreement with previous findings, PCP induced a number of complex behaviours in the animals including hyperlocomotor activity, increased speed of movements, stereotyped behaviours such as head-weaving and head-bobbing, back-peddling, and circling behaviours (Fessler *et al.* 1980; Castellini and Adams 1981b; Moghaddam and Adams 1998). Phencyclidine-induced hyperlocomotor activity peaked within 10-20 minutes of administration (between the 40 and 50 min time intervals tested), and increased the number of photo-beam breaks measured within the first 10 minutes of the injection. As predicted, the use of clozapine at 10mg/kg and risperidone at 1mg/kg alone were both able to significantly attenuate the locomotor effects of PCP within the first 10 min of administration, reducing beam-breaks by 72% and 59% respectively as compared to the saline-treated animals. This effect has been demonstrated in a number of other studies investigating clozapine's (Freed *et al.* 1984; Maurel-Remy *et al*. 1995) and risperidone's (Maurel-Remy *et al*. 1995) effect on PCP-induced locomotor activity in animals.

The findings reported here suggest that the administration of Arg alone (at all of the 3 doses tested) had no significant effect on PCP-induced hyperlocomotor activity. Of interest, Arg did increase the total number of beam breaks measured in the Arg 1500mg/kg + PCP treatment group as compared to the vehicle + PCP treatment group. Hyperlocomotor activity was also marginally increased when Arg was combined with clozapine (as compared to clozapine treatment alone). Previous studies have demonstrated enhanced locomotor effects in PCP-treated animals by the use of NOS inhibitors (Noda *et al.* 1995; Noda *et al.* 1996; Bujas-Bobanovic *et al.* 2000b), and others have used NO donors to antagonize locomotor effects in PCP and MK-801 in animal models (Bujas-Bobanovic *et al.* 2000a; Pitsikas *et al.* 2006; Pitsikas *et al.* 2009). The increase of the substrate Arg availability to the nNOS enzyme in theory may regulate the production of NO and perhaps be able to reverse the downstream NO signaling deficit induced by

the NMDA receptor blockade. As demonstrated in a similar experiment, Noda *et al*. (1995) found that the enhancing effects of the NOS inhibiting drug NG-nitro-L-arginine methyl ester (L-NAME) on PCP (3 mg/kg)-induced hyperlocomotion were significantly prevented by Arg administered at 1000mg/kg i.p. These findings suggest that Arg may have attenuated the hyperlocomotor effects of PCP in that experiment by increasing the generation of endogenous NO (Noda *et al.* 1995).

The ability of PCP to stimulate locomotor activity is used as a behavioural index of the drug to potentiate psychosis in humans (Adams and Moghaddam 1998; Castellani and Adams 1981b; Steinpreis and Salamone 1993). Hyperlocomotion generated in the animals from PCP most likely reflects enhanced presynaptic prefrontal cortex and mesolimbic dopamine release (Doherty *et al*. 1980; Carboni *et al*. 1989; Steinpreis and Salamone 1993; Hondo *et al*. 1994; Adams and Moghaddam 1998). The release of dopamine may involve an increase in synaptic availability of Glu at non-NMDA receptors as studies have also reported increases in Glu concentrations following administration of PCP in animals (Liu and Moghaddam 1995; Moghaddam *et al*. 1997). Also, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate receptor antagonists have been able to reduce hyperlocomotion following administration of other NMDA receptor antagonist drugs such as dizocilpine and MK-801 (Hauber and Anderson 1993; Willins *et al*. 1993).

The results found in the experiments here are consistent with results from the clinical trial (discussed in Chapter 2) where we also found no significant changes in psychotic symptoms measured by the Positive and Negative Syndrome Scale (PANSS) when augmenting antipsychotic treatment with Arg in patients with schizophrenia. Of interest, the overall reduction of PCP-induced locomotor activity was more effective with the risperidone + Arg treatment combination than the clozapine + Arg treatment combination, which is contrary to what was predicted in Chapter 2. The greater reduction in PCP-induced hyperlocomotion may be directly influenced by risperidone

being a much stronger dopamine receptor-2 (D2)-blocking agent as compared to clozapine and having a greater ability to antagonize D2 receptors within the mesolimbic dopaminergic circuits activated by increased dopamine release by PCP.

