

ACUTE CARDIOMETABOLIC SIDE EFFECTS OF CLOZAPINE IN RODENTS

by

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Abstract

Clozapine (CLZ) is considered the treatment of choice for treatment resistant schizophrenia. Although highly efficacious, the widespread use of CLZ is limited by metabolic side effects, fatal agranulocytosis and cardiovascular complications such as myocarditis via unknown mechanisms. As the autonomic nervous system (ANS) is known to regulate metabolic and cardiovascular function, we sought to assess the involvement of the ANS in the cardiometabolic effects of CLZ in rodent models. Glucose dysregulation from acute CLZ treatment was evaluated using the hyperinsulinemic euglycemic clamp (HIEC) and the intraperitoneal glucose tolerance test (IGTT). Plasma catecholamine (CAT) levels were measured using high performance liquid chromatography (HPLC) to determine autonomic activity. We also assessed the metabolic liability of the principal metabolite of CLZ, norclozapine (NOR), and compared the results to its parent compound. In a separate series of experiments, we measured cardiovascular parameters in response to CLZ in a novel rodent model of CLZ-induced tachycardia. We attempted to reverse CLZ-induced tachycardia with mecamylamine (MEC), a ganglionic blocker, and propranolol (PRO), a β -adrenoceptor blocker. Lastly, we performed a study to evaluate the use of adrenoceptor ligands in treating CLZ-induced glucose dysregulation.

Our results indicate NOR does not induce metabolic dysregulation at the same severity as CLZ, despite substantial retention of NOR in plasma. NOR induced insulin resistance but not glucose intolerance at the highest dose compared to vehicle. CLZ induced tachycardia and depressor effects immediately following injection. MEC and PRO reversed the tachycardia, where MEC caused significant depressor effects. The effects were not noted

with PRO. There was a significant elevation in plasma NE levels from CLZ treatment which was reversible with MEC. Our pilot study showed β -adrenoceptor blockers reversed insulin resistance associated with CLZ, whereas α -adrenoceptor antagonists had no effect. The results suggest both β_1 - and β_2 -adrenoceptor blockade contribute to insulin sensitivity. In conclusion, the present series of experiments indicate the ANS mediates CLZ's cardiometabolic side effects and warrants further studies focusing on autonomic dysregulation as a potential target for treating these adverse effects.

Lay summary

Clozapine is an antipsychotic used to treat schizophrenia. It is particularly effective in patients who do not respond adequately to conventional treatment. However, it is often used as a last resort because of potentially fatal side effects, such as heart failure and lowered white blood cell count. Clozapine is also known to cause obesity and diabetes. At present, it is unknown how clozapine causes these serious side effects. Therefore, it is extremely important to understand how clozapine acts in the body. The goal of this thesis is to replicate clozapine's side effects in animals and to try to reverse these effects by introducing appropriate treatments. Animal models are essential in studies that require testing of novel treatments that will ultimately aid in the development of new treatments for human use. Our results will help with the safe use of clozapine in patients who have no other choice of treatment.

Preface

This dissertation contains excerpts that have been published in peer-reviewed journals.

Individual contributions toward these publications are indicated below.

Chapter 1 – Introduction

Yuen JWY, Kim DD, Procyshyn RM, White RF, Honer WG, Barr AM (2018) Clozapine-Induced Cardiovascular Side Effects and Autonomic Dysfunction: A Systematic Review. *Front Neurosci* 12:203.

J.W.Y.Y. conducted the literature search, analyzed the search results, designed all figures and tables and wrote the manuscript. D.D.K. assisted in revising the manuscript. R.M.P. and W.G.H. revised the manuscript. A.M.B. wrote the manuscript.

Chapter 2 – Acute metabolic side effects of clozapine and norclozapine in rodents

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Chapter 4 – Evaluation of the acute cardiovascular side effects of clozapine in adult rats

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List of abbreviations

5-HT	serotonin receptor
5-HT _{2A} HT ₂	serotonin 2A receptor
ACh	acetylcholine
AMPK	adenosine monophosphate-activated protein kinase
ANS	autonomic nervous system
ATEN	atenolol
AUC	area under the curve
BP	blood pressure
BPRS	Brief Psychiatric Rating Scale
BUT	butoxamine
Ca ²⁺	calcium
CAD	coronary artery disease
CAT	catecholamine
CAT	catecholamine
CLZ	clozapine
COMT	catechol- <i>o</i> -methyltransferase
CRP	C-reactive protein
CVD	cardiovascular disease
D _{2/3}	dopamine 2/3 receptor
DA	dopamine
DHPG	dihydroxyphenylglycol
E	epinephrine
EPS	extrapyramidal symptoms
FGA	first generation antipsychotic
GADPH	glyceraldehyde-3 phosphate dehydrogenase
GIR	glucose infusion rate
H ₁	histamine H ₁ receptor
HDL	high density lipoprotein
HDL	high density lipoprotein
HIEC	hyperinsulinemic euglycemic clamp
HPLC	high performance liquid chromatography
HR	heart rate
i.p.	intraperitoneal
IDA	idazoxan
IGTT	intraperitoneal glucose tolerance test
IL-6	interleukin 6
M ₁₋₅	muscarinic receptor M ₁₋₅
MEC	mecamylamine
MHPG	methoxyhydroxyphenylglycol
nAChR	nicotinic acetylcholine receptor
NE	norepinephrine
NET	norepinephrine transporter
NMN	normetanephrine

NO	nitric oxide
NOR	norclozapine
NTS	nucleus tractus solitarius
OGTT	oral glucose tolerance test
PEG	polyethylene glycol
PMNT	phenylethanolamine methyltransferase
PNS	parasympathetic nervous system
PRA	prazosin
PRO	propranolol
RVLM	rostral ventrolateral medulla
SGA	second generation antipsychotic
SNS	sympathetic nervous system
TD	tardive dyskinesia
TNF- α	tumor necrosis factor- α
VLDL	very low density lipoprotein
$\alpha_{1,2}$	alpha 1,2 adrenoceptor
$\beta_{1,2}$	beta 1,2 adrenoceptor

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Dedication

I dedicate this dissertation in loving memory to my mother, Mrs. Susanna Tze Kuen Yuen. You were my first and greatest teacher. Thank you for everything you've given me. I miss you.

“One never notices what has been done; one can only see what remains to be done.”

- Marie Skłodowska Curie

Chapter 1: Introduction

1.1 Schizophrenia

Schizophrenia is a devastating mental illness marked by cognitive dysfunction as well as positive and negative symptoms. The diagnosis of schizophrenia is made in the presence of 2 or more of the following symptoms: delusions, hallucinations, disorganized speech, disorganized or catatonic behavior and negative symptoms (American Psychiatric Association, 2013). The symptoms must persist for a minimum of 6 months, of which the symptoms are in the active-phase for at least 1 month (American Psychiatric Association, 2013). Positive symptoms refer to characteristics present in individuals with schizophrenia that are absent in the general population. Examples of positive symptoms include hallucinations, delusions and disorganized thought and behavior. Negative symptoms are characteristics absent in patients with schizophrenia that would otherwise be present in the general population. Examples include the lack of speech and motivation, anhedonia, and attention deficits (Andreasen and Olsen, 1982). In 2004, there was an estimated 234 305 people with schizophrenia in Canada, with an associated economic burden of \$6.85 billion mainly attributed to decreased productivity (Goeree et al., 2005).

Early mortality is a major concern for persons living with schizophrenia. Suicidality rate is markedly higher in affected individuals, with lifetime risk of suicide at an estimated 5% (Palmer et al., 2005; Hor and Taylor, 2010). In a systematic review investigating the suicidal rate amongst patients with schizophrenia, Hor and Taylor identified unemployed, young males with higher levels of education as having the highest risk of suicide (2010).

Other strongly associated factors include depression, positive symptoms, family history, history of suicide attempt and substance abuse (Hor and Taylor, 2010).

Apart from suicidality, lifestyle is also a known contributor, with smoking, unhealthy diet and reduced physical activity identified as factors that potentially exacerbate premature mortality (Olfson et al., 2015). Cardiovascular disease (CVD) is consistently identified as one of the leading causes of death in patients with schizophrenia (Casey et al., 2004; Olfson et al., 2015). Risk factors for CVD include hypertension, diabetes mellitus, obesity, hyperlipidemia and coronary heart disease (Olfson et al., 2015). While suicide risk is largely reduced by treatment with antipsychotics, the use of antipsychotics is associated with increased cardiovascular mortality involving cardiomyopathy, myocarditis and sudden cardiac death. In particular the second generation antipsychotics (SGAs) have the greatest risk of leading to cardiometabolic sequelae. The SGAs are increasingly prescribed due to their minimal risk for extrapyramidal symptoms (EPS), better efficacy and safety profiles, and potentially therapeutic effects on cognition (Meltzer, 2004). However, any benefits of prescribing SGAs must be weighed against the range of cardiovascular side effects associated with SGA use (Leung et al., 2012).

1.1.1 Pathogenesis of schizophrenia

The pathogenesis of schizophrenia is complex and varies in both severity and time course of deterioration among patients (Lieberman et al., 2001). The prodromal phase marks the beginning of the clinical course of schizophrenia, although the symptoms and behaviors observed within this phase (e.g. illusions, anxiety, lack of concentration, social withdrawal)

lack diagnostic value (Lieberman et al., 2001). First episode schizophrenia follows, with official diagnosis occurring when the patient requires medical treatment, after approximately 1 year of psychotic symptoms and 3 years from the first occurrence of prodromal symptoms. While patients may recover from this first episode of schizophrenia, persistent relapses of psychotic symptoms and failure to respond to treatment eventually lead to chronic illness.

1.1.2 Structural anomalies of schizophrenia

Progression of illness is believed to be the result of aberrant synaptogenesis, maturation of neuronal connections and loss of cellular function of cortical-limbic striatal neurons (Lieberman et al., 2001). Growing evidence obtained via brain imaging has revealed characteristic changes in the neuroanatomy of patients with schizophrenia. Namely, patients with schizophrenia have a significant decrease in hemispheric volume and left cerebral ventricular enlargement in comparison to age-matched controls (DeLisi et al., 1997). The latter finding has been corroborated by numerous studies and is considered one of the more established neuroanatomical changes associated with schizophrenia (Shenton et al., 2001; Del Re et al., 2016). Other less consistent changes include whole brain volume reduction, whole temporal lobe volume reduction, frontal lobe volume reduction, parietal lobe volume reduction and asymmetry, cerebellum hemisphere asymmetry and volumetric changes in subcortical structures (Shenton et al., 2001).

Progressive changes in the brain anatomy of patients with schizophrenia are linked to multiple clinical features of schizophrenia (Andreasen et al., 1982; Sanfilippo et al., 2000; Mathalon et al., 2001; Sigmundsson et al., 2001; Ho et al., 2003; Pantelis et al., 2005).

Ventricular enlargement has been associated with negative symptoms and cognitive deficits in patients with schizophrenia, while patients without ventricular enlargement predominantly displayed positive symptoms (Andreasen et al., 1982). A 7 year longitudinal imaging study identified volumetric changes in the frontotemporal regions as key areas in correlation to clinical symptom severity as measured by the Brief Psychiatric Rating Scale (BPRS) and duration of hospitalization between scans (Mathalon et al., 2001). Cerebrospinal fluid volume expansion in frontal sulcus was correlated to higher BPRS global and positive scores, and increased hospitalization time between scans (Mathalon et al., 2001). Prefrontal sulcus enlargement and reduction in gray matter volume, and gray matter decline in the posterior temporal lobe were correlated with higher BPRS negative symptom scores (Mathalon et al., 2001). However, results from imaging studies have been inconsistent in the past. For instance, in another study, prefrontal and temporal gray matter volume reduction has been suggested to be a structural anomaly associated with schizophrenia and unrelated to clinical symptoms (Sanfilipo et al., 2000). Patients displaying negative symptoms for schizophrenia had anatomical abnormalities in the white matter tracts associated with neocortical and limbic regions in the left hemisphere (Sigmundsson et al., 2001). While prefrontal white matter volume reduction was statistically insignificant in patients, white matter decrements in this region are strongly associated with negative symptoms (Sanfilipo et al., 2000). When patients are divided into sub-populations depending on progression of cognitive and social deterioration, it was found patients with poor outcome displayed greater deficits in prefrontal white matter volume (Dusi et al., 2017). Reductions in frontal lobe volume are evident in patients with schizophrenia compared to healthy controls over time, where white matter volume was significantly decreased (Ho et al., 2003). Lateral ventricle enlargement and

reduction in frontal lobe gray matter volume were associated with poor outcome in patients (Ho et al., 2003). More recently, ventricular enlargement was found to be inversely related to volumetric changes in the central corpus callosum, although only the former was correlated with lower global functioning (Del Re et al., 2016). Furthermore, chronic treatment with antipsychotics is associated with global white matter volume reductions and thus may exacerbate brain tissue volume deficits in patients with schizophrenia (Ho et al., 2011). Another study found global gray and white matter deficits in patients with chronic schizophrenia and these were negatively correlated with antipsychotic exposure (Torres et al., 2016).

Collectively, the abovementioned evidence points towards enlarged ventricles and volumetric changes in prefrontal and medial temporal lobe as consistently reported structural abnormalities of schizophrenia. It has been proposed these structural anomalies occur before birth and subsequently renders patients susceptible to schizophrenia during early adulthood (Weinberger, 1987). One concern with this “neurodevelopmental” proposal is that the specific deficits are assumed to be present prior to birth and remain stagnant for the remainder of life, thus progressive changes during the disease process are not accounted for (Pantelis et al., 2005). A newer view is that early neurodevelopmental insults increase susceptibility to neuronal deficits later in life, and in combination with specific factors, will lead to onset of schizophrenia. Specifically, there is initial loss of gray matter volume and dysfunction of the prefrontal cortex; secondly, these anomalies in the presence of causative factors including substance abuse, an unhealthy lifestyle, stress and hypothalamic pituitary adrenal axis dysregulation contributes to progressive neurodegeneration in the medial temporal and orbital prefrontal regions, and ultimately leads to full disease onset (Pantelis et

al., 2005). Indeed, working memory and executive function are both impaired when prefrontal cortical activity is reduced in patients with schizophrenia (Tan et al., 2007).

Clinical deterioration coupled with MRI imaging studies showing progressive, heterogeneous changes in brain volume suggest a complex interplay between genetic, environmental and neurobiological factors in the pathogenesis of schizophrenia (DeLisi et al., 1997; McGrath et al., 2003; Fatemi and Folsom, 2009; Davis et al., 2016; Lieberman et al., 2019). Complications in pregnancy and birth possibly affect fetal brain development and thereby increase the risk of developing schizophrenia later in life (Geddes et al., 1999; Cannon et al., 2002; Piper et al., 2012). Additional factors linked to risk of schizophrenia include paternal age (Malaspina et al., 2001; Malaspina et al., 2002; Zammit et al., 2003; Naserbakht et al., 2011; Fountoulakis et al., 2018), seasonality of birth (Gallagher et al., 2007; Schwartz, 2011; Modinos et al., 2013) and urbanicity (van Os et al., 2010; Vassos et al., 2012; Haddad et al., 2015).

Substance abuse, particularly cannabis, contributes to higher risk of schizophrenia in a dose-dependent manner (Marconi et al., 2016; Murray et al., 2016; Murray et al., 2017). More recently, a causal link was established between cannabis use and the risk of schizophrenia by using Mendelian randomization (Vaucher et al., 2018). Changes in cannabinoid receptor distribution and cortical maturation associated with cannabis use are postulated to contribute to the increased risk (Vaucher et al., 2018). However, it should be noted that there is supporting evidence for the opposite (i.e. schizophrenia risk predicts likelihood cannabis consumption) and thus causal estimates should be interpreted with caution (Gage et al., 2017; Khokhar et al., 2018; Krebs et al., 2019).

1.1.3 Genetics of schizophrenia

Genetics may also be influential in brain development as those with family history of schizophrenia are more susceptible to the abovementioned structural anomalies, in particular the prefrontal regions (Pantelis et al., 2005; Tan et al., 2007). Family history itself is the best-known risk factor for schizophrenia (Pedersen and Mortensen, 2001; Mortensen et al., 2010). First-degree relatives of patients with schizophrenia are 13 times as likely to develop schizophrenia than controls, having a lifetime risk of 6.5% for schizophrenia (Kendler et al., 1993). Twin studies are essential when studying the genetic transmission of schizophrenia, as they allow for the estimation of heritability by calculation of concordance rates in monozygotic and dizygotic twins (Torrey and Yolken, 2019). Heritability estimates in literature may vary depending on methodology (Cardno and Gottesman, 2000), where inadequate screening and sampling of subjects can lead to higher estimates of concordance in monozygotic twins (Torrey and Yolken, 2019). The heritability liability for schizophrenia, or the risk for the disease attributable to genetics in the general population (Tandon et al., 2008), is estimated to be at 79 – 81% (Sullivan et al., 2003; Hilker et al., 2018). The reported probandwise concordance rate for monozygotic twins is approximately 33% and 7% for dizygotic twins (Hilker et al., 2018). However, twin studies assume that the twins are exposed to homogenous environmental factors and that genes have limited interactions with the environment (Torrey and Yolken, 2019). In addition, the low concordance rate for identical twins suggest a genetic basis for the risk of disease, and additional interactions from the environment is required for illness penetrance (Hilker et al., 2018). Thus gene-environment interactions may account for a significant proportion of the liability for

schizophrenia and the combined influence of candidate genes with non-genetic contributors may warrant further research.