The lack of behavioural effect of Arg may also be directly related to PCP's ability to bind to an inhibitory site within the ion channel pore of the NMDA receptor and reduce Ca^{2+} entry into the cell (Zukin and Zukin 1979). The nNOS enzyme is dependent on Ca^{2+} binding to calmodulin for activation exclusively when the neuron depolarizes and Ca^{2+} enters the cell. Calmodulin binding enhances the electron transfer in the reductase domain and increases nNOS activity and NO production (Zhou and Zhu 2009). At the 5mg/kg dose of PCP used in this study, PCP may have reduced nNOS activity and the ability of the NMDA receptor to allow enough Ca^{2+} to enter the cell to activate the nNOS enzyme.

The results discussed here suggest that treatment with the NO precursor Arg is not an effective augmentation strategy when treating psychoses. The modulation of NMDA receptor function and clozapine's effect on NMDA mechanisms coupled with the effect of increased Arg substrate availability to the nNOS and NO signaling cascades was not able to preferentially reduce hyperlocomotion in PCP-treated rats. The anxiolytic benefits of Arg in schizophrenia reported in Chapter 2 may be better modeled in future experiments by the use of an anxiolytic paradigm such as the Elevated Plus-Maze test to replicate these particular effects of Arg in animals.

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CHAPTER 7.

GENERAL DISCUSSION

7.1 GENERAL DISCUSSION

There has not been any human or animal research to date that has investigated the direct relationship of L-arginine (Arg)-induced neuronal NO production in the brain to improve antipsychotic effectiveness in schizophrenia. L-Arginine used at 6g per day proved to be a safe and effective augmenting strategy for improving intrinsic residual anxiety in the illness. Results from the clinical trial were able to demonstrate the anxiolytic effect of Arg treatment when augmented with antipsychotic medication. This was an unexpected but welcomed finding since many patients with schizophrenia continue to experience persistent psychic anxiety that is an integral part of their illness despite compliance to adequate doses of antipsychotic medications.

It was also of interest that 80% of responders were also on clozapine, the drug of choice for treatment-resistant illness (Kane *et al.* 1988; Lieberman *et al.* 1994; Meltzer 1997). Clozapine's effect on *N*-methyl-D-aspartate (NMDA) receptor functioning coupled with the effect of increased Arg substrate availability to the neuronal nitric oxide synthase (nNOS) enzyme and NO signaling cascades were mechanisms that were considered that may have improved NMDA receptor functioning and promoted a response to Arg treatment in the participants.

Patients who responded to Arg therapy had reduced anxiety symptom scores that were measured within the general psychopathology subscale of the Positive and Negative Syndrome Scale (PANSS) as compared to those patients on placebo. The mechanisms that are involved in the anxiolytic effect of Arg remain unknown, however an increase in endogenous central NO production generated by increasing Arg availability to the nNOS enzyme may have initiated cyclic guanosine monophosphate (cGMP)-mediated signaling cascades and contributed to the anxiolytic effect.

Nitric oxide has been investigated for its role in modulating neurotransmission in animal models. The effect of a variety of NO-producing drugs in microdialysis experiments as well as in synaptosome preparations have confirmed that the release and also the reuptake of several neurotransmitters, including catecholamines, acetylcholine, and excitatory and inhibitory amino acids, may be regulated by NO (Pogun and Kuhar 1994; Lonart *et al.* 1992; Guevera-Guzman *et al.* 1994; Prast and Philippu 2001). Increased glutamate (Glu) release from presynaptic glutamatergic neurons mediated by Arg and NO production may have enhanced the activation of post-synaptic NMDA receptors on gamma-aminobutyric acid (GABA)-producing neurons and contributed to the anxiolytic effect of Arg. The administration of Arg in previous studies has been shown to increase brain GABA concentrations and also reduce the activity of gamma-aminobutyric acid transaminase (GABA-T), the enzyme that degrades GABA to succinic semialdehyde (Jayakumar *et al.* 1999; Paul and Jayakumar 2000). The anxiolytic effect of Arg may have also been related to the modulation of the tonic concentrations of serotonin and norepinephrine in the brain. In animal models, it has been reported that increased levels of NO can block the reuptake of monoamines (Pogun and Kuhar 1994; Lonart *et al.* 1992; Kiss and Vizi 2001).

Finally, the generation of agmatine (the intermediate that is produced by the decarboxylation of Arg via arginine decarboxylase [ADC]) may have also contributed to the anxiolytic effect of Arg supplementation. Agmatine has been shown to have both antidepressant (Zomkowski *et al*. 2002) and anxiolytic (Aricioglu and Altunbas 2003; Lavinsky *et al*. 2003; Gong 2006) properties in animal models, and concentrations of agmatine have been found to be elevated in human plasma (Halaris 1999) and increased in post-mortem brain tissue samples in untreated patients with depression (Bernstein 2012). The increase in both plasma and brain concentrations of agmatine as well as upregulation of agmatinase (AGMAT) in hippocampal tissue samples in subjects with affective disorders may be homeostatic

response mechanisms that support the theory that agmatine may be an endogenous antidepressant in the illness (Bernstein 2012). Although we did not measure agmatine as part of the amino acid assay in this thesis, both human plasma and whole brain levels of agmatine in animal samples following Arg supplementation would be worth investigating to confirm if the concentration of agmatine is indeed increased following Arg supplementation.

The addition of Arg to antipsychotic drugs also proved to be a safe augmenting strategy in the illness. L-Arginine did not exacerbate any of the hemodynamic side-effects, neurological side-effects, or metabolic side-effects that were already present as a result of ongoing antipsychotic therapy. There was no significant weight gain or change to body mass index (BMI) following treatment with Arg. It is of interest that Arg has been investigated for its specific effect on adiposity in both animal and human studies, and the production of NO does play an important role in fat metabolism (Fu *et al*. 2005; Lucotti *et al.* 2006). L-Arginine has been studied for its ability to reduce body weight, fat mass, waist circumference, and other metabolic markers in animal models of obesity and also in human studies with type 2 diabetes mellitus (Fu *et al*. 2005; Lucotti *et al*. 2006). The consideration of augmenting agents to antipsychotic drugs that will not exacerbate weight gain, a side-effect that is linked to an increased risk for diabetes and cardiovascular disease that is common in schizophrenia should be a desirable adjunctive strategy to antipsychotic therapy in the illness (Allison *et al*. 1999).

7.2 SUMMARY OF NOVEL FINDINGS

In terms of Arg metabolism, we have confirmed that in the post-absorption phase of Arg pharmacokinetics, the concentration of Arg in human plasma is physiologically maintained at trough conditions. A novel finding was that plasma glutamine (Gln) levels decrease following long-term supplementation of Arg. To our knowledge, this finding has never been

reported in human studies until now. This finding is most likely related to the differential function of the hepatocytes of the liver (Jungermann and Kietzmann, 1996; Gebhardt 1992). Following oral dosing, Arg is absorbed from the intestine and transported to the portal vein of the liver via the mesenteric veins. The cells surrounding the portal vein (periportal cells) contain an increased number of urea cycle enzymes and favour urea synthesis particularly at high ammonia concentrations (Haussinger 1983; Gaasbeek *et al.* 1984; Miyanaka *et al.* 1998). Cells found closer to the central vein are part of the perivenous zone and favour Gln synthesis (Haussinger 1983; Gebhardt and Mecke 1983; Haussinger and Gerok 1983). Although we did not measure arginase activity directly, it is speculated that Arg supplementation in this study may have activated arginase enzymes within the periportal area of the liver acinus and reduced Gln production as an adaptive response to high ammonia concentrations.