A mutation from valine to methionine in the catechol-*o*-methyltransferase (COMT) gene is linked to significantly reduced dopamine (DA) catabolism, where regions such as the prefrontal cortex with limited DA transporters are affected the most (Egan et al., 2001; Tan et al., 2007). There is evidence that the COMT Val^{108/158}Met genotype is linked to executive cognition deficits as shown through significantly worse performance on the Wisconsin Card Sorting Test in comparison to controls (Egan et al., 2001). The decline in cognitive function has been attributed to the COMT Val allele, which leads to a reduction of synaptic DA in the prefrontal cortex and thereby impacting postsynaptic DA responses and ultimately working memory (Egan et al., 2001). As DA is central to neuronal signal transduction, it has been postulated that DA contributes to the various symptoms of schizophrenia and will be discussed further in the following sections.

Recent evidence suggests another component, the complement system, in addition to the ‘two hit’ models that suggest genetic and environmental risk factors lead to schizophrenia. With roles in synaptic formation and elimination during neurodevelopment, the complement system has been a subject of interest in the pathophysiology of schizophrenia. In particular, complement components such as C1, C2, C3, C3C and C4 are expressed at abnormal levels in patients with schizophrenia (Nimgaonkar et al., 2017). The abnormal activation of complement proteins can disrupt neuronal connections by altering dendritic spine and synaptic density, possibly activated in response to external factors (e.g. instance maternal infections) and presumably increases susceptibility to schizophrenia (Nimgaonkar et al., 2017). Animal studies may prove beneficial in establishing the link

between brain dysfunction and immune responses in schizophrenia as present epidemiological studies alone are inconclusive (Nimgaonkar et al., 2017).

1.1.4 Dopamine hypothesis of schizophrenia

Despite having its weaknesses, the “DA hypothesis” has been the most enduring and influential hypothesis for the pathophysiology of schizophrenia to date. The original DA hypothesis spawned from the finding that antipsychotics increased norepinephrine (NE) neurotransmission (Carlsson and Lindqvist, 1963) and the crucial identification of a correlation between clinical efficacy and blockade of dopaminergic receptors (Seeman and Lee, 1975). Seeman and Lee’s experiments provided the first evidence that antipsychotic activity and the corresponding inhibition of impulse-evoked DA release are directly correlated, presumably through interference of calcium (Ca^{2+})-mediated presynaptic dopaminergic transmission (1975). Following this discovery, the DA hypothesis has evolved into one of describing excessive DA transmission as responsible for the symptoms associated with schizophrenia. Supporting evidence for the DA hypothesis include the use of drugs such as amphetamine to replicate the symptoms of schizophrenia by altering DA levels.

Amphetamine is known to increase DA presence in the synaptic cleft and elicits amphetamine psychosis that resembles acute paranoid schizophrenia (Young and Scoville, 1938; Snyder, 1976). Cocaine also reproduces symptoms of schizophrenia at high doses, possibly through inhibition of catecholamine (CAT) reuptake in the synaptic cleft (Snyder, 1976). Importantly, all antipsychotics bind to and block dopamine 2 (D_2) receptors and these receptors are upregulated in the striata of patients with schizophrenia (Seeman, 1987). An

imaging study revealed more striatal D₂ receptors are occupied in patients with schizophrenia as compared to controls and positive symptoms were reduced with the use of antipsychotics, as a result of a decline in DA transmission (Abi-Dargham et al., 2000).

It is now increasingly clear that DA levels are not uniform throughout the brain and the resultant DA deficits associated with schizophrenia varies depending on the pathway involved (Perez-Costas et al., 2010). The most widely studied DA tracts involved in schizophrenia are the mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular pathways. The mesolimbic DA pathway, commonly known as the “reward pathway”, consists of projections originating from cell bodies in the ventral tegmental area to the limbic striatum (Stevens, 1979; Green et al., 1999), where hyperstimulation of D₂ receptors results in hallucinations and delusions (Meltzer and Stahl, 1976; Epstein et al., 1999). For this reason, blockade of dopaminergic receptors with antipsychotics is beneficial for managing positive symptoms in the mesolimbic tract (Reynolds, 2004).

The mesocortical DA tract consists of cell bodies that are located in the A10 group and substantia nigra, which project to the frontal cortex and the anterior cingulate cortex, respectively (Meltzer and Stahl, 1976). The mesocortical DA pathway is involved in cognitive function where dopaminergic neurotransmission in this tract is hypoactive in patients with schizophrenia, hence administration of antipsychotics may exacerbate these symptoms. Of interest, targeted inhibition of norepinephrine transporter (NET) and α_2 -adrenoceptors selectively increases dopaminergic neurotransmission in the prefrontal cortex (Masana et al., 2011). Combined administration of NET inhibitors and antipsychotics possessing α_2 -adrenoceptors inhibitory properties such as clozapine (CLZ) may be beneficial

for treating negative symptoms and improving cognitive function, yet increased NE levels may lead to side effects to be discussed in later chapters of this thesis.

The nigrostriatal dopamine tract mediates motor function and is largely linked to EPS (Reynolds, 2004). The cell bodies of this widely studied tract are localized to the pars compacta, also known as A9, of the substantia nigra and projects to the caudate-putamen of the neostriatum (Meltzer and Stahl, 1976). EPS arising from antipsychotic treatment results from blockade of the D₂ receptors in the neostriatum, leading to movement dysfunction resembling Parkinson's disease (Meltzer and Stahl, 1976). Over time, chronic inhibition of DA receptors of the nigrostriatal tract can induce tardive dyskinesia (TD) in 5% of the patients treated with first generation antipsychotics (FGAs) (Serretti et al., 2004). Ensuing hypersensitivity of postsynaptic DA receptors from the chronic blockade is thought to underlie TD pathophysiology (Margolese et al., 2005).

The tuberoinfundibular pathway is separate from the previous DA tracts in that it projects from the hypothalamus to the pituitary gland (Serretti et al., 2004). Blockade of this tract raises the risk for hyperprolactinemia, since hypothalamic DA inhibits production and secretion of prolactin in the anterior pituitary (Kapur and Remington, 2001; Kinon et al., 2003a, b; Montgomery et al., 2004). Hyperprolactinemia mainly affects sexual and reproductive function, such as disrupted galactorrhea, menstrual cycles and impaired spermatogenesis, with long term effects including osteoporosis and CVD (Dickson and Glazer, 1999). Symptoms can be alleviated by reducing dosage or discontinuation of prolactin-elevating antipsychotics (FGAs and risperidone), or switching to a SGA, such as olanzapine (Marken et al., 1992; Keller and Mongini, 2002; Kinon et al., 2003b; Kinon et al., 2006). Of interest, aripiprazole successfully lowered prolactin levels in over 80% subjects

with schizophrenia when administered in conjunction with haloperidol (Shim et al., 2007). It has been postulated alleviation of hyperprolactinemia is due to aripiprazole's partial agonistic effects on D₂ receptors and higher affinity for the D₂ receptor compared to haloperidol (Shim et al., 2007).

1.2 Antipsychotics

The primary treatment for schizophrenia is the prescription of antipsychotics (Kane, 1987; American Diabetes Association, 2004). As discussed in the preceding sections, all antipsychotics have antagonistic properties at the D₂ receptor with varying affinities and thus induce a range of side effects. Based on this commonality, antipsychotics have been conventionally classified as either FGAs or SGAs. FGAs are the first medications brought into use for managing schizophrenia. Administration of FGAs can result in EPS. While EPS may be intolerable to some patients and result in noncompliance, FGAs are still administered for the treatment of positive symptoms such as delusions and hallucinations (American Diabetes Association, 2004). With the introduction of SGAs and the realization that they have higher affinity for the serotonin receptor 5-HT_{2A} relative to the D₂ receptor, antagonism of 5-HT_{2A} receptors became one of the main distinguishing features between FGAs and SGAs (Meltzer et al., 1989). Another defining characteristic is the lower propensity to induce EPS, TD, dystonia and akathisia with SGAs (Rummel-Kluge et al., 2012). Higher affinity for 5-HT_{2A} and lower affinity for D₂ receptors was postulated to be key to the minimal liability for EPS, hyperprolactinemia and TD of SGAs (Meltzer et al., 1989). In recent years, however, there is evidence that the distinction between FGAs and SGAs cannot be made

solely on binding affinities and risk for EPS. In a head-to-head comparison of SGAs, it was shown that SGAs have different propensities of inducing EPS as reflected by the prescription of antiparkinson medication to treat these symptoms (Rummel-Kluge et al., 2012). Thus, SGAs are heterogeneous as a class and have varying drug profiles, where risk of EPS should be balanced with other side effects to optimize efficacy (Leucht et al., 2009; Rummel-Kluge et al., 2012). SGAs with complex pharmacological profiles are believed to possess superior clinical efficacy, as with CLZ, the gold standard for managing treatment resistant schizophrenia (Roth et al., 2004).

1.2.1 Clozapine

CLZ is the treatment of choice for treatment resistant schizophrenia, producing therapeutic responses in 30% of patients previously refractory to neuroleptic treatment (Kane et al., 1988). Discovered in 1959, CLZ has superior efficacy in the treatment of schizophrenia with minimal risk for EPS and hyperprolactinemia (Crilly, 2007). It is well established that CLZ effectively reduces violent and aggressive behavior in patients with schizophrenia (Ratey et al., 1993; Glazer and Dickson, 1998; Spivak et al., 1998; Volavka et al., 2004; Frogley et al., 2012). The mortality rate associated with CLZ is the lowest among all antipsychotics, largely due to the significantly lower suicidal risk associated with CLZ treatment (Tiihonen et al., 2009; Vera et al., 2012). It has been reported chronic CLZ treatment substantially reduced suicidal behavior by 3.3-fold compared to other treatments (Hennen and Baldessarini, 2005). The substantial reduction in suicidal behavior has been attributed to CLZ's efficacy in lowering aggression and impulsiveness (Spivak et al., 2003).

Yet the concern of causing adverse effects such as agranulocytosis (Griffith and Saameli, 1975; Idanpaan-Heikkila et al., 1975; Ronaldson et al., 2015), myocarditis (Haas et al., 2007; Swart et al., 2016), dyslipidemia, hypertension (Henderson et al., 2004), hypotension (Gerlach et al., 1974; Koren et al., 1997; Pereira et al., 2010) and tachycardia (Leo et al., 1996b; Koren et al., 1997; Cohen et al., 2001; Krentz et al., 2001; Baciewicz et al., 2002; Thomas and Pollak, 2003; Pereira et al., 2010; Akinsola and Ong, 2011) through ill-defined mechanisms has limited its widespread use in the clinic. Importantly, premature death in schizophrenia is primarily caused by CVD (Hennekens et al., 2005; Lahti et al., 2012; Sweeting et al., 2013; Ifteni et al., 2014), of which risk factors such as obesity, dyslipidemia, diabetes mellitus and hypertension are exacerbated by the use of CLZ (Henderson et al., 2005). As CLZ is often the only option for treatment resistant schizophrenia, it is imperative to understand the underlying mechanisms leading to CLZ-induced side effects.

1.2.2 The actions and side effects of clozapine

The “DA hypothesis” in schizophrenia proposes that excessive synaptic DA, caused by elevating presynaptic DA release and/or postsynaptic D₂ receptor number or sensitivity, increases D₂ receptor occupancy and thereby results in overstimulation of these receptors (Abi-Dargham et al., 2000). Importantly, the resultant hyperactive D₂ receptors in the mesolimbic dopaminergic system contribute to positive symptoms and antipsychotics exert therapeutic effects by blockade of the D₂ receptors (Meltzer and Stahl, 1976). As with all antipsychotics, CLZ binds to and antagonizes D₂ receptors (Creese et al., 1976), but with a

weaker affinity compared to FGAs and thus has limited liability for EPS and prolactin elevation (Fitton and Heel, 1990; Baldessarini and Frankenburg, 1991; Pilowsky et al., 1992; Pickar, 1995).

Several other models have emerged in the past years to explain CLZ's unique pharmacologic profile and efficacy. CLZ's weak antagonism of D₂ receptors combined with its high affinity for serotonin 5-HT_{2A} receptors are center to the "serotonin-dopamine hypothesis", where increased 5-HT and DA neurotransmission is thought to be the reason for CLZ's superior efficacy (Meltzer, 1989). The higher affinity for 5-HT₂ receptors as compared to D₂ receptors minimizes complications of prolactin elevations and EPS, while modifying the mesocortical dopaminergic pathway to produce antipsychotic effects (Meltzer et al., 2003). An alternative explanation for CLZ's unique actions is the "fast-off hypothesis", which postulates CLZ's rapid dissociation from D₂ receptors allows for sufficient dopaminergic neurotransmission and thus clinical efficacy is driven solely by this fast dissociation (Kapur and Seeman, 2001). The weak affinity for D₂ receptors allows for quicker response to DA surges due to easy displacement of CLZ by endogenous DA (Kapur and Seeman, 2001).

Both theories have their limitations, namely the classification of antipsychotics based on binding affinities and the clinical relevance of 5-HT₂. The serotonin-dopamine hypothesis suggests the SGAs have the distinctive feature of blockade of both 5-HT₂ receptors and D₂ receptors and the blockade produces clinical efficacy. However, binding studies revealed antagonism of 5-HT_{2A} receptors is irrelevant in determining the D₂ occupancy threshold for antipsychotic action and the emergence of EPS, thus contradicting the hypothesis (Seeman, 2002). Furthermore, the SGAs remoxipride and amisulpride are notable exceptions as they do

not potently antagonize 5-HT receptors despite being clinically effective (Seeman, 2002). Likewise, the fast-off hypothesis is not without limitations, as the fast dissociation from D₂ receptors is applicable only to CLZ and quetiapine (Meltzer et al., 2003). Other SGAs such as olanzapine, have comparatively slower dissociation rates despite being clinically effective (Meltzer et al., 2003). Loose binding to D₂ receptors thus lacks consistency as a classifying criterion of atypical antipsychotics. The uncertainty regarding which specific receptors define atypicality and clinical efficacy led to the newer view to model antipsychotics after CLZ's actions on multiple receptors (Roth et al., 2004). This hypothesis is based on the observation that the most effective antipsychotics are those that non-selectively target multiple receptors.

Indeed, CLZ is a potent antagonist for multiple receptors including dopamine receptors D₁ (Farde et al., 1992; Nordstrom et al., 1995) and D₄ (Van Tol et al., 1991), adrenergic receptors α_1 (Cohen and Lipinski, 1986) and α_2 (Tandon et al., 1999), serotonergic receptors 5-HT_{1A} (Mason and Reynolds, 1992; Newman-Tancredi et al., 1996) and 5-HT_{2A} (Canton et al., 1994), muscarinic receptors M₁₋₅ (Bolden et al., 1991; Zorn et al., 1994; Roth et al., 2004) and histamine receptor H₁ (Peroutka and Synder, 1980). CLZ's actions at these receptors in relation to cardiovascular and metabolic side effects will be discussed below.

1.3 Insulin resistance and glucose intolerance

Insulin is a hormone essential in the maintenance of glucose homeostasis, where it has inhibitory effects on hepatic glucose production and stimulatory effects on glucose uptake in peripheral tissue (Muniyappa et al., 2008). Insulin resistance refers to decreased sensitivity to insulin, leading to impaired uptake and metabolism of glucose in target organs (Guyton and

Hall, 2006). Hyperinsulinemia results as β -cells of the pancreas secrete excessive amounts of insulin to compensate for reduced insulin sensitivity in target tissues and is a hallmark of type II diabetes (Guyton and Hall, 2006). SGAs, especially CLZ, have a high propensity to induce insulin resistance and subsequently hyperglycemia (American Diabetes Association, 2004). The combination of insulin resistance and hyperglycemia is of concern, as both are both important risk factors for CVD (Newcomer and Haupt, 2006).

To test for insulin resistance in the present series of experiments, the hyperinsulinemic euglycemic clamp (HIEC) and intraperitoneal glucose tolerance test (IGTT) were performed. The HIEC is a direct method for testing insulin sensitivity, where it is assumed the hyperinsulinemic state will inhibit hepatic gluconeogenesis and there is no net change in plasma glucose concentrations, thus exogenous infusion of glucose is indicative of the amount of glucose needed to compensate for the elevated insulin levels (Tam et al., 2012). Whole body tissue insulin sensitivity and response of β -cells to glucose are measured with this method (DeFronzo et al., 1979).