It is unknown if long-term oral Arg supplementation would also reduce brain Gln concentrations. Whole brain Gln levels measured in both dose- and time-response preclinical experiments following acute Arg administration (discussed in Chapter 4) were no different than Gln levels measured in vehicle-treated animals. In the brain, circulating Arg is able to enter into both neurons and glia through system y^+ active transport carriers (Oldendorf and Szabo 1976; Closs and Mann 1999; Malandro and Kilberg 1996). Once inside the cell, the majority of Arg is catabolized by the enzyme arginase to produce ornithine (Orn) and urea (Wu and Morris 1998; Morris 2004). Ornithine does have a close metabolic relationship with glutamate (Glu) as it is metabolized to Glu by ornithine transaminase (ORN-T). In terms of the effect on brain Gln production, it is unknown whether Arg-induced increases in Orn would also increase Glu synthesis via ORN-T activation. Subsequent downstream influence on Gln synthetase activity within glial cells or influence on glutaminase once transported back inside the neuron as a result of increased Glu levels are also unknown. It has been suggested that brain Glu and Gln levels probably have no relationship to plasma Glu and Gln

levels. A proton magnetic resonance spectroscopy (^{1}H-MRS) study in healthy volunteers has suggested that peripheral (plasma) and central (medial prefrontal cortex) Glu and Gln levels have no correlation between each other (Shulman *et al.* 2006). To our knowledge, there have been no clinical imaging studies that have measured changes in any brain amino acid levels following oral Arg supplementation.

Preclinical studies to examine central Arg metabolic pathways in the brain were also intriguing. We were able to confirm in both dose- and time-response experiments that Arg administered i.p. does cross the BBB (measured within 15 minutes of administration). Intracellular pools of Arg within neurons and glia cells in the brain are dependent on local protein breakdown within the cells or by active transport mechanisms that can carry circulating plasma Arg across the BBB and across plasma cell membranes. Metabolic enzymes that use Arg as a substrate have been localized within neurons that also contain nNOS, suggesting that Arg biosynthesis and catabolism are important mechanisms required for normal brain metabolism as well as NO production (Nakamura *et al.* 1991; Braissant *et al.* 1999).

The competing actions of NOS and arginase for Arg substrate binding that were indirectly determined by measuring the concentrations of Orn and citrulline (Cit) following dose- and time-response studies were also interesting. The immediate potentiation of whole brain Cit levels within 15 minutes of Arg administration confirmed that a much lower concentration of Arg is required to achieve half-saturation of the NOS enzymes than the concentration of Arg that is required for the arginase enzymes (Griffith and Stuehr 1995). Although not measured directly in this experiment, it is assumed that NO was produced in the brain quickly following Arg supplementation because of the increase in production of Cit (a byproduct of the NO pathway).

This thesis was also able to support a genetic influence on brain development in schizophrenia and may add further insight with regard to NMDA receptor functioning and neurodevelopmental factors in the illness

(Chapter 5). An Neuronal Period-Arylhydrocarbon Nuclear Translocator (ARNT)-Single-minded (PAS) domain containing 3(*NPAS3*) gene variant previously associated with schizophrenia was found in two of the clinical trial participants. The biosynthesis or functioning of NPAS3 protein may have been altered in early brain development in the carriers of this variant, a process that may have impacted the female carrier in this study which has also been reported previously in females with schizophrenia (Wong *et al.* 2013).

The reduction of NPAS3 protein function in both of the patients with the *NPAS3* variant may have also impacted the integrity of the Glu NMDA receptor and response to Arg therapy. The postsynaptic density (PSD-95) protein is the primary protein that determines the functional connection and integrity between the Glu NMDA receptor and the NO system (Brenman *et al.* 1996). In *NPAS3* knockout mice, reduced expression of the PSD-95 has been found in the frontal-parietal cortex of these animals (Brunskill *et al.* 2005). Therefore, a deficient PSD-95 protein may explain the outcome of the effects of Arg therapy in the two *NPAS3* carriers, both of whom had no response to the treatment.

Genotyping of catechol-O-methyltransferase (*COMT*), a schizophrenia-associated gene, in this clinical sample of patients also revealed that no one had 22q11 deletion syndrome. The *COMT* genotype frequencies found in this clinical trial were as would be predicted and did not deviate from Hardy-Weinberg equilibrium. In this study, the gene-gene interaction of *NPAS3* and *COMT* and their combined impact on treatment response to Arg therapy was not significant. Genotyping and identification of the *NPAS3* c.1654G>C variant with an added *COMT* homozygous *Val* allele expression (thought to be detrimental in schizophrenia) was a clinical consideration that in combination did not predict worse clinical outcomes for patients. None of the study participants had combined *NPAS3* and *COMT* variants of interest. A summary of these findings is illustrated in Figure 7-1.

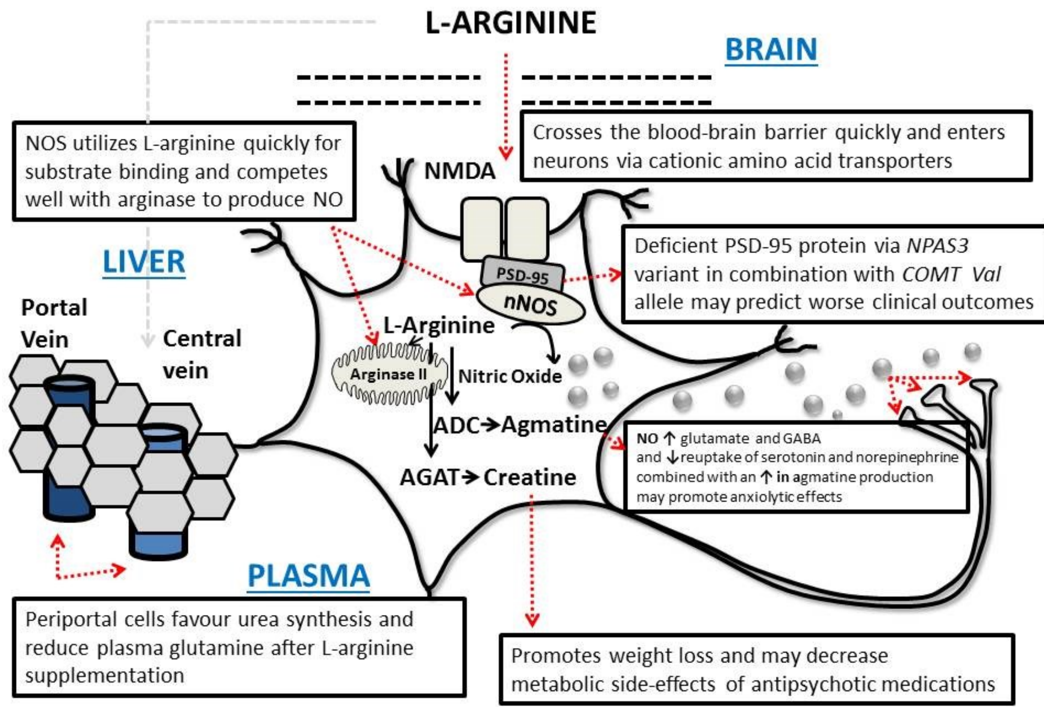

Figure 7-1. A summary of the putative therapeutic effects and the central and peripheral metabolic mechanisms of L-Arginine supplementation in both human plasma and rat brain.

Finally, the animal behavioural studies conducted in Chapter 6 were able to confirm the utility of phencyclidine (PCP) as a pharmacological model of psychosis as well as the ability of clozapine and risperidone to reduce PCP-induced hyperlocomotor activity. The ability of PCP to stimulate locomotor activity is used as a behavioural index of the drug to potentiate psychosis in humans (Adams and Moghaddam 1998; Castellani and Adams 1981b; Steinpreis and Salamone 1993). The administration of
Arg alone (at all of the 3 doses tested) had no significant effect on hyperlocomotor activity and it also did not further enhance the locomotor-reducing effects of clozapine or risperidone. L-Arginine was able to reduce the total distance moved in the animals when combined with risperidone. These findings were consistent with results from the clinical trial (discussed in Chapter 2) where we also found no significant changes in psychotic symptoms measured by the PANSS when augmenting antipsychotic treatment with Arg in patients with schizophrenia.

The lack of behavioural effect of Arg may have also been related to PCP's ability to bind to an inhibitory site within the ion channel pore of the NMDA receptor and reduce Ca^{2+} entry into the cell (Zukin and Zukin 1979). The nNOS enzyme is dependent on Ca^{2+} binding to calmodulin for activation. These results suggest that treatment with the NO precursor Arg is not an effective augmentation strategy to improve psychoses; however it does have anxiolytic properties (as reported in Chapter 2). The modulation of NMDA receptor function and clozapine's effect on NMDA mechanisms coupled with the effect of increased Arg substrate availability to the nNOS and NO signaling cascades was not able to preferentially reduce hyperlocomotion in PCP-treated rats. The anxiolytic benefits of Arg in schizophrenia may be better modeled in future experiments by the use of an anxiolytic paradigm such as the Elevated Plus-Maze test to evaluate this effect of Arg in animals.