Conversely, the IGTT assesses both insulin resistance and anomalies in β -cell function and primarily tests for glucose intolerance (Andrikopoulos et al., 2008). Typically, a glucose challenge is introduced after overnight fasting and blood samples are collected at selected time points to measure plasma glucose. While it is conducted with ease compared to the HIEC, it should be noted that the IGTT is an indirect measure of insulin resistance and is more useful as an indicator of glucose intolerance (Muniyappa et al., 2008). This is due to the inability to attribute the increase in plasma glucose levels to hepatic gluconeogenesis or insulin resistance without additional measures (Boyda et al., 2010a).

1.4 The metabolic syndrome

The metabolic syndrome, known previously as “syndrome X”, consists of multiple variables that contribute to the development of coronary artery disease (CAD) (Reaven, 1988). Central components of syndrome X include impaired insulin-mediated glucose uptake, glucose intolerance, hyperinsulinemia, hypertension, elevated plasma very low density lipoprotein (VLDL) triglycerides and reduced plasma high density lipoprotein (HDL) cholesterol. At the time, obesity and physical activity were considered environmental factors that influence insulin resistance, but were not causative of the syndrome (Reaven, 1988). However, in consideration of the importance of obesity in association with type 2 diabetes and CVD, visceral obesity has since been included in updated definitions of syndrome X, now commonly known as the metabolic syndrome (Alberti et al., 2005; Eckel et al., 2005; Gallagher et al., 2008).

The clinical definition of metabolic syndrome has varied over past years depending on whether the emphasis was on insulin resistance, obesity or cardiovascular anomalies (Alberti et al., 2005). In addition to Reaven’s initial description of hypertension, dysglycemia and dyslipidemia as factors that raise the risk for CVD and type 2 diabetes, the metabolic syndrome is also known to consist of abnormalities in coagulation and inflammation (Reaven, 1988; Gallagher et al., 2008; Grundy, 2016; Han and Lean, 2016). These risk factors are frequently associated with obesity (Grundy, 2016). Clinically, the metabolic risk factors themselves are not routinely measured for a diagnosis of metabolic syndrome. Instead, a diagnosis is made if 3 of the 5 following criteria are met: 1) waist circumference at or above 102 cm in men and 88 cm in women, 2) triglyceride levels at or above 150 mg/dl, 3)

HDL cholesterol below 40 mg/dl in men and 50 mg/dl in women, 4) hypertension (blood pressure at or above 130/85 mm Hg) and 5) glucose levels at or above 100 mg/dl (Grundy, 2004). It remains debatable whether the metabolic syndrome arises as a cumulative result from the abovementioned risk factors or as the result of individual, unrelated components (Gallagher et al., 2008).

1.4.1. Metabolic syndrome and antipsychotics

Interestingly, the prevalence of diabetes and obesity is estimated to be 1.5 to 2 times higher in patients with schizophrenia compared to the general population (American Diabetes Association, 2004). Antipsychotics may contribute to this higher prevalence as there is increasing evidence of metabolic side effects associated with their use in treating schizophrenia (De Hert et al., 2008; Meyer et al., 2008). Despite the difficulty of attributing metabolic disturbances to psychotic treatment, there are evident trends suggestive of higher metabolic risk associated with SGAs (American Diabetes Association, 2004). One study reported a 5-fold increase in the prevalence of the metabolic syndrome with SGA treatment compared to baseline (De Hert et al., 2008). In contrast, FGA treatment resulted in a 2-fold increase from baseline (De Hert et al., 2008). Other studies have also found FGAs to be associated with less metabolic liability compared to SGA and polypharmacy treatment groups (Cerit et al., 2010; Chadda et al., 2013; Hammoudeh et al., 2020). Along with olanzapine, CLZ is known to induce more weight gain than any other SGA (Rummel-Kluge et al., 2010). CLZ is also known to elevate blood glucose and cholesterol, both of which are established risk factors of CVD (Castelli et al., 1986; Sarwar et al., 2010). For the purposes of the present thesis, individual components of the metabolic syndrome in relation to CLZ

treatment will be discussed separately.

1.4.2 Hypertension

Elevated blood pressure (BP) is common in both diabetic and obese individuals, present in 85% of patients diagnosed with metabolic syndrome (O'Neill and O'Driscoll, 2015). It was suggested the hypertension observed in these individuals is a result of compensatory mechanisms to the lack of response to insulin at the cellular level (Sowers, 1990).

Specifically, impaired insulin-mediated calcium (Ca^{2+}) metabolism and subsequent elevations in intracellular Ca^{2+} lead to enhanced smooth muscle tone and hypertension, as demonstrated using insulin resistant Zucker rats (Sowers, 1990). Furthermore, poor response to insulin in insulin resistant individuals is also a contributing factor, where insulin normally induces production of nitric oxide (NO) for vascular relaxation (Sowers, 2004). Decreased insulin sensitivity leads to hyperinsulinemia as a compensatory mechanism, ultimately causing hypertension via activation of the renin angiotensin aldosterone system (O'Neill and O'Driscoll, 2015). Finally, an overactive sympathetic nervous system (SNS) can lead to hypertension, often present in individuals with obesity and insulin resistance (Ginsberg, 2000). Elevated plasma CAT levels induced by hyperinsulinemia may possibly contribute to the rise in BP (Reaven, 1988).

In comparison to the incidence rates of diabetes mellitus, dyslipidemia and obesity, there are less reported cases of hypertension in patients treated with CLZ (Henderson et al., 2004). A claims-based approach study found no significant difference in the incidence of hypertension in patients treated with CLZ as compared to patients receiving FGAs (Lund et

al., 2001). However, another chart review comparing patients treated with FGAs (n = 56), SGAs other than CLZ (n = 102) or CLZ (n = 82) had contradictory results, where hypertension was strongly associated with CLZ use (Henderson et al., 2004). At the end of the 5 year follow-up period, the CLZ group had significantly increased BP, resulting in 22% of these patients requiring medication for hypertension, as compared to 4% of the FGA group and 9% of the SGA group (Henderson et al., 2004). BP increased as early as 6 months of initiating CLZ treatment, signifying the need to routinely monitor BP as a prevention for CVD (Henderson et al., 2004).

1.4.3 Abdominal obesity

Obesity and weight gain, commonly estimated by the body mass index (Newcomer and Haupt, 2006), potentially contribute to increased risk of CVD, yet available evidence has singled out waist circumference as 1 of 5 defining characteristics of the metabolic syndrome (Despres and Lemieux, 2006). Past studies have identified excess abdominal fat as an independent risk factor for CVD, where abdominal fat distribution in particular is a better predictor of CVD than body mass index (Larsson et al., 1984; Reeder et al., 1997; Dagenais et al., 2005). Of note, obesity does not necessarily coincide with insulin resistance, diabetes mellitus nor risk for CVD, since weight gain can be similar between patients but visceral fat distribution can vary, thus emphasizing the importance of abdominal adiposity as an independent risk factor for CVD (Despres and Lemieux, 2006; Van Gaal, 2006; Henderson et al., 2009). The causal relationship, if any, of visceral adiposity with the metabolic syndrome remains poorly understood (Ritchie and Connell, 2007). One proposed theory is the

inflammatory state has a direct role in obesity and insulin resistance, where cytokines are believed to influence adipocyte metabolism (Hotamisligil et al., 1993; Xu et al., 2003). Proinflammatory molecules including interleukin 6 (IL-6) and tumor necrosis factor- α (TNF- α), and the inflammatory marker C-reactive protein (CRP) are released as excess energy is directed to visceral adipose tissue in obese individuals (Despres and Lemieux, 2006). Elevated production of cytokines from adipocytes influences insulin function and results in decreased lipolysis, thereby causing insulin resistance in adipose tissue (Grundy, 2004). Experimental evidence has indicated expression of TNF- α is specifically elevated in adipose tissue of obese and insulin resistant mice, while neutralization of TNF- α with a recombinant TNF- α receptor protein resulted in greater insulin sensitivity to glucose infusion (Hotamisligil et al., 1993). IL-6 and CRP are also elevated in relation to obesity, and are both independent predictors of risk for type 2 diabetes (Pradhan et al., 2001). Elevation of CRP precedes diabetes mellitus, where insulin resistance is believed to be the underlying cause of the disease (Haffner et al., 2000; Festa et al., 2003; Haffner, 2006).

The propensity for CLZ to cause weight gain and obesity is well documented in patients with schizophrenia (Allison et al., 1999; Allison and Casey, 2001; Haupt, 2006; Lamberti et al., 2006; Van Gaal, 2006). Numerous studies have reported weight gain in patients receiving CLZ treatment (Cohen et al., 1990; Lamberti et al., 1992; Umbricht et al., 1994; Henderson et al., 2000). CLZ was associated with the largest amount of weight gain during the first 12 months of treatment and up to 46 months, with 30.5% of patients subsequently developing type 2 diabetes mellitus (Henderson et al., 2000). A follow-up study performed by the same authors revealed patients gained approximately 13.6 kg over a 10 year period of CLZ administration, with the risk of CVD and diabetes mellitus increasing

over time in this cohort (Henderson et al., 2005). Significant weight gain is a concern as an increase of >7% of desirable weight (the midpoint of a weight range for a specific height) can predispose patients at risk for CVD (Hubert et al., 1983; Nasrallah, 2003).

In support of the inflammatory theory, CLZ increased production of proinflammatory cytokines in insulin responsive cells, where monocyte infiltration and the inflammatory state possibly contributes to obesity and insulin resistance (Contreras-Shannon et al., 2013). In addition, waist circumference is positively correlated with insulin resistance in nondiabetic patients with schizophrenia who received CLZ treatment (Henderson et al., 2009). Of interest, increased waist circumference is the strongest predictor of insulin resistance among commonly used anthropometric measurements (e.g. body mass index, insulin sensitivity index, lipid levels) in CLZ-treated patients but the same association was absent in patients treated with olanzapine (Henderson et al., 2009).

CLZ's blockade of multiple receptors may also contribute to weight gain, where the histamine H₁-receptor is believed to play a significant role (Kroeze et al., 2003). Weight gain was previously demonstrated in H₁-receptor knock out mice in which intake of high fat diets caused quicker body fat deposition as compared to wild type mice (Masaki et al., 2001b). Leptin is potentially key to the observed increase in adiposity, likely acting by disinhibition of the negative feedback loop involving histamine neurons that normally suppress food intake (Masaki et al., 2001b). Blockade of H₁-receptors would therefore promote food intake and fat deposition. In support of this, animal experiments have shown CLZ reversed leptin's effects via selective augmentation of hypothalamic adenosine monophosphate-activated protein kinase (AMPK) activity (Kim et al., 2007). As AMPK stimulation in the hypothalamus is known to increase food intake (Minokoshi et al., 2004), CLZ potentially

removes leptin's anorexigenic effects by activating AMPK. In addition to suppressing appetite, decreased lipolysis resulting from inhibition of H₁-receptors (Masaki et al., 2001a) may also contribute to CLZ-induced weight gain.

1.4.4 Hyperglycemia

Hyperglycemia is the defining characteristic of metabolic dysfunction linked to diabetes mellitus (Nathan et al., 2009). Individuals with fasting blood glucose levels between 5.6 – 6.9 mmol/l or 2-hour plasma glucose values of 7.8 – 11.0 mmol/l in the oral glucose tolerance test (OGTT) are considered to have impaired fasting glucose and impaired glucose tolerance, respectively (Association, 2010). These individuals are considered to have elevated risk for developing diabetes, commonly known as the pre-diabetic stage. For diagnosis of diabetes, the standard biomarker for glycemic control, hemoglobin A1C, is used. The A1C assay measures the average plasma glucose over a span of 2-3 months and a value of $\geq 6.5\%$ is used to diagnose diabetes (American Diabetes Association, 2010). The A1C assay, coupled with a fasting plasma glucose of ≥ 7.0 mmol/l or a 2 hour plasma glucose of ≥ 11.1 mmol/l in the OGTT, form the diagnostic criteria for diabetes (Association, 2010).

The possibility of hyperglycemia as an independent risk factor for CVD is demonstrated not only in diabetic patients, but also in healthy individuals. A meta-analysis revealed CVD risk is increased by at least 27% in nondiabetic individuals with post challenge blood glucose levels in the 8.3 – 10.8 mmol/l range as compared to the lowest 3.8 – 5.9 mmol/l range, where the threshold for the increased risk is a fasting blood glucose of 5.6

mmol/l (Levitan et al., 2004). Hyperglycemia causes cardiovascular damage by activating pathways that lead to excessive oxidative stress (Brownlee, 2001). Four mechanisms have been proposed to underlie glycemia-related vascular damage: decreased production of the antioxidant glutathione via activation of the polyol pathway, excessive generation of advanced glycation end products, activation of protein kinase C and increased activation of the hexosamine pathway (Brownlee, 2005). The common link between these 4 mechanisms is the inhibition of the glycolytic enzyme, glyceraldehyde-3 phosphate dehydrogenase (GADPH), by excessive superoxide production. Inhibition of GADPH results in increased upstream glycolytic intermediates and glucose, subsequently activating the aforementioned damaging pathways (Du et al., 2003; Brownlee, 2005). Several regions are susceptible to hyperglycemia-induced vascular damage, including the peripheral nerve, renal glomerulus and retina, as well as arteries in the brain, heart and lower limbs (Brownlee, 2001). Exposure to reactive oxygen species can adversely affect vascular contractile function, result in cardiomyopathy and atherosclerosis, and cause renal dysfunction (Cai et al., 2002; Touyz, 2004; Fiorentino et al., 2013).

Of the SGAs, CLZ has the highest propensity to induce hyperglycemia that usually can be resolved upon discontinuation (Kamran et al., 1994; Yazici et al., 1998; Koller et al., 2001; Lindenmayer et al., 2001; Sernyak et al., 2003). Glucose intolerance associated with CLZ treatment contributes to the development of newly onset diabetes mellitus and exacerbates pre-existing cases (Kamran et al., 1994; Wirshing et al., 1998), both of which can occur independently of weight gain (Popli et al., 1997; Yazici et al., 1998). Weight gain is not present in all patients receiving CLZ treatment, and is consequently considered a contributing factor rather than the sole mechanism underlying insulin resistance (Wirshing et

al., 1998; McIntyre et al., 2001; Muench and Carey, 2001; Newcomer et al., 2002). As a causal relationship between CLZ use and the development of diabetes mellitus cannot simply be attributed to excessive adiposity (Sernyak et al., 2002), there has been increasing attention given to weight-independent mechanisms to explain glucose dysregulation. One area of focus is CLZ's antagonistic properties at receptors mediating glucose homeostasis, namely muscarinic, serotonergic and dopaminergic receptors (Hahn et al., 2011). Acute antagonism of M₃ and 5-HT_{2A} receptors, known to directly affect pancreatic β -cell function and insulin secretion (Gautam et al., 2006), was found to decrease insulin secretion during the hyperglycemic clamp (estimates peripheral insulin sensitivity and secretory capacity of β -cells following a glucose challenge) in animals whereas blockade of D₂/D₃ receptors had the opposite effect (Hahn et al., 2011). In a follow up study, α_1 antagonism with prazosin inhibited insulin secretion and glucose infusion rates after a glucose challenge, indicative of impaired β -cell function (Guenette et al., 2013). As CLZ is known to rapidly reduce insulin sensitivity and hepatic glucose production (Chintoh et al., 2009), whether antagonism of the above receptors is responsible for CLZ-induced impairment of β -cell function remains to be determined.

1.4.5 Dyslipidemia

Excessive plasma triglycerides ≥ 1.7 mmol/L and/or HDL-cholesterol levels below 40 mg/dl in men (< 50 mg/dl in women), are part of the diagnostic criteria for the metabolic syndrome (Alberti and Zimmet, 1998; Grundy, 2004; Han and Lean, 2016; Lamster and Pagan, 2017). Dyslipidemia has detrimental effects on endothelial function and significantly increases the

risk of coronary artery disease particularly in individuals with diabetes (Stamler et al., 1993; Seligman et al., 2000). Endothelial injury arises through the accumulation of excessive lipids which eventually leads to atherosclerosis. Specifically, the infiltration of monocytes and T helper type 1 cells between dysfunctional endothelial cells results in the proliferation of smooth muscle cells and lipid-filled macrophages to form fibrous plaques characteristic of atherosclerosis (Ross, 1993; Gistera and Hansson, 2017). Evidence for the development of this fibrous cap can be found in both animal and human studies, where subsequent rupture and loosening of the plaque leads to acute cardiovascular events (Libby and Aikawa, 2002). In hypercholesterolemic animals, hyperlipidemia caused proliferation of smooth muscle cells in the intima and induced morphological changes in the cells by increased lipid deposition (Ross and Harker, 1976; Nakashima et al., 1994). The lipid-laden smooth muscle cells combine with foamy macrophages to form advanced lesions, where calcification of the fibrous plaques and necrosis can occur (Nakashima et al., 1994). Similar fibrous caps overlying foamy lesions are present in human atherosclerosis (Gown et al., 1986), where plaque rupture accounts for > 75% of coronary thrombotic events (Falk, 1991). Ideally, plaques that are unstable and are at high risk to occlude arteries should be identified for preventive strategies. Detection of protein changes in human atherosclerotic samples have given rise to a multi-panel of biomarkers for plaque instability, comprised of proteins involved in processes such as inflammation and apoptosis (Eslava-Alcon et al., 2020). Inflammation is particularly important as its resolution through lowering LDL cholesterol can reduce cardiovascular events (Back et al., 2019).