7.3 FUTURE DIRECTIONS

Drug targets specific to the glutamate-NO pathway may offer a promising new approach to treatment in schizophrenia and be of therapeutic benefit to those that have developed resistance to current available treatments. Treatment-resistant schizophrenia is common. In a meta-analysis of treatment response, 60% of patients failed to achieve a response after 23 weeks on drug therapy (Hegarty *et al.* 1994). For those patients who do not achieve adequate symptom control or maintenance of response to dopamine receptor-2 (D2)-blocking agents, clozapine is the drug of choice (Kane *et al.* 1988; Lieberman *et al.* 1994; Meltzer 1997). Treatment-resistance can be identified much earlier than the time it takes for consideration of clozapine therapy and it is not always associated with chronic pathology. Early non-response to antipsychotic treatment (within two weeks) has been shown to be a robust predictor of subsequent non-response (Correll *et al.* 2003; Leucht *et al*. 2007; Kinon *et al.* 2008). Evaluating and identifying non-responding patients as early as two weeks into antipsychotic treatment may receive benefit from an early start of NO-based therapy. This would help to avoid multiple unsuccessful treatments, including clozapine, and the likelihood to develop antipsychotic drug associated side-effects.

The potential clinical advantage of using an amino acid such as Arg to augment antipsychotic therapy in schizophrenia is that it has proven to be a safe strategy (in the clinic and in an animal model of psychosis) for residual anxiety with few side-effects reported. Much of the side-effect burden of the antipsychotic drugs is caused by reduced signaling through the D2 receptor. An augmenting agent like Arg that interacts (intracellularly) with Glu NMDA receptors may be a clinical advantage as a non-dopaminergic approach to treatment. Arginine is able to cross the BBB (within 15 minutes of administration as confirmed in an animal model in this thesis) and it has also been previously shown to influence brain activation and deactivation in a pharmacologic magnetic resonance imaging (phMRI) study in human healthy

volunteers (within 30 minutes following a 0.5mg/kg infusion) (Lees 2007). This indicates that Arg is probably necessary to maintain normal brain functioning and may modulate central nervous system (CNS) function in brain regions that have not only been directly linked to schizophrenia but also have been compromised by reduced Glu NMDA receptor functioning in the illness (Lees 2007).

Although Arg was effective at reducing intrinsic residual anxiety in some patients with schizophrenia, it was also shown to have no benefit in improving these residual symptoms in others. This may be an indication that the glutamate-NO pathway is disrupted in those who had no response to Arg and that the ability to produce adequate levels of NO in the brain via an intact NMDA receptor may be impaired by the disease processes of the illness.

A major disadvantage of Arg in this clinical trial is that it did not improve the antipsychotic effects of the medications that the participants were prescribed. Positive symptoms are often the first to respond to antipsychotic treatments but often never completely resolve and can remain quite disabling in treatment-resistive patients (Kane *et al.* 1988; Lieberman *et al.* 1994; Meltzer 1997). As an augmenting treatment, Arg at 6g per day was unable to improve the positive symptoms of the illness. Although NO is thought to modulate dopamine activity in schizophrenia and play a role in the effectiveness of antipsychotic drugs (Ebstein *et al.* 1976; Lee and Kim 2008; Maia-de-Oliveira *et al.* 2012), Arg may not be an optimal NO precursor in the illness. This may be related to the high demand for Arg as a substrate for many other enzymes (eg. arginase) within other metabolic pathways in the body (Wu and Morris 1998; Morris 2004).

Arginine also demonstrated poor efficacy against the primary negative symptoms of schizophrenia. The negative symptoms usually do not respond as well to antipsychotic medications as the positive symptoms do. This indicates that these symptoms may involve additional neurotransmitters and associated pathways other than dopamine and serotonin (Tamminga *et al.* 1992; for review see Chue and Lalonde 2014). The negative symptoms of

schizophrenia also cause a significant amount of the disability that is associated with the illness. The presence and severity of negative symptoms have the greatest effect on the functional decline in the illness. Reduced emotional response, alogia, avolition, anhedonia, social isolation, and other negative features usually emerge early in the course of the illness and often persist throughout the disease. Up to 58% of patients on antipsychotic medications continue to experience negative symptoms (Bobes *et al.* 2010). Unresponsive negative symptoms are also highly correlated with the cognitive deficits of the illness (Buchanan *et al.* 1994; Buchanan *et al.* 1997; Bryson *et al.* 2001). These negative and associated cognitive symptoms continue to be the true unmet needs of schizophrenia and it is unfortunate that Arg was also not effective at addressing these features. This will continue to be the primary focus of any future augmenting therapy to antipsychotic drug treatment in the illness.

To date, both animal and human studies that have investigated the benefit of NO-based therapies in pharmacological models of psychosis and also in schizophrenia have suggest that sodium nitroprusside (SNP) may produce the best therapeutic effect (Bujas-Bobanovic *et al.* 2000; Hallak *et al.* 2013; Maia-de-Oliveira *et al.* 2014; Maia-de-Oliveira *et al.* 2015; Kandratavicius *et al.* 2015). Sodium nitroprusside is a potent nitrovasodilator drug that has been used for many years for acute hypertensive crisis. When administered, SNP reacts with oxyhemoglobin molecules within erythrocytes to form methemoglobin. This causes the SNP molecule (a sodium salt with 5 cyanide ions that are complexed to an iron molecule) to become very unstable and quickly release NO into the bloodstream (Friederich and Butterworth 1995). In comparison to other NO donor drugs, the quick release of NO seems to give SNP an advantage over other organic nitrate drugs such as glyceryl trinitrate (GTN) for the immediate production of central cGMP in preclinical models of migraine and

to also improve behavioral effects in animal models of psychosis (Berkelmans *et al.* 1989; Bujas-Bobanovic *et al.* 2000; Tassorelli *et al.* 2004; Kandratavicius *et al.* 2015).

Glyceryl trinitrate (GTN), another potent nitrovasodilator drug has also been used for many years to treat angina and other cardiac conditions including myocardial infarct and congestive heart failure. The metabolic conversion of GTN involves both enzymatic and nonenzymatic pathways that complicate its pharmacokinetic and pharmacodynamics properties (Hashimoto and Kobayashi 2003). The complex biotransformation of GTN may also affect the downstream effects of NO production. In preclinical studies that have measured the time-course of changes in cGMP production, SNP was able to induce the upregulation of cGMP within 10 minutes of administration while GTN-induced cGMP production occurred 2 hours after drug administration and only reached its maximal intensity at 4 hours post injection (Berkelmans *et al.* 1989; Tassorelli *et al.* 2004). This delayed effect on the production of cGMP may have been a limitation in the therapeutic and preventive behavioural effects measured following pre-and post-treatment with GTN as compared to effects of SNP in a ketamine rat model of psychosis (Kandratavicius *et al*. 2015).

Although Arg therapy was unable to improve the positive and negative symtoms of schizophrenia, work presented in this thesis does support the use of Arg as a safe and effective augmenting strategy that may improve intrinsic anxiety in treatment-refractory schizophrenia. Future clinical trials involving the use of NO-based therapies in schizophrenia should use NO donor drugs such as SNP that can quickly restore deficient NO concentrations and improve the multitude of behavioural domains of the illness. Sodium nitroprusside has been demonstrated to be beneficial in both early stage schizophrenia and treatment-refractory disease and did improve a spectrum of symptoms, including the positive, negative, and anxiety features of the illness (Hallak *et al*. 2013; Maia-de-Oliveira *et al*. 2014). Rapid-acting therapies that can control acute psychotic episodes and

improve treatment resistance within a reasonable timeframe are needed for the treatment of schizophrenia. Nitric oxide donors may offer a well-tolerated augmenting strategy for early treatment, neuroprotection, and prevention of further neurobiological and functional decline in the illness. It will be exciting to see the full potential of this molecular target to improve treatment for those with this debilitating disease.

7.4 REFERENCES

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