Clinical studies indicate lowering plasma lipids are beneficial not only to patients with existing CVD (Scandinavian Simvastatin Survival Study Group, 1994), but can also be

used as a primary prevention for CVD. In one study, 20,536 individuals with CVD were randomly selected to receive the lipid lowering medication simvastatin or placebo over a span of 5 years (Heart Protection Study Collaborative Group, 2002). Results indicate simvastatin had protective effects by lowering myocardial infarction, stroke and reoccurrence of vascular events by approximately 25% (Heart Protection Study Collaborative Group, 2002). This was in line with previous studies where risk of coronary heart disease was lowered by 30% with the use of lipid lowering drugs (colestyramine, gemfibrozil, pravastatin and lovastatin) over a duration of 5 to 7 years (Lipid Research Clinics Program, 1984; Frick et al., 1987; Shepherd et al., 1995; Downs et al., 1998; Pignone et al., 2000). Recently, a reduction of 55 to 60% in LDL cholesterol has been reported when statins are administered at maximal dosages (Newman et al., 2019). Yet adverse events such as myopathy have been attributed to statins leading to its discontinuation in up to 10% of patients (Newman et al., 2019). It was estimated that there would be 5 cases of myopathy, 5 – 10 cases of hemorrhagic strokes and 50 – 100 cases of diabetes for every 10,000 patients given statins on over a 5 year period (Collins et al., 2016). Of note, placebo-controlled randomized trials failed to find a causal link between statin treatment and the aforementioned adverse events (Collins et al., 2016).

Despite evidence for the beneficial effects of lipid lowering agents on CVD, their use as a prevention for CVD has been largely overlooked in medium to high risk individuals (Goff et al., 2006). Undertreatment of dyslipidemia is particularly common in patients with schizophrenia, with up to 88% of the large scale Clinical Antipsychotic Trials of Intervention Effectiveness schizophrenia trial subjects reportedly not receiving treatment (Nasrallah et al., 2006). The percentage of patients not receiving treatment for dyslipidemia have since been

updated to range from 69% to 95% (Ko et al., 2013; Kraal et al., 2017). Inadequate access to medical care can be a cause of increased mortality among these patients (Green et al., 2003; Nasrallah et al., 2006).

There is increasing evidence associating the use of antipsychotics with dyslipidemia, especially in patients treated with SGAs (Casey, 2004; Olfson et al., 2006; Newcomer, 2007; Tse et al., 2014). In particular, multiple studies have reported CLZ significantly raises serum triglyceride levels in patients (Spivak et al., 1998; Gaulin et al., 1999; Baymiller et al., 2003; Henderson et al., 2005; Procyshyn et al., 2007; Lappin et al., 2018; Kim et al., 2019). Administration of CLZ for a period of 1 year increased serum triglycerides and is significantly correlated with weight gain, possibly mediated through inhibition of H₁-receptors and thus increasing appetite (Baymiller et al., 2003). The increase in serum triglycerides and total cholesterol occurred as early as the first month of initiating CLZ treatment and persisted throughout the study (Baymiller et al., 2003). However, other studies have noted that dyslipidemia can also occur independent of weight gain (Newcomer, 2007; Procyshyn et al., 2007). The association of CLZ treatment with hyperlipidemia is further demonstrated when the increase in triglyceride levels is resolved upon discontinuation of CLZ and returns when treatment is resumed (Ghaeli and Dufresne, 1999). Of interest, elevated triglycerides are associated with improved outcome in patients with schizophrenia, as indicated by decreased Positive and Negative Syndrome Scale scores (Procyshyn et al., 2007) and this is independent of weight gain (Kim et al., 2019). This raises the possibility of serum lipids influencing the efficacy of CLZ, warranting future research.

Treatment options for CLZ-induced dyslipidemia include the use of statins that effectively lower cholesterol and triglyceride levels (De Hert et al., 2006; Hanssens et al.,

2007; Landry et al., 2008; Ojala et al., 2008; Tse et al., 2014; Skrede et al., 2015; Winckel and Siskind, 2017). Considered the standard treatment for lowering cholesterol, statins act to antagonize hydroxymethylglutaryl-coenzyme A reductase to primarily lower LDL and total cholesterol levels, with a lesser effect on triglycerides (Ojala et al., 2008). While displaying promising results for treating SGA-associated dyslipidemia, it should be noted statins can have adverse side effects. Notably, statins can elevate the risk of developing diabetes (Sattar et al., 2010). Therefore, clinical benefits of improving cardiovascular health should be weighed against the increase in the incidence of diabetes from statin use, especially in patients treated with SGAs.

The underlying mechanism for CLZ-induced dyslipidemia remains unknown and no receptor targets have been identified to date (Tse et al., 2014). Given its involvement in cardiovascular function and regulating metabolism, the autonomic nervous system (ANS) and its individual branches are potential candidates for mediating the cardiometabolic side effects of CLZ. In particular, the SNS, where its heightened activity has known contributions to glucose dysregulation and cardiovascular anomalies (Bravo, 1989; Tentolouris et al., 2008; Boyda et al., 2013a). Indeed, plasma levels of the major SNS neurotransmitter NE is significantly elevated in association with CLZ-induced tachycardia and hyperlipidemia (Spivak et al., 1998). The role of the ANS in cardiovascular health and metabolism in relation to CLZ use will be discussed in the sections to follow.

1.5 Cardiovascular side effects of CLZ

CLZ's diverse receptor profile produces a range of side effects, including adverse cardiovascular effects such as tachycardia and orthostatic hypotension through antagonism of muscarinic and α -adrenoceptors (Richelson, 1999). Muscarinic M₂ receptors of the heart have known involvement in slowing heart rate (HR), mediated through acetylcholine (ACh) at parasympathetic nerves (Gomez et al., 1999; Buckley and Sanders, 2000). The involvement of M₂ receptors in vagal inhibition has been demonstrated in mutant mice without functional M₂ receptors, where administration of muscarinic agonists no longer induced bradycardia (Gomez et al., 1999). This mechanism possibly underlies CLZ-induced tachycardia, as CLZ is a potent antagonist of M₂ receptors. In addition, both α_1 and α_2 -adrenoceptors mediate cardiovascular function through binding of its endogenous agonists NE and epinephrine (E). Binding of NE to vascular α_1 -adrenoceptors causes vasoconstriction to increase blood BP and stimulates cardiac myocytes to contract (Yu and Han, 1994; Endoh, 1996; Nagashima et al., 1996; Civantos Calzada and Alexandre de Artinano, 2001), whereas stimulation of α_2 -adrenoceptors provides negative feedback to inhibit presynaptic NE release and results in hypotension (MacMillan et al., 1996). Antagonism of α_1 -adrenoceptors therefore is likely to be involved in CLZ's hypotensive effects, through which the vasodilation can lead to reflex tachycardia (Buckley and Sanders, 2000). A more in-depth discussion of CLZ-induced cardiovascular side effects and the role of the ANS in mediating these adverse events will be provided below.

1.5.1 Autonomic regulation of cardiovascular function

BP and HR changes are regulated in part by the ANS, which is divided into the SNS and parasympathetic nervous system (PNS). A third branch, the enteric nervous system, will not be discussed in detail as it mainly regulates gastrointestinal function. Determinants of BP (cardiac output and vascular resistance) is primarily controlled by the SNS, whereas both the SNS and PNS determine contractility and HR (Guyenet, 2006). Information from peripheral receptors are relayed via afferent nerves to the rostral ventrolateral medulla (RVLM), where preganglionic neurons with cell bodies located in the RVLM project to postganglionic neurons that innervate and stimulate effector organs via release of neurotransmitters (Dampney, 1994). The RVLM is best known as a cardiovascular center situated in the medulla, where increased activity of sympathetic neurons exiting the RVLM is relayed to the heart, kidney and arterioles to increase BP (Kumagai et al., 2012). Increases in BP are opposed by stimulation of baroreceptor afferent nerves projecting to the cell bodies of interneurons in the nucleus tractus solitarius (NTS), activating neurons in the caudal ventrolateral medulla to release inhibitory gamma-aminobutyric acid to suppress the RVLM and ultimately lower BP (Pilowsky and Goodchild, 2002; Kumagai et al., 2012). Clinically, neurovascular compression of the RVLM is associated with essential hypertension and physical stimulation of the RVLM leads to hypertension in animals (Morimoto et al., 1997; Morimoto et al., 1999).

HR is determined by autonomic control of the sinoatrial node, where parasympathetic influence is dominant over sympathetic activity. Parasympathetic fibers are inhibitory and lowers HR, hence elevating HR requires a decrease in parasympathetic activity and a simultaneous increase in sympathetic activity (Yamamoto et al., 1991). Cell bodies of preganglionic parasympathetic fibers reside in the nucleus ambiguus and dorsal motor

nucleus of the vagus nerve (Stuesse, 1982; Ter Horst et al., 1996), synapsing with cholinergic postganglionic fibers within cardiac ganglia in the atria (Loffelholz and Pappano, 1985). Negative inotropic and chronotropic effects are mediated via binding of ACh, the PNS neurotransmitter, to muscarinic receptors in cardiac cells. Positive inotropy in ventricles and atria is the result of sympathetic activation and release of the neurotransmitter NE, which preferentially binds and stimulates β_1 -adrenoceptors in the heart.

1.5.2 Mechanisms and interventions for clozapine-induced cardiovascular side effects

The most commonly reported cardiovascular side effects of CLZ are tachycardia and orthostatic hypotension, occurring in 25% and 9% of patients, respectively (Safferman et al., 1991). Tachycardia refers to a HR above 100 bpm and a mean HR of over 90 bpm within a 24 hour period (Sheldon et al., 2015; Yuen et al., 2018). HR can reportedly reach 150 bpm after 3 weeks of CLZ treatment at 100 – 150 mg/day (Baciewicz et al., 2002). Tachycardia is reversible upon lowering the dosage of CLZ or administering β -adrenoceptor blockers such as metoprolol (Safferman et al., 1991; Baciewicz et al., 2002). Persistently elevated HR is of concern particularly when tachycardia can lead to cardiomyopathy and sudden cardiac death (Shinbane et al., 1997; Jones et al., 2014). It has been suggested CLZ-induced tachycardia results from sympathetic hyperactivity, prompting the prescription of β -adrenoceptor blockers to lower sympathetic contribution while increasing vagal activity (Stryjer et al., 2009). Past clinical studies have demonstrated decreased vagal activity and increased HR with the use of CLZ (Zahn and Pickar, 1993; Agelink et al., 2001; Kim et al., 2007). Of the SGAs, only CLZ has known involvement in inducing tachycardia via blockade of muscarinic

M₂ receptors and subsequent inhibition of parasympathetic activity (Buckley and Sanders, 2000; Michelsen and Meyer, 2007). In addition, reflex tachycardia can occur when blockade of α_1 -adrenoceptors leads to vasodilation (Michelsen and Meyer, 2007).

The use of β -adrenoceptor blockers (i.e. conventional blockers such as atenolol, propranolol and metoprolol), although effective in lowering HR, can also lead to detrimental metabolic side effects such as dyslipidemia and insulin resistance through unknown mechanisms (Fonseca, 2010). These effects can be exacerbated with CLZ treatment due to CLZ's high risk for weight gain and metabolic abnormalities (De Hert et al., 2012; Tse et al., 2014). This is demonstrated by a double-blinded trial investigating serum glucose and lipid levels in patients with schizophrenia prescribed CLZ (n = 38, 400 mg/day), followed by propranolol (PRO) in the event of tachycardia. Results showed significant elevation of serum triglycerides, glucose and cholesterol when CLZ was administered in combination with PRO, compared to CLZ alone (Baymiller et al., 2003).

Early stages of antipsychotic treatment is associated with a high incidence of hypotension, with CLZ having the highest propensity to induce hypotension among the SGAs (Tandon, 2002). Hypotension occurs in 9% of patients receiving CLZ and is attributed to antagonism of adrenoceptors (Safferman et al., 1991). Treatment options include dihydroergotamine (Thulesius and Berlin, 1986; Marinkovic et al., 1994) and fludrocortisone (Testani, 1994). Dihydroergotamine administered at 10 mg/day in a double blinded clinical trial successfully prevented postural hypotension (Thulesius and Berlin, 1986). Orthostatic hypotension from CLZ treatment is due to strong antagonism of α_1 -adrenoceptors, in which hypotensive effects are worsened in the event of autonomic failure that prevents the release of NE (Gugger, 2011). Syncope, especially in the elderly, can result from α_1 -adrenoceptor

blockade due to failure to maintain sympathetic tone during postural changes (Young et al., 1998b; Michelsen and Meyer, 2007).

1.5.3 Catecholamines and their metabolites

Catecholamines (CATs) are a class of neurotransmitters that are essential in cardiovascular homeostasis. DA, NE and E are the 3 primary CATs of biological importance, and plasma levels of these CATs and their respective metabolites are indicative of origin and function of their source organs (Goldstein et al., 2003). NE is synthesized from DA, a reaction catalyzed by dopamine- β -hydroxylase (DBH) in secretory granules of sympathetic neurons, and is converted into E by phenylethanolamine methyltransferase (PMNT) in the adrenal medulla (Kopin, 1985; Esler et al., 1990). While the majority of E enters the circulation via direct secretion from the adrenal medulla, NE undergoes a much more complex fate as only a minority of NE enters the circulation unchanged (Goldstein et al., 2003). Instead, NE is mostly taken back up into sympathetic nerve terminals and metabolized. The resulting metabolites released into plasma reflect specific functions such as NE turnover (i.e. dihydroxyphenylglycol, DHPG), of which together with plasma NE levels, lends insight to exocytosis from sympathetic nerves (Goldstein et al., 2003). The production of other metabolites such as normetanephrine (NMN) and methoxyhydroxyphenylglycol (MHPG) further elucidates the fate of NE. NMN is mainly derived from *O*-methylation of NE in the adrenal glands by COMT and MHPG is the product of DHPG metabolism by COMT in non-neuronal cells (Eisenhofer et al., 1995; Goldstein, 2010). NMN can therefore be viewed as an indicator of non-neuronal NE metabolism and MHPG is a marker of peripheral NE turnover

(Goldstein et al., 2003). Indeed, plasma levels of CAT metabolites such as MHPG and DHPG have been measured in clinical studies associating CLZ with clinical efficacy and cardiovascular parameters (Breier et al., 1994; Brown et al., 1997).

Plasma NE level is a function of “spillover” from sympathetic nerves into plasma and the rate of clearance from circulation, as well as regional differences in sympathetic innervation (Goldstein et al., 2003). When interpreted and measured correctly, plasma NE levels are a valuable means of assessing sympathetic activity in pathological states (Hjemdahl, 1993). For instance, hyperactivity of the SNS has been implicated in hypertension and congestive heart failure, and is associated with a characteristic increase in plasma NE levels (Goldstein, 1983; Hasking et al., 1986; Solt et al., 1990; Masuo et al., 2003). In the case of congestive heart failure, elevated plasma NE is possibly caused by impaired clearance of NE in addition to increased release of NE into the circulation (Hasking et al., 1986). Given the importance of NE in cardiovascular function, the current thesis will be focused on the role of NE in CLZ’s cardiovascular side effects.

1.5.4 Clozapine and elevation of plasma norepinephrine

A hallmark of CLZ treatment in both humans and animals is a significant increase in plasma NE. An early clinical study showed treatment of CLZ at 175 – 600 mg/day for 30 days resulted in a significant elevation of plasma NE levels, as well as HR, in patients with psychosis compared to healthy controls (Sarafoff et al., 1979). Subsequent studies in patients with schizophrenia produced similar results (Pickar et al., 1992; Breier et al., 1994; Brown et al., 1997; Elman et al., 1999). It was initially thought the increase in plasma NE was due to

inhibition of α -adrenoceptors and the NE transporter, since the levels of the intraneuronal metabolite DHPG remained unchanged (Breier et al., 1994). A follow up study refuted this hypothesis, since radiolabeled DHPG concentrations remained unchanged in plasma and thus indicated normal reuptake and metabolism of NE (Elman et al., 1999). The authors suggest increased NE vesicular fusion with the sympathetic nerve membrane accounts for the unchanged plasma DHPG levels and increased plasma NE. The mechanism through which CLZ elevates plasma NE and whether this is associated with improved psychotic symptoms remains debatable (Brown et al., 1997). A possible reason for the discrepancy is the small sample numbers ($n < 14$) and short duration of available trials measuring plasma NE, with the longest published trial lasting 6 weeks (Breier et al., 1994; Brown et al., 1997).

Surprisingly, only 1 of these studies included BP and HR measurements taken at baseline and at the end of 5 weeks of treatment (Breier et al., 1994). Furthermore, cardiovascular events resulting from CLZ use is often left untreated. A systematic review of CLZ-induced autonomic dysfunction and cardiovascular side effects showed that only 16 out of 37 published studies reported using interventions for cardiovascular side effects (Yuen et al., 2018). Since failure to treat CLZ-induced cardiovascular anomalies can lead to grave consequences such as myocarditis and cardiomyopathy, further studies are needed to discern the underlying mechanisms and to introduce relevant interventions to treat these complications.

1.5.5 Adrenoceptor activation and inhibition

Adrenoceptors are generally divided into α and β subtypes (Bylund et al., 1994), where $\alpha_{1,2}$ - and $\beta_{1,2}$ -adrenoceptors are of paramount importance in the regulation of cardiometabolic functions (Figure 1.1). In vasculature, NE predominantly binds α_1 -adrenoceptors on smooth muscle to induce vasoconstriction (Klabunde, 2012). Activation of α_1 -adrenoceptors in cardiac myocytes by NE results in increased chronotropy, dromotropy and inotropy (Klabunde, 2012). In addition, α_1 -adrenoceptors contribute to glucose homeostasis by promoting glucose uptake into the heart, white adipocytes and skeletal muscle (Boyda et al., 2013a; Shi et al., 2017).

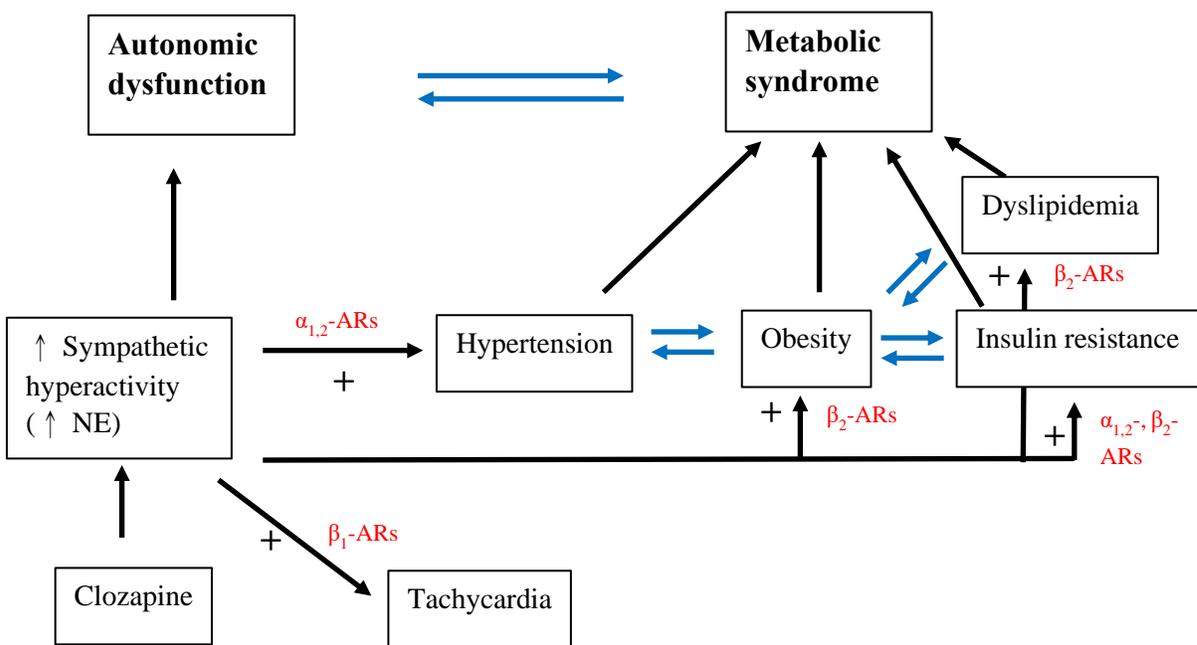


Figure 1.1: Clozapine-induced cardiometabolic side effects and the involvement of the sympathetic nervous system. Cardiometabolic side effects from CLZ treatment results from heightened sympathetic activity and mediated via α , β -adrenoceptors.

α_2 -adrenoceptors on pancreatic β -cells play a critical role in regulating blood glucose by inhibiting insulin release and raising blood glucose levels by binding to NE and E (Savontaus et al., 2008; Fagerholm et al., 2011). Insulin secretion is restored by stimulation of α_2 -adrenoceptors which also serves as a negative feedback mechanism to inhibit release of E and NE, in the adrenal medulla and sympathetic nerve terminal, respectively (Fagerholm et al., 2011). Furthermore, NE can activate postjunctional α_2 -adrenoceptors on smaller vessels to cause vasoconstriction, whereas activation of prejunctional α_2 -adrenoceptors inhibits NE release (Klabunde, 2012).

The role of β_1 -adrenoceptors in regulating chronotropy and inotropy in the heart is well-established (Skomedal et al., 1997; Boyda et al., 2013a). These effects are primarily mediated by NE (and E depending on adrenoceptor distribution and affinity) which preferentially binds to β_1 -adrenoceptors in cardiac tissue (Klabunde, 2012; Espinoza et al., 2019). In comparison to its cardiovascular effects, β_1 -adrenoceptors have a less prominent role in regulating glucose homeostasis. Instead, it acts in combination with $\beta_{2,3}$ -adrenoceptors to modulate insulin levels (Philipson, 2002; Boyda et al., 2013a).

Stimulation of β -adrenoceptors in the liver increases breakdown of glycogen whereas in the skeletal muscle and adipose tissue glucose utilization is inhibited (Asensio et al., 2005). In support of these observations, it was shown knockout mice lacking $\beta_{1,2,3}$ -adrenoceptors displayed glucose intolerance, defective insulin secretion and increased gluconeogenesis (Asensio et al., 2005). It was further established that the $\beta_{2,3}$ subtypes are of higher functional importance in terms of glucose regulation and are abundantly expressed in human and rodent adipose tissue (Boyda et al., 2013a).

Of importance, β_2 -adrenoceptors are highly expressed in adipose tissue, liver, pancreas and skeletal muscle, reflecting their function in these tissues: increased lipolysis, gluconeogenesis, glycogenolysis and inhibition of insulin-mediated glucose uptake (Boyda et al., 2013a). The actions of CATs on glucose homeostasis is mediated primarily by the β_2 subtype (Shrayyef and Gerich, 2010). E is known to increase hepatic glucose production and decrease glucose clearance from the circulation by activating β_2 -adrenoceptors (Rizza et al., 1980; Vardeny et al., 2008). The effects of NE on β_2 -adrenoceptor mediated glucose production is transient and smaller in comparison (Sacca et al., 1980). The β_2 subtype is also present in cardiac tissue yet play a comparatively minor role in moderating cardiovascular function (Klabunde, 2012).

The present series of experiments will employ several α - and β -adrenoceptor antagonists to investigate the effects of inhibiting adrenoceptors on cardiometabolic function in CLZ-treated animals. Focus will be placed on butoxamine (BUT), idazoxan (IDA), prazosin (PRA) and propranolol (PRO) based on the abovementioned critical roles that $\alpha_{1,2}$ - and $\beta_{1,2}$ -adrenoceptors play in regulating cardiometabolic function. PRA and IDA are antagonists for the α_1 - and α_2 -adrenoceptor, respectively. BUT selectively inhibits the β_2 -adrenoceptor, whereas PRO inhibits both $\beta_{1,2}$ subtypes.

1.6 Summary

There is increasing evidence for CLZ-induced metabolic side effects, with numerous research groups reporting cardiometabolic side effects associated with CLZ treatment. Consequently, the metabolic liability, risk of fatal agranulocytosis and cardiovascular complications such as

myocarditis have hampered the widespread use of CLZ. As CLZ is exceptionally efficacious in managing treatment resistant schizophrenia, further studies delving into the etiology and interventions for CLZ-induced adverse effects are warranted. For this reason, the use of animal models is increasingly important in providing mechanistic insight for CLZ's actions. Existing models for investigating insulin resistance and glucose tolerance include the HIEC and IGTT, of which the former is considered the "gold standard" for insulin resistance studies (Ayala et al., 2006; Tam et al., 2012; Remington et al., 2015b). As glucose dysregulation emerges shortly after starting CLZ treatment and is a major risk factor of CVD (Henderson et al., 2000; Henderson et al., 2015), it is of paramount importance to develop an animal model that can reliably reproduce CLZ's cardiometabolic effects. While multiple studies have used HIEC and IGTT to assess antipsychotic-induced glucose dysregulation in animals (Chintoh et al., 2009; Boyda et al., 2010b; Boyda et al., 2012a; Boyda et al., 2013b; Boyda et al., 2014b; Hahn et al., 2014; Remington et al., 2015b), assessment of cardiovascular events arising from antipsychotic use is less established in comparison. Therefore, we aimed to develop a rodent model that displays the cardiometabolic side effects associated with CLZ use, allows for the investigation of the etiology of these adverse events and subsequently introduce relevant treatments.

1.7 Specific aims and hypotheses

The specific aims for the metabolic experiments in this thesis are:

AIM 1: Replicate acute clinical symptoms of CLZ-induced cardiometabolic side effects in a rodent model. The animal model will be used to assess glucose tolerance, insulin sensitivity and cardiovascular parameters following acute CLZ treatment.

AIM 2: Determine whether norclozapine (NOR), the active metabolite of CLZ, has comparable metabolic effects to CLZ. Plasma drug levels of CLZ and NOR in association with CLZ-induced changes in metabolic indices will be measured.

AIM 3: The effects of the α -adrenergic antagonists idazoxan (IDA) and prazosin (PRA), and the β -adrenergic antagonists atenolol (ATEN) and butoxamine (BUT), are evaluated for the possibility of reversing metabolic disturbances induced by CLZ. Mecamylamine (MEC) will be administered to determine the effects of blocking neuronal transmission in the ANS on metabolic indices

AIM 4: Determine whether the SNS has a role in regulating cardiometabolic side effects following CLZ administration. Plasma NE levels will be measured at pre-determined time points to reflect sympathetic activity. PRO is evaluated as a treatment for CLZ-induced tachycardia. Tachycardia is induced by a single intraperitoneal injection of CLZ, where changes in various cardiovascular parameters are measured in real time. PRO is administered after tachycardia is established to decrease the elevation in HR.

We employed several techniques to investigate the metabolic dysregulation. In addition to HIEC and IGTT, high performance liquid chromatography (HPLC) was used to analyze plasma CAT and drug levels at designated time points. We hypothesize that: 1) acute administration of CLZ will induce insulin resistance and glucose intolerance in rats shown through HIEC and IGTT experiments, 2) the SNS plays a pivotal role in mediating these

metabolic effects, where its heightened activity following CLZ administration is reflected through elevated plasma NE levels, 3) pharmacological intervention using adrenergic blockers will have beneficial effects in increasing insulin sensitivity following CLZ administration, 4) MEC will block neuronal transmission and consequently reverse the glucose dysregulation associated with CLZ use and 5) NOR will have a lesser effect on metabolic dysregulation in comparison to its parent compound, CLZ.

As a novel approach, we administered CLZ to induce acute tachycardia in rats and recorded the changes in cardiovascular parameters over time. PRO is then administered to reverse the tachycardia. In a separate experiment, plasma samples are collected to measure CAT levels following the administration of CLZ and MEC. We hypothesize that: 1) CLZ induces acute tachycardia that will gradually subside over time, 2) PRO can reverse CLZ-induced tachycardia, 3) sympathetic activity reflected via plasma NE levels is increased following CLZ administration, 4) plasma NE levels will decrease immediately after administration of MEC due to blockade of neuronal transmission.

Chapter 2: Acute metabolic side effects of clozapine and norclozapine in rodents

2.1 Introduction

The SGA drug CLZ is the preferred pharmacological agent for treatment-resistant schizophrenia, as it displays superior efficacy to other SGAs in approximately 30% of patients who are persistently unresponsive or under-responsive to other antipsychotic drugs (Honer et al., 2015; Remington et al., 2015a). Furthermore, CLZ can reduce suicidality in patients with treatment-resistant schizophrenia (Meltzer, 1999; Kerwin and Bolonna, 2004), being the only pharmacotherapy approved by the U.S. Food and Drug Administration to manage suicidal behavior in patients with schizophrenia or schizoaffective disorder (Citrome et al., 2016). Additional clinical benefits of clozapine include its low incidence of neurological side-effects, with the lowest risk among all antipsychotics for EPS (Miller, 2000; Divac et al., 2014), and hyperprolactinemia (Haddad and Wieck, 2004; Melkersson, 2005).

However, the clinical benefits of CLZ are countered by a number of severe adverse drug reactions (Remington et al., 2016). These include a wide range of cardiovascular and immune side-effects, which can be lethal in some patients (Leung et al., 2012; Roge et al., 2012; Tse et al., 2015; Yuen et al., 2018). More commonly, CLZ is associated with metabolic complications, which include both glycemic dysregulation and insulin resistance (Tse et al., 2014; Whitney et al., 2015; Zimbron et al., 2016). The mechanisms underlying these effects remain largely unknown, and were initially believed to be in part due to CLZ's high

propensity to cause weight gain (Allison et al., 1999; Wirshing et al., 1999; Whitney et al., 2015). However, preclinical studies in rodents have shown these metabolic effects can occur acutely, even in the absence of weight gain (Houseknecht et al., 2007; Chintoh et al., 2009; Boyda et al., 2010a; Boyda et al., 2013c). Animal studies offer the advantage of precisely controlled intervention, and clozapine patients – in particular – often have complicated and chronic medical histories, with extensive previous drug trials and drug polypharmacy being common (Lee et al., 2018), which could act as potential confounds for metabolic effects. In addition, preclinical studies allow the use of more invasive tests, including the glucose tolerance test, which measures glucose clearance after a glucose challenge, and the HIEC, which measures whole body insulin resistance (Muniyappa et al., 2008).

The metabolism of CLZ is of interest, as there is evidence to suggest that individual metabolites may possess therapeutic properties (Young et al., 1998a; Maehara et al., 2011b; Maehara et al., 2011a). The primary focus has been on the active metabolite, NOR, and its interaction with M₁ muscarinic receptors (Sur et al., 2003; Weiner et al., 2004; Li et al., 2005). NOR is a more potent M₁ receptor agonist than CLZ (Davies et al., 2005), and its ratio to CLZ in serum predicts therapeutic outcome (Weiner et al., 2004) and working memory performance (Rajji et al., 2015; Molins et al., 2017). Indeed, clinical studies have demonstrated that co-administration of CLZ and selective serotonin reuptake inhibitors, such as fluvoxamine, improve therapeutic efficacy via modification of the CLZ to NOR ratio (Goff et al., 1990; Hiemke et al., 1994; Szegedi et al., 1995) in which they reduce NOR formation by inhibiting the CYP450 1A2 isoenzyme (Legare et al., 2013). Importantly, a clinical trial by (Lu et al., 2004) observed that co-treatment of CLZ with fluvoxamine, which decreased NOR levels, prevented the significant increase in glucose and triglyceride levels

reported with CLZ monotherapy, and changes in weight, serum glucose and triglyceride levels were correlated with the plasma concentration of NOR, but not with levels of CLZ. The goal of the present study was therefore to directly test the metabolic effects of NOR and CLZ head-to-head for the first time. By using both the glucose tolerance test and the HIEC, we sought to provide a comprehensive assessment of whether NOR contributes significantly to the adverse metabolic effects associated with CLZ use.

2.2 Materials and methods

Animals

Female Sprague Dawley rats (Charles River Laboratories, Montreal, QC, Canada) weighing 300–350 g were habituated in the University of British Columbia's (UBC) Animal Research Unit facility for at least 7 days prior to use, and kept under a 12-hour light/dark cycle (lights on at 07:00h) at $22 \pm 1^\circ\text{C}$. Animals were pair-housed in a temperature and humidity controlled environment with free access to food and water. Female rats were used as they tend to show antipsychotic-drug induced metabolic disturbances that are more consistent and robust than males (Davey et al., 2012). All experimental procedures involving animals were approved by the UBC's Animal Care Committee and in compliance with Canadian Council on Animal Care guidelines.

Pharmacological agents and solutions

CLZ and NOR were obtained from Toronto Research Chemicals Inc. (Toronto, ON, Canada) and prepared immediately before use. Drugs were dissolved in a vehicle polyethylene glycol (PEG) solution consisting of 50% PEG, 40% distilled water and 10% ethanol, as previously (Boyda et al., 2012a; Wu et al., 2014). Pharmacological agents were administered via intraperitoneal (i.p.) injections at volumes of 1 ml/kg. Glucose solutions were comprised of 50% dextrose in 0.9% saline w/v. Human insulin (Eli Lilly, Indianapolis, IN, USA) was prepared in 0.9% w/v saline.

Intraperitoneal Glucose Tolerance Test (IGTT)

A week prior to commencing the IGTT, rats (n = 40) were subjected to a washout IGTT to determine total glucose levels, as per (Boyda et al., 2010b; Boyda et al., 2012b; Boyda et al., 2014a). Briefly, rats were fasted overnight and weighed on the day of the washout IGTT. Blood was obtained from the lateral saphenous vein at baseline and blood glucose was measured using handheld glucometers (One Touch Ultra) (Boyda et al., 2014b). Animals were immediately subjected to a glucose challenge (1 g/kg/ml, i.p.) and blood glucose readings were taken after 15 mins, then every 30 mins thereafter for a further 90 mins. Rats were ranked in order according to total glucose levels (area under the curve) and then matched to 1 of 10 treatment groups at random. Each rat was assigned either vehicle or different doses of CLZ or NOR (0.5, 2, 8, 20 mg/kg/ml, i.p.) for the first week of testing, then randomized to a different drug following a 1 week washout period (i.e. each rat only received 2 treatments); no carryover effects were observed between weeks (i.e. the mean levels of glucose in the glucose tolerance test for all groups were not significantly different in the

second test than in the first test). Mean rat body weights were 248.7 ± 1.9 g during the first test, and 254.9 ± 1.9 g during the second test. The procedure of the drug challenge test is similar to the pre-screen test, except that after the baseline blood glucose measure, the drugs were immediately administered and a second fasting blood glucose measure was taken after 60 mins. Subsequently, rats were subjected to a glucose challenge (1 g/kg/ml, i.p.) and glucose measures were taken every 15 mins thereafter for another 120 mins.

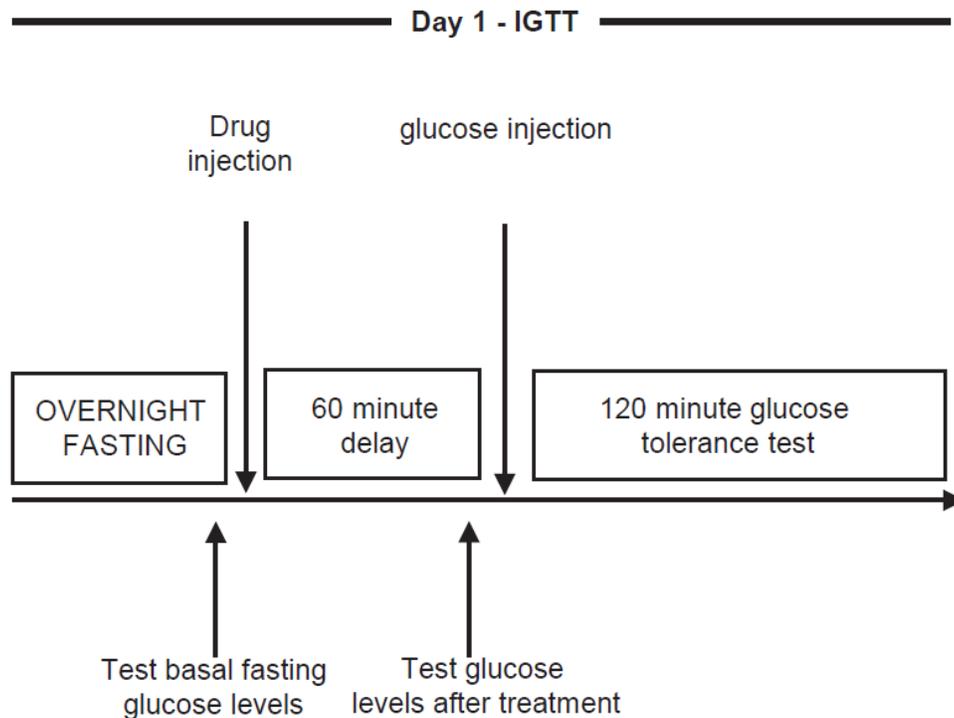


Figure 2.1: Overview of the experimental procedure for the intraperitoneal glucose tolerance test (IGTT).

Surgical procedures

Animals were anesthetized with isoflurane and given ketoprofen (5 mg/kg, s.c.) prior to surgery. The right common carotid artery and both exterior jugular veins were exposed and cannulated with polyethylene cannulae (PE50). The cannulae were tunneled subcutaneously from the ventral neck to the dorsal neck and exteriorized. The arterial cannula was used to sample blood for determination of blood glucose, while the jugular veins were for constant infusion of insulin and variable infusion of dextrose. Animals were allowed to recover for 24 hours before commencing the HIEC experiment.

Hyperinsulinemic-Euglycemic Clamp (HIEC)

The HIEC procedure was described previously (Boyda et al., 2013b; Wu et al., 2014). Experiment-naïve rats were individually housed and fasted overnight for 16 ± 2 hours with free access to water prior to and during the experiment. Venous cannulae were connected to infusion-only pumps (Harvard Apparatus, Holliston, MA, USA) for infusion of dextrose (50% w/v) and insulin (3 mU/kg/min). Blood samples from the arterial cannula were obtained every 10 minutes to determine blood glucose levels. Insulin infusions were kept at a constant rate for the duration of the experiment and glucose infusion rate (GIR) was adjusted every 10 minutes to maintain blood glucose concentrations at 5.6 – 6.4 mmol/L, where euglycemia refers to 3 consecutive measures within this range. Twenty-nine rats were randomly assigned to 1 of 5 treatments: PEG vehicle (n = 5), 2 or 20 mg/kg/ml CLZ (n = 6, each dose) or 2 or 20 mg/kg/ml NOR (n = 6, each dose). Rats received i.p. injections of vehicle or drug once euglycemia was reached and the experiment was continued for an additional 120 mins. Animal handlers were blinded to drug treatments.

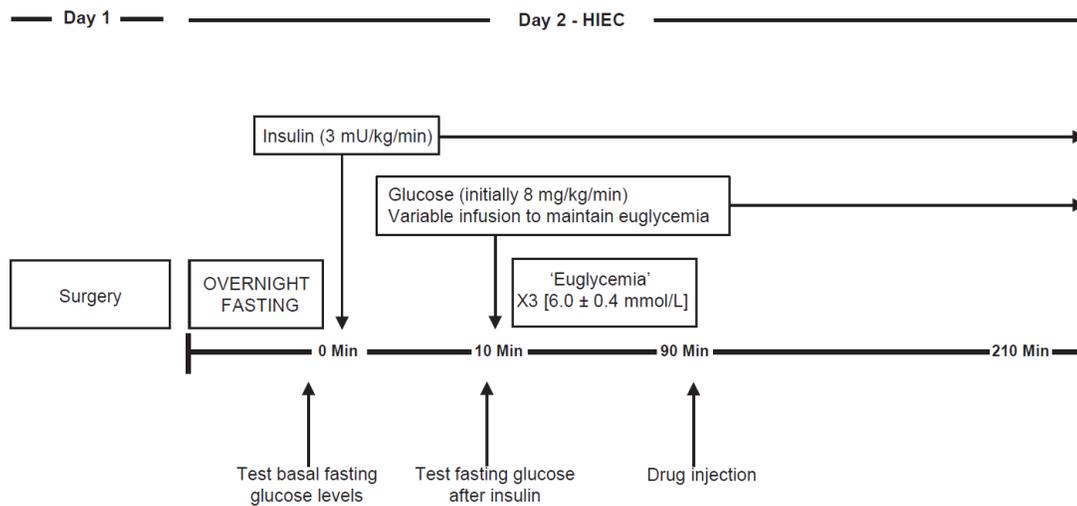


Figure 2.2: Overview of the experimental procedure for the hyperinsulinemic-euglycemic clamp (HIEC).

Sample preparation for determination of plasma clozapine and norclozapine concentrations

In order to determine the effects of treatment with clozapine on plasma levels of CLZ and NOR, a separate group of rats ($n = 6$) were treated with the higher (20 mg/kg) dose of clozapine from the clamp study. Female rats were injected with the drug i.p. as per the prior experiments, and blood was collected from the saphenous vein (200 μ l) every 60 mins until 300 mins after treatment. Whole blood was centrifuged (10,000 rpm, 10 min, 4 $^{\circ}$ C) to obtain plasma, and stored at -80° C. Standard curves for CLZ and NOR were generated by dilutions of 2 mg/ml stock solutions with 0.1 M orthophosphoric acid (OPA). Risperidone was diluted in 0.1 M OPA to obtain a 10 μ g/ml internal standard. Eppendorf tubes containing 45 μ l of plasma, 5 μ l of 10 μ g/ml risperidone and 55 μ l of 0.5 M dibasic sodium phosphate

were vortex mixed and 800 μl of 70:30 (v/v) diisopropyl ether:pentane was added. Solutions were vortex mixed, centrifuged (1400 rpm, 10 min) and the organic layer was transferred to new microcentrifuge tubes containing 100 μl of 0.1 M OPA. This was followed by vortex mixing, centrifugation and removal of the organic layer. This was followed by addition of 40 μl of 70:30 (v/v) diisopropyl ether:pentane, vortex mixing, centrifugation and the organic layer was discarded. The remaining solution was vacuum centrifuged for 1 min at 1400 rpm and 40 μl was transferred to individual vial inserts for HPLC-UV analysis.

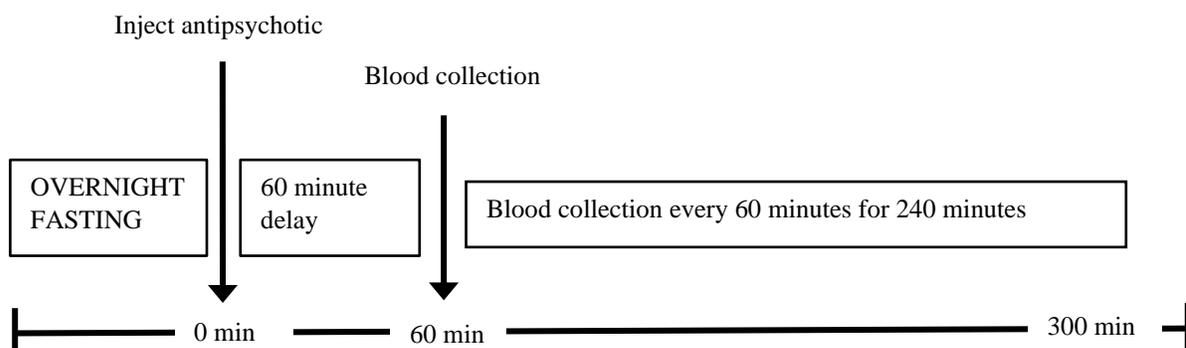


Figure 2.3: Overview of the experimental procedure for blood sample collection.

High Performance Liquid Chromatography (HPLC)-Ultraviolet (UV) Detection

CLZ and NOR levels were determined using a Thermo Scientific Dionex UltiMate 3000 HPLC system coupled to a VWD-3100 UV/Vis detector (Thermo Scientific, Sunnyvale, California). Mobile phase (1.9 L double-distilled water, 1.1 L acetonitrile, 6 ml triethylamine, pH 3.3) was pumped through the system at a flow rate of 0.3 ml/min and samples were injected in 20 μl volumes. Analytes were separated on a Thermo Scientific Synchronis aQ 250 x 4.6 mm aQ HPLC column and quantified by the UV-detector (detection at 245 nm), and

concentrations were determined using Chromeleon software (Thermo Scientific, Sunnyvale, California).

Statistical Analyses

Glucose indices from the IGTT were analyzed using one-way analysis of variance (ANOVA), with drug treatment as the between group factor. Glucose data for all time points after glucose injection were summed as the area under the curve. The clamp results were analyzed using a between-within subject analysis, with drug treatment as the between subjects factor and clamp indices (blood glucose levels, GIR over time, and change in GIR over time from baseline levels) as the within subjects factor. Level of significance was set at $p < 0.05$ and Fisher's least significant difference (LSD) post-hoc tests were conducted when a main effect or interaction between main effects was significant. Data were analyzed with SPSS software, Chicago, IL, version 21.

2.3 Results

IGTT

The analysis of the data revealed that none of the groups differed from each other at the initial fasting baseline glucose measurement, indicating that the groups were well matched on glucose levels [$F_{(8,79)} = 1.27$, NS]. Following treatment with the drugs, there was a significant main effect on pre-challenge glucose levels [$F_{(8,79)} = 6.69$, $p < 0.001$] with post-hoc analyses

indicating that the 8 and 20 mg/kg/ml doses of CLZ, as well as the 20 mg/kg/ml dose of NOR, caused a significant increase in glucose levels compared to vehicle treated animals. Following the glucose challenge injection, all animals showed a large and sustained increase in glucose levels throughout the 120 min test period (Figure 2.4). The analysis of the cumulative glucose levels during the 120 mins test, as the area under the curve, revealed a significant main effect of drug treatment [$F_{(8,79)} = 10.75$, $p < 0.001$]. Post-hoc analysis specified that both the 8 and 20 mg/kg/ml CLZ doses showed large and highly significant ($p < 0.001$) increases in the area under the curve relative to vehicle; while this same measure showed an increase in the 20 mg/kg NOR dose, this effect was only a non-significant trend ($p = 0.09$). Exploratory analyses indicated that the 8 and 20 mg/kg/ml NOR doses significantly increased glucose levels compared to vehicle in the first 15 min, but this effect diminished by 30 min after glucose challenge. By contrast, the increase in glucose levels with the two higher doses of CLZ was sustained, and glucose levels always remained significantly higher than vehicle glucose levels for the duration of the test.

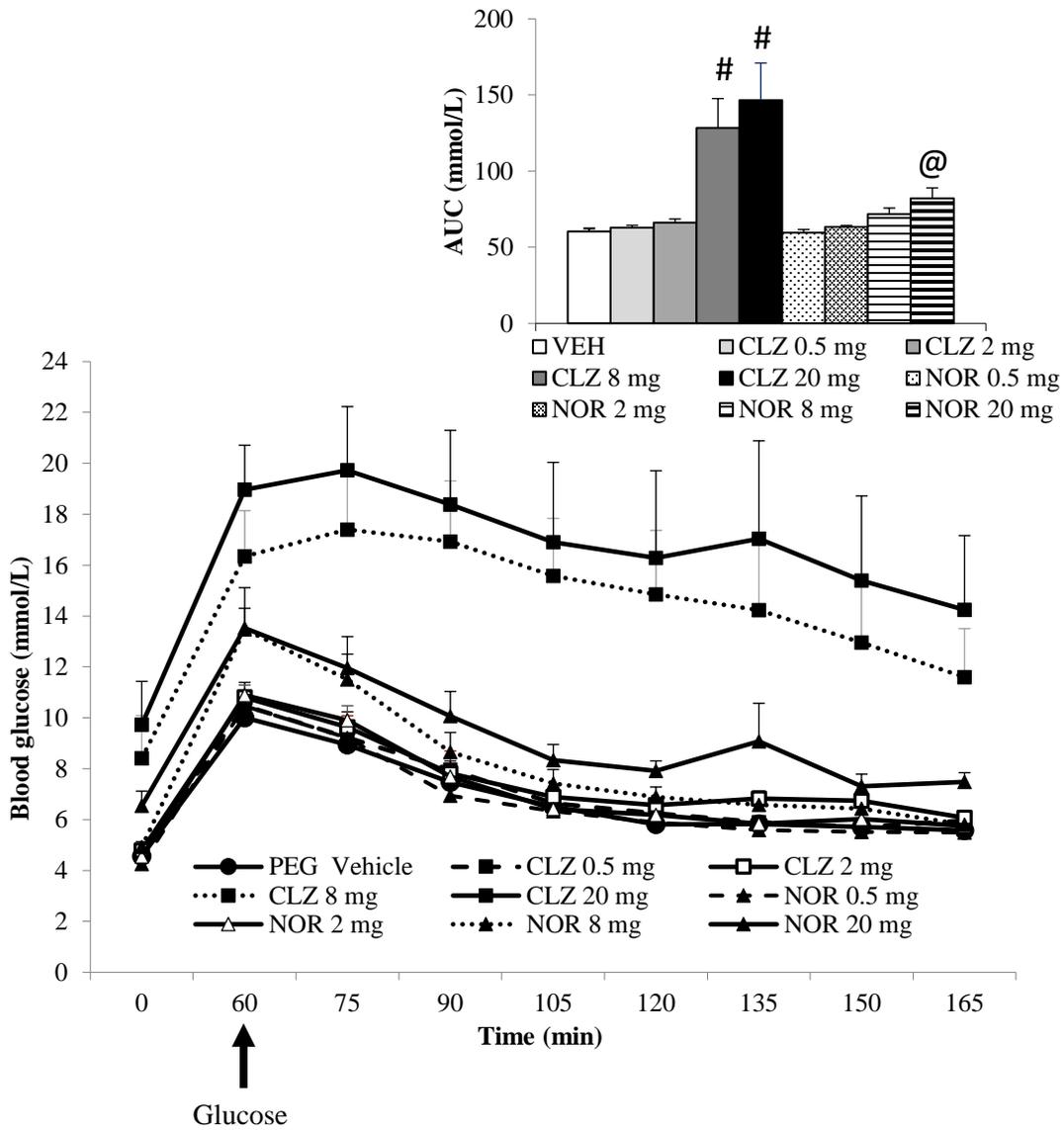


Figure 2.4: Acute effects of CLZ and NOR on glucose levels in adult female rats in the IGTT. Animals (n = 8 per group) received a single injection of vehicle, CLZ or NOR (0.5, 2, 8, 20 mg/kg i.p.) in a volume 1 ml/kg. Glucose levels were recorded prior to drug treatment in overnight-fasted rats, and then 60 min following drug administration (x-axis). Immediately following this glucose measurement, all rats were subjected to an IGTT by receiving an i.p. challenge injection of 1 g/ml/kg of glucose, and blood glucose levels were measured every 15 mins for the next 120 mins. Total cumulative glucose levels for each treatment group are summed as the “area-under-the-curve” (AUC) during the glucose tolerance test by graph inset (top right). Values represent group means \pm SEM; * indicates fasting glucose levels higher than vehicle-treated animals, $p < 0.05$; # indicates area-under-the-curve greater than vehicle-treated animals, $p < 0.001$; @ indicates area-under-the-curve non-significant trend greater than vehicle-treated animals, $p < 0.1$.

HPLC

Levels of both CLZ and NOR were well within the limit of detection at all time points measured. Plasma levels of both compounds had peaked by the first 60 min, and demonstrated a predicted gradual decline out to 300 mins after treatment. Mean group levels of both compounds are presented in Table 1.

Table 2.1: Mean plasma levels of CLZ and NOR after acute treatment of CLZ in drug-naïve adult female rats.

Time after treatment (mins)	Clozapine levels (ng/ml)	Norclozapine levels (ng/ml)
60	172 ± 31	152 ± 38
120	101 ± 18	96 ± 22
180	58 ± 16	38 ± 15
240	53 ± 9	32 ± 23
300	44 ± 10	28 ± 17

Drug levels were measured every 30 mins after a single i.p. injection of 20 mg/kg/ml CLZ in a group (n = 6) of drug-naïve adult female rats. Values are expressed as group mean ± SEM.

HIEC

Average basal glucose levels were similar for all groups prior to euglycemia and administration of the two drugs. Whole body insulin resistance during the HIEC is indicated by a reduction in the GIR, and therefore the primary analysis compared the effects of antipsychotics on change in the GIR over 120 min, as is standard for such studies (Hahn et al., 2011; Boyda et al., 2013b; Wu et al., 2014). For the overall ANOVA, drug treatment represented the between-subjects factor, while the change in GIR over time from the baseline

value at $t = 0$ mins (i.e. at administration of the drug) was the within-subjects factor. The ANOVA indicated a significant main effect of drug treatment [$F_{(4,2323)} = 39.90, p < 0.001$], a main effect of time [$F_{(1212,276)} = 25.48, p < 0.001001$], and a significant interaction of drug treatment \times time [$F_{(48,276)} = 5.80, p < 0.001$]. The vehicle treated group maintained a constant GIR over the 120 mins clamp, whereas all drug treated groups showed a rapid and sustained decrease in the GIR (Figure 2.5). This peaked for most animals between 20 and 30 min after drug treatment, followed by a slow increase in the GIR to control values. Post-hoc analysis revealed that for the entire 120 min clamp, all four drug treated groups displayed a significantly reduced GIR compared to the vehicle treated animals. Drug effects appeared to be dose dependent, as the high dose of each drug produced significantly greater decreases in the GIR than the low dose ($p < 0.01$). The 2 mg/kg doses of CLZ and NOR did not differ significantly in their effects on the GIR, while the 20 mg/kg dose of CLZ produced a greater decrease in the GIR than all other groups ($p < 0.05$), evident when represented by the area under the curve (Figure 2.5 inset).

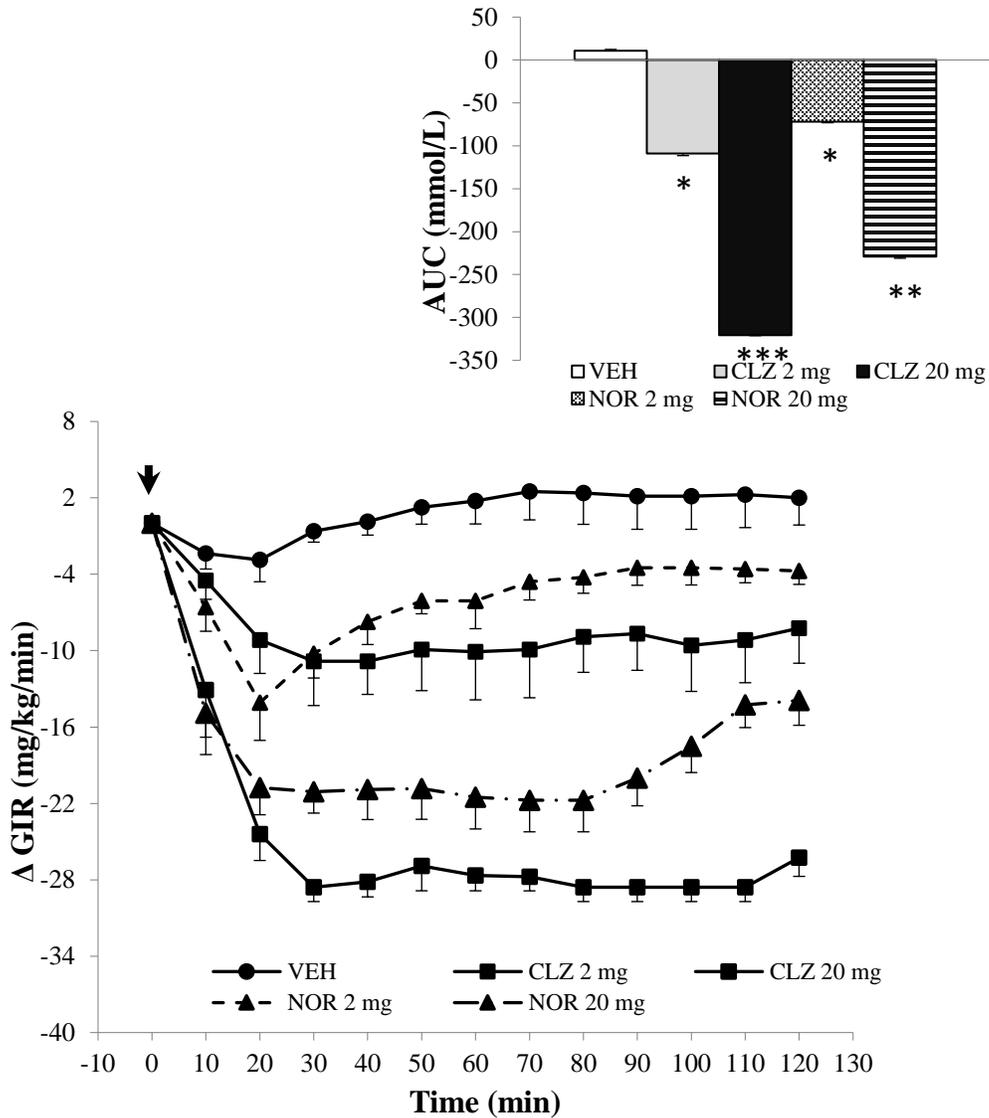


Figure 2.5: Acute effects of CLZ and NOR in adult female rats in the HIEC. Animals (n = 5–6 per group) were fasted overnight and subjected to the HIEC. After animals reached euglycemia (3 consecutive blood glucose readings of 6.0 ± 0.4 mmol/L), rats were treated with vehicle, CLZ or NOR (2 or 20 mg/kg, i.p.) in a volume of 1 ml/kg. Glucose levels were recorded every 10 mins and the glucose infusion rate was adjusted as needed. Glucose infusion rates for each treatment group are presented as change in GIR from euglycemia. Values represent group means \pm SEM. Total cumulative change in GIR for each treatment group are summed as the “area-under-the-curve” (AUC) over the HIEC from t = 0 min. * indicates significantly lower than vehicle-treated animals, $p \leq 0.01$; ** indicates significantly lower than vehicle-treated, 2 mg/kg CLZ and 2 mg/kg NOR animals, $p < 0.001$; *** indicates significantly lower than all other groups $p < 0.005$.

2.4 Discussion

In the current study, we assessed the metabolic side-effects of the primary CLZ metabolite NOR for the first time, as well as CLZ itself for reference as a positive control. Using two separate, well-established techniques, we were able to evaluate metabolic dysregulation with both the HIEC and the IGTT. Consistent with a handful of previous rodent studies, CLZ caused dose-dependent glucose intolerance in the glucose tolerance test and insulin resistance in the HIEC (Houseknecht et al., 2007; Tulipano et al., 2007; Chintoh et al., 2009; Smith et al., 2009; Boyda et al., 2010b; Boyda et al., 2013c). In comparison, the effects of NOR were not as pronounced in the glucose tolerance test, with only the highest dose having an effect on fasting glucose levels and a non-significant trend to increase glucose levels throughout the 120 mins tolerance test. By contrast, both doses of NOR caused a robust and sustained decrease in the GIR in the HIEC, indicating that they induced potent whole body insulin resistance. The present findings reconfirm the strong metabolic effects of the antipsychotic drug CLZ, and also demonstrate for the first time that its primary metabolite NOR has potent metabolic effects, when tested using specific procedures. The 2 techniques used in this study provide complementary indices of metabolic dysregulation, and represent the most reliable and accurate methods of assessing glucose tolerance and insulin resistance. In previous studies where we compared the SGAs olanzapine, asenapine and lurasidone concurrently with both the IGTT and the HIEC, there was a strong but not perfect consensus between metabolic effects with the 2 techniques (Boyda et al., 2013b; Wu et al., 2014). Presently, both techniques consistently revealed metabolic dysregulation with higher doses of CLZ. But there was a clear disparity for NOR, where effects were proportionally much weaker in the IGTT than in the HIEC. The explanation for this is not immediately apparent. In the clamp,

insulin levels are fixed, at a high level for the duration of the test, while in the IGTT, levels of both insulin and glucose are free to vary. It is feasible that both CLZ and NOR cause insulin resistance and therefore elevate glucose levels, but in the in the case of NOR, the hyperglycemia associated with initial glucose intolerance could be overcome by greater release of insulin from pancreatic beta-cells, to cause increased glucose uptake by the body. By contrast, CLZ may directly inhibit beta cell function, thereby attenuating insulin release in response to a glucose challenge, in a manner similar to that seen with the related SGA olanzapine (Chintoh et al., 2009; Park et al., 2010; Hahn et al., 2011). Indeed (Chintoh et al., 2009), previously demonstrated, using the hyperglycemic clamp, that both CLZ and olanzapine impaired beta cell function, evident as decreased insulin secretion. Future studies with NOR should therefore use the hyperglycemic clamp to determine whether it also impairs beta cell function, although the present findings would suggest that it does not. Both olanzapine and CLZ are potent antagonists at the muscarinic M₃ receptor, which has been linked to decreased insulin release from beta cells (Weston-Green et al., 2013). NOR, in contrast, is an M₃ agonist (Davies et al., 2005) and therefore unlikely to inhibit insulin release in the same manner from beta cells. In addition, it would be of strong interest to compare the effects of both CLZ and NOR on serum insulin and C-peptide concentrations prior to, and at the time point corresponding to peak glucose, during the IGTT.

An alternative explanation for the difference in effects observed for NOR between the euglycemic clamp and the tolerance test is that the drug was administered at time zero for the clamp, but glucose levels were not measured until 60 mins after drug administration in the IGTT; this period was chosen to be consistent with previous studies from our laboratory (Boyda et al., 2010b; Boyda et al., 2013c). However, this is unlikely to account for the

discrepancy, as data from the current HIEC experiment clearly demonstrated that NOR still exerted potent metabolic effects after 60 mins, even with the lower 2 mg/kg/ml dose. Also, the plasma elimination half-life of NOR in the rat is approximately the same as that of clozapine (Baldessarini et al., 1993), and so levels of both drugs would be expected to be similar at the 60 mins time point, when CLZ was still causing notable glucose intolerance.

Neither the mechanisms by which both CLZ and NOR cause whole body insulin resistance, nor whether these physiological pathways shared by the two drugs are the same, are known. Data from studies with olanzapine, which is closely structurally related to CLZ, suggest that both peripheral and central insulin mechanisms may be at play (Hahn et al., 2014; Kowalchuk et al., 2017),, as well as enzymes involved in cellular energy homeostasis, such as 5' AMP-activated protein kinase (Bush et al., 2018), but further mechanistic studies are needed.

Dosing for the present study was based on previous reports using CLZ and NOR by ours and other laboratories. Previous rat metabolic studies with CLZ have observed effects with CLZ doses from 1 to 10 mg/kg (Houseknecht et al., 2007), 10 mg/kg (Smith et al., 2009), 7.5 mg/kg (Tulipano et al., 2007) and 2–20 mg/kg (Boyda et al., 2010b; Boyda et al., 2013c). To our knowledge, there are no studies, with any species, that have administered NOR to directly examine its metabolic effects. Of the relatively few preclinical studies in literature that administered NOR, the focus was on discriminative stimulus properties to assess antipsychotic efficacy (Prus et al., 2009; Wiebelhaus et al., 2012) at doses from 0.25 to 20 mg/kg, while (Maehara et al., 2011c) observed behavioral effects in rats using doses between 10 and 30 mg/kg. Data from the group of rats in the present study treated with the 20 mg/kg dose of CLZ that were assayed for plasma levels of the drug indicated that levels of

both CLZ and NOR had peaked by 60 mins. Levels of CLZ at this time point for the group were 172 ± 31 ng/ml, and 152 ± 38 ng/ml for NOR. While comparison of plasma levels between rats and humans bears certain caveats, previous studies in humans who are at steady state with CLZ indicate that treatment with a 100 mg/day dose of CLZ results in levels of 150 ng/mL plasma CLZ, and NOR levels about half as much (Chang et al., 1997). As the current animals were not at steady state (which would result in higher plasma levels), these findings strongly suggest that the current doses of CLZ resulted in plasma levels of CLZ and NOR homologous to those observed in patients treated with clinically relevant doses of CLZ.

Clinical data on the direct metabolic effects of NOR are scant. Although the drug was tested as a potential antipsychotic drug by Acadia Pharmaceuticals (as ACP-104; ClinicalTrials.gov Identifier: NCT00490516), we are not aware of any reported data from those trials on metabolic effects. Perhaps the most relevant clinical study was a recent report by (Lu et al., 2018), where in a 12-week randomized, double-blinded study, fluvoxamine treatment combined with low dose CLZ resulted in reduced gains in body weight, and levels of insulin, glucose, and triglycerides, compared to higher dose CLZ monotherapy. The fluvoxamine combination group had lower NOR levels and higher CLZ–NOR ratios. While this may suggest that decreasing NOR levels is a good metabolic strategy, the study was confounded by several key issues, including different CLZ doses between the two groups (CLZ's metabolic effects are dose dependent (Simon et al., 2009), although plasma levels remained similar between both groups) and the addition of an SSRI, which could affect appetite (Carter et al., 2003; Lu et al., 2018). Nevertheless, the findings are consistent with our current results, where NOR acutely produces potent insulin resistance. Given that both CLZ and its primary metabolite NOR cause insulin resistance, decreasing NOR levels

through CYP1A2 inhibition could hypothetically decrease the additive effects of both compounds on insulin resistance. Although CLZ appears to have greater metabolic effects than NOR, the summed effects of both compounds should be considered.

In conclusion, the results of the present study indicate that NOR, when administered acutely, produces mild effects on glucose intolerance, but potent dose-dependent effects on insulin resistance. Further study is required to determine the physiological pathways that are involved, and understand how the two compounds differ in their metabolic effects. An important limitation of this study was the absence of data on the effects of CLZ and NOR on insulin and C-peptide levels in the glucose challenge tests, and should be a priority for future studies in animals and humans. Overall, the present observations may be cautiously considered relevant to human studies, and progress in this field may hope to better understand how CLZ and other SGAs exert their harmful metabolic side-effects.

Chapter 3: Adrenoceptor antagonists in the treatment of clozapine-induced acute glucose dysregulation

3.1 Introduction

Sympathetic hyperactivity has been implicated in the pathogenesis of multiple metabolic disturbances leading to the metabolic syndrome and ultimately cardiovascular disease.

Individual components of the metabolic syndrome, as discussed in Chapter 1, can arise from abnormalities of the sympathetic nervous system (SNS) by: 1) direct interaction with adrenoceptors and/or changes in regional blood flow, 2) sympathetic hyperactivity and 3) damage to organs (i.e. arterial stiffening, cardiac hypertrophy and hyperplasia, arterial wall thinning) (Mancia et al., 2007). The metabolic effects of heightened sympathetic activity are most notable with hypertension and obesity (Moreira et al., 2015).

Sympathetic hyperactivity is a common characteristic of obese individuals, as reflected by elevated plasma norepinephrine (NE) levels (Tuck, 1992). Obesity is strongly associated with the development of hypertension, type 2 diabetes and dyslipidemia that lead to the metabolic syndrome (Mancia et al., 2007; Zalesin et al., 2008). In patients with metabolic syndrome, sympathetic activity in muscles has been shown to be positively correlated with the homeostasis model assessment index (indicator of insulin resistance) and the body mass index (Grassi et al., 2005). The sympathetic overactivity is further potentiated in hypertensive obese subjects (Grassi et al., 2000), attributable to factors such as impaired baroreflex sensitivity and increased leptin release (Aneja et al., 2004; Grassi et al., 2007; Moreira et al., 2015). Importantly, an overactive SNS elicits structural changes in the heart,

namely hypertrophy and lesions that are also present in atherosclerosis, which possibly explains the increased incidence of sudden death in obese patients with hypertension (Grassi et al., 2000).

Insulin resistance is associated with increased sympathetic activity, commonly measured by the rate of which radioactively labeled NE is released into plasma or “NE spillover” (Esler et al., 2001; Mancia et al., 2007). In skeletal muscle, insulin resistance is believed to be secondary to hemodynamic defects, as insulin-mediated glucose uptake is dependent upon regional blood flow (Baron et al., 1990). The hypertensive state resulting from increased sympathetic outflow contributes to insulin resistance, where vasoconstriction of skeletal muscle is known to reduce glucose uptake and insulin availability (Julius et al., 1992). In support of this, β -adrenoceptor blockers that reduce cardiac output and raise vascular resistance are known to exacerbate insulin resistance (Julius et al., 1992). In another randomized double-blinded clinical study, patients with hypertension who were treated with the β_1 -adrenoceptor antagonist atenolol (ATEN) displayed impaired insulin sensitivity followed by elevated serum lipids after 48 weeks of treatment (Reneland et al., 2000). Abnormalities in insulin sensitivity can impair vasodilation and decrease skeletal muscle blood flow via a decrease in nitric oxide production and bioavailability, thus limiting insulin-mediated glucose uptake (Manrique et al., 2014).

Moreover, there is extensive evidence of NE-mediated lipolysis in white adipose tissue by activation of β -adrenoceptors (Langin, 2006; Wang et al., 2008b; Bartness et al., 2014). The breakdown of triglycerides into glycerol and free fatty acids is increased in obesity and influences the risk of developing diabetes and the metabolic syndrome (Arner, 2005; Guilherme et al., 2008). In obese subjects, the elevation in the basal rate of lipolysis is

tightly linked to the size of fat cells (Engfeldt and Arner, 1988). The continuous exposure of skeletal muscle to circulating fatty acids eventually causes hypertrophy of adipocytes, thereby disrupting insulin-dependent glucose transport to lower insulin sensitivity and ultimately leads to insulin resistance (Guilherme et al., 2008).

The abovementioned abnormalities are especially concerning in patients with schizophrenia who are treated with clozapine (CLZ), as CLZ is strongly associated with elevated plasma NE levels (Green et al., 1993b; Breier et al., 1994; Brown et al., 1997; Elman et al., 1999; Yuen et al., 2019). Our results from the previous chapter suggest a role of the ANS in the mediation of the metabolic side effects of CLZ. In particular, adrenoceptors have been implicated in glucose dysregulation resulting from CLZ treatment, although the underlying mechanism remains unclear (Guenette et al., 2013). It is possible that CLZ-induced insulin resistance and glucose dysregulation directly result from perturbances to adrenoceptors situated on tissues regulating glucose homeostasis (Boyda et al., 2013a). Known examples include the α_1 -adrenoceptors of adipose tissue and liver, α_2 -adrenoceptors of the pancreas and β_2 -adrenoceptors of skeletal muscle and liver (Boyda et al., 2013a). The involvement of α_1 -adrenoceptors was demonstrated preclinically, as blockade using prazosin (PRA) significantly reduced insulin secretion in rodents (Guenette et al., 2013). Conversely, idazoxan (IDA), an α_2 -adrenoceptor antagonist, increased insulin sensitivity during the hyperglycemic clamp (Guenette et al., 2013).

To further elucidate CLZ's influence on the ANS and subsequent metabolic disturbances at the receptor level, we selectively administered α - and β -adrenoceptor antagonists in rats treated with CLZ and assessed insulin resistance using the HIEC. PRA, IDA, ATEN and butoxamine (BUT) were randomly administered to specifically inhibit α_1 -

α_2 -, β_1 - and β_2 -adrenoceptors, respectively, 50 mins following CLZ injection. The effects of CLZ-induced glucose dysregulation and its possible reversal by adrenoceptor blockade was further assessed through ganglionic inhibition by administration of MEC in a separate group of animals. Of note, for this study, the dose of CLZ was adjusted to 10 mg/kg/ml to match HIEC studies in literature (Chintoh et al., 2009). To our knowledge, this is one of the first studies to investigate the possibility of adrenoceptor blockade as an intervention to reverse CLZ-induced glucose dysregulation.

3.2 Materials and methods

Animals

Female Sprague Dawley rats (Charles River Laboratories, Montreal, QC, Canada) weighing approximately 300 – 350 g were habituated in UBC's Animal Research Unit facility for at least 7 days prior to use and kept under a 12-hour light/dark cycle. Animals were pair-housed in a temperature ($22 \pm 1^\circ\text{C}$) and humidity controlled environment with free access to food and water. All experimental procedures involving animals were approved by the UBC's Animal Care Committee and in compliance with Canadian Council on Animal Care guidelines.

Pharmacological agents and solutions

CLZ was obtained from Toronto Research Chemicals Inc. (Toronto, ON, Canada) and prepared immediately before use. Drugs were dissolved in a vehicle PEG solution consisting of 50% PEG, 40% distilled water and 10% ethanol. Pharmacological agents were administered via i.p. injections at volumes of 1 ml/kg. Glucose solutions were comprised of 50% dextrose in 0.9% saline w/v. Human insulin (Eli Lilly, Indianapolis, IN, USA) was prepared in 0.9% w/v saline.

Surgical procedures

Animals were anesthetized with isoflurane and given ketoprofen (5 mg/kg, s.c.) prior to surgery. Polyethylene cannulae (PE50) containing heparinized saline were inserted into the right carotid artery and both exterior jugular veins. The cannulae were tunneled subcutaneously from the ventral neck to the dorsal neck and exteriorized. Blood samples were obtained from the arterial cannula for glucose measurements, while dextrose and insulin were separately infused via the venous cannulae. Animals were allowed to recover for 24 hours before commencing the HIEC experiment.

Hyperinsulinemic Euglycemic Clamp (HIEC)

The HIEC procedure was described previously (Boyda et al., 2013b; Wu et al., 2014). Rats were individually housed and fasted overnight for 16 ± 2 hours with free access to water prior to and during the experiment. Venous cannulae were connected to infusion-only pumps

(Harvard Apparatus, Holliston, MA, USA) for infusion of dextrose (50% w/v) and insulin (3 mU/kg/min). Blood samples from the arterial cannula were obtained every 10 minutes to determine blood glucose levels. Insulin infusions were kept at a constant rate for the duration of the experiment and GIR was adjusted every 10 minutes to maintain glucose concentrations at 5.6 – 6.4 mmol/L.

We wished to use an intermediate dose of CLZ in between the low (2 mg/kg/ml) and higher (20 mg/kg/ml) doses of the drug that were used in the previous study, to be consistent with previous HIEC studies in the literature, and we were not certain in which direction the adrenoceptor ligands could modify insulin resistance, despite our hypotheses. We therefore first aimed to replicate the dose-dependent effects of CLZ on HIEC measures of insulin resistance in 12 rats, while including a new intermediate dose of 10 mg/kg. Once these replications were complete, the aim was to use this new intermediate dose to test the effects of the adrenoceptor ligands.

Thirty-eight rats were randomly assigned to 1 of 5 treatments: PEG vehicle (n = 6), 10 mg/kg/ml CLZ (n = 6), 10 mg/kg/ml CLZ and 1 mg/kg/ml MEC (n = 5), 10 mg/kg/ml CLZ and 15 mg/kg/ml ATEN (n = 6), 10 mg/kg/ml CLZ and 15 mg/kg/ml BUT (n = 5), 10 mg/kg/ml CLZ and 15 mg/kg/ml IDA (n = 5), or 10 mg/kg/ml CLZ and 15 mg/kg/ml PRA (n = 5). Rats received a single injection of vehicle or CLZ once euglycemia was reached at t = 0 min and either received MEC at t = 30 mins or one of the adrenoceptor antagonists at t = 50 mins. Due to the novelty of these experiments, the adrenoceptor antagonists and MEC were administered at time points and in dose ranges that were known to yield significant effects from previous studies in our laboratory. This was to ensure maximal safety and survivability of the animals.

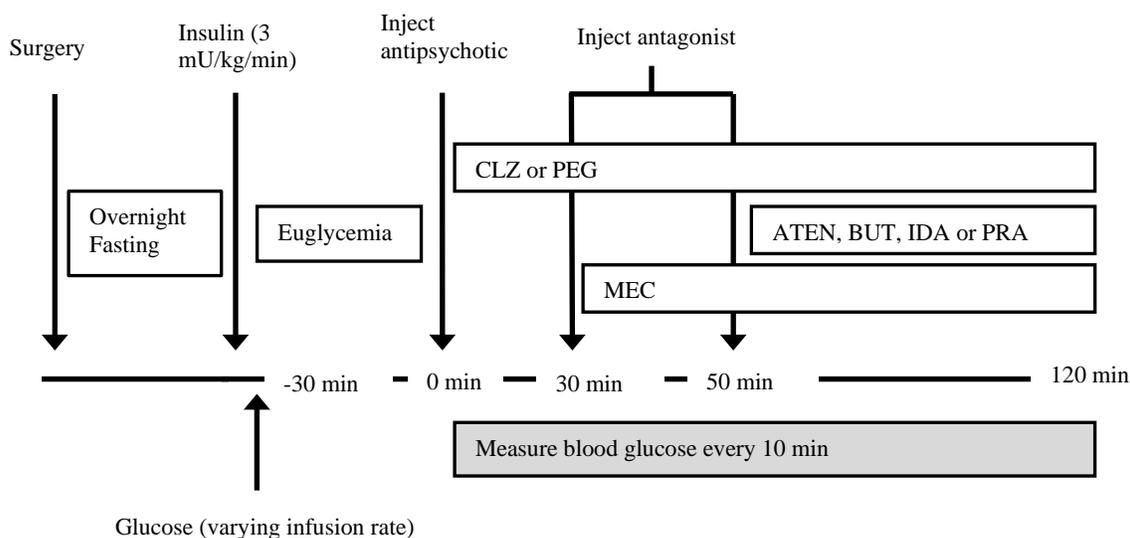


Figure 3.1: Overview of the hyperinsulinemic-euglycemic clamp procedure for acute administration of clozapine and reversal with selected adrenoceptor and ganglionic blockers. Drug-naïve adult female rats were fasted overnight and infused with insulin at 3 mU/kg/min and dextrose at varying rates. Upon reaching euglycemia (5.6 – 6.4 mmol/L), rats were injected with clozapine (CLZ) at t = 0 min. Depending on the randomly assigned treatment group, this was followed by another i.p. injection of mecamylamine (MEC) at t = 30 mins or an adrenoceptor antagonist at t = 50 mins.

Plasma preparation for High Performance Liquid Chromatography (HPLC)

CAT (NE, E, DA) levels were determined in plasma samples collected from fasted rats treated with 10 mg/kg CLZ (n = 8) or 10 mg/kg CLZ and 1 mg/kg MEC (n = 6). Blood was collected from the saphenous vein in heparinized collecting tubes at baseline, then 15, 60 and 120 minutes following CLZ administration (Figure 3.2). MEC was administered at t = 30 mins. Blood samples were centrifuged (10,000 rpm, 10 min, 4°C) and the plasma was extracted and stored at -80°C. A catecholamine standard curve was derived from diluting a stock 1 mg/kg catecholamine solution with catecholamine standard diluent, both purchased from Thermo Fisher Scientific Inc. (Chelmsford, Massachusetts). The internal standard was

diluted from a 1.0 mg/ml 3,4-Dihydroxybenzylamine (DHBA) stock solution (Thermo Fisher Scientific Inc., Chelmsford, Massachusetts). Electroactive species were removed using a preliminary acid-washed alumina extraction procedure (Lucot et al., 2005). Eppendorf tubes containing 5 mg of alumina, 50 μ l plasma, 90 μ l 3 M Tris-5% ethylene-diamine-tetraacetic acid (EDTA) buffer and 10 μ l 20 ng/ml DHBA were vortex mixed, centrifuged (10,000 rpm, 5 min, 4°C), and the supernatant discarded. Samples were washed with 400 μ l double-distilled water, centrifuged (5000x g, 1 min, 4°C) and aspirated for a total of 3 washes. After the third wash, 50 μ l of 0.1 M perchloric acid was added to the samples. These were vortex mixed, centrifuged and 40 μ l of the acidic aqueous layer was transferred into individual vials for analysis.

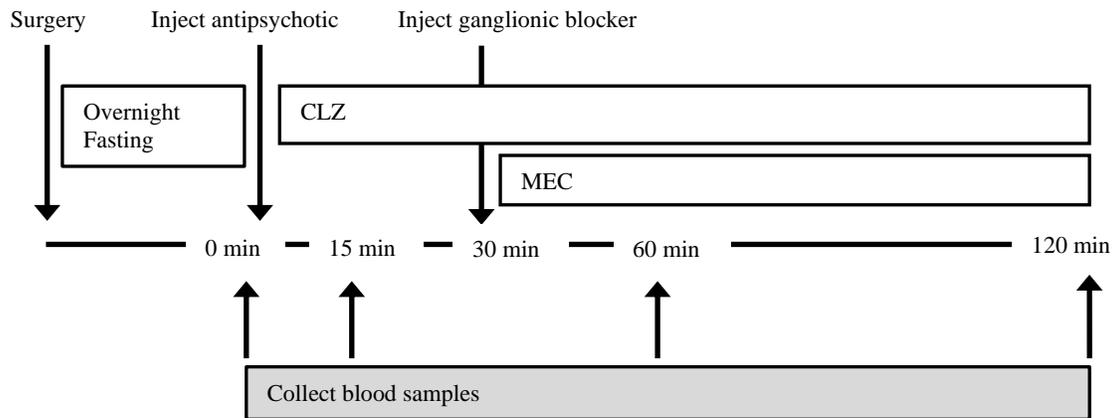


Figure 3.2: Overview of the experiment procedure for plasma collection in animals acutely treated with clozapine and mecamlamine. Drug-naïve adult female rats were fasted overnight and injected with clozapine (CLZ) at t = 0 min, followed by another i.p. injection of mecamlamine (MEC) at t = 30 mins. Whole blood samples were collected at t = 0, 15, 60 and 120 mins.

Plasma catecholamine levels

A Shimadzu series HPLC system (Shimadzu Corporation, Kyoto, Japan) coupled to a Coulochem III Electrochemical Detector (ESA Biosciences, Inc., Chelmsford, Massachusetts) was used for determining plasma catecholamine levels. Mobile phase (3 L double-distilled water, 20.7 g sodium phosphate, 88.2 g sodium citrate, 0.6 g EDTA, 6.6 g diethylamine HCl, 2.94 g 1-octanesulfonic acid, 144 ml acetonitrile, 66 ml dimethylacetamide, pH 3.1) was delivered at a flow rate of 0.4 ml/min, sample injection volumes were set at 20 μ l and analytes were separated on an ESA 80x4.6 mm column packed with 3 μ m C18 resin. The resultant peaks were analyzed using LCsolution software (Shimadzu Corporation, Kyoto, Japan).

Statistical Analysis

HIEC results were analyzed using a between-within subject analysis, with drug treatment as the between subjects factor and change in GIR from the baseline as the within subjects factor. Baseline fasting glucose immediately prior to treatment was measured at a single time point with an one-way ANOVA. Following drug treatment, glucose data were summed as area under the curve. Level of significance was set at $p < 0.05$ and LSD post-hoc tests were conducted when a main effect or interaction between main effects was significant. HPLC data were analyzed using ANOVA with significance set at $p < 0.05$ and LSD post-hoc tests were used for follow-up analyses when significant effects were present. Data were analyzed

with SPSS software, Chicago, IL, version 21.

3.3 Results

HIEC

For the initial study where we compared the effects of the 3 doses of CLZ (2, 10 and 20 mg/kg/ml) on glucose indices in the HIEC, there was no difference in fasting glucose levels between groups in the final glucose measurement immediately prior to drug treatment [$F_{(3,18)} = 0.71$, NS]. Following treatment with the 3 different doses of CLZ, glucose levels showed an initial increase in some groups until restored to euglycemia following change of GIR. There was thus a main effect of drug treatment [$F_{(3,18)} = 6.10$, $p < 0.01$], time [$F_{(12,216)} = 5.84$, $p < 0.001$] and treatment \times time [$F_{(36,216)} = 1.50$, $p < 0.05$]. Post-hoc analysis indicated that this effect was primarily due to an increase in glucose levels by the 2 higher doses of CLZ – this was significant for the 20 mg/kg/ml dose only (Figure 3.3a).

When we analyzed the change in GIR following CLZ drug treatment, we observed a significant main effects of drug treatment [$F_{(3,18)} = 26.17$, $p < 0.0001$], time [$F_{(12,216)} = 47.38$, $p < 0.0001$] and treatment \times time [$F_{(36,216)} = 7.32$, $p < 0.0001$]. Post-hoc analysis indicated that this was due to significant reductions in the GIR in all 3 CLZ treated groups compared to vehicle. Both the 10 and 20 mg/kg/ml groups exhibited significantly lower glucose infusion rates than the 2 mg/kg dose of CLZ (Figure 3.3b).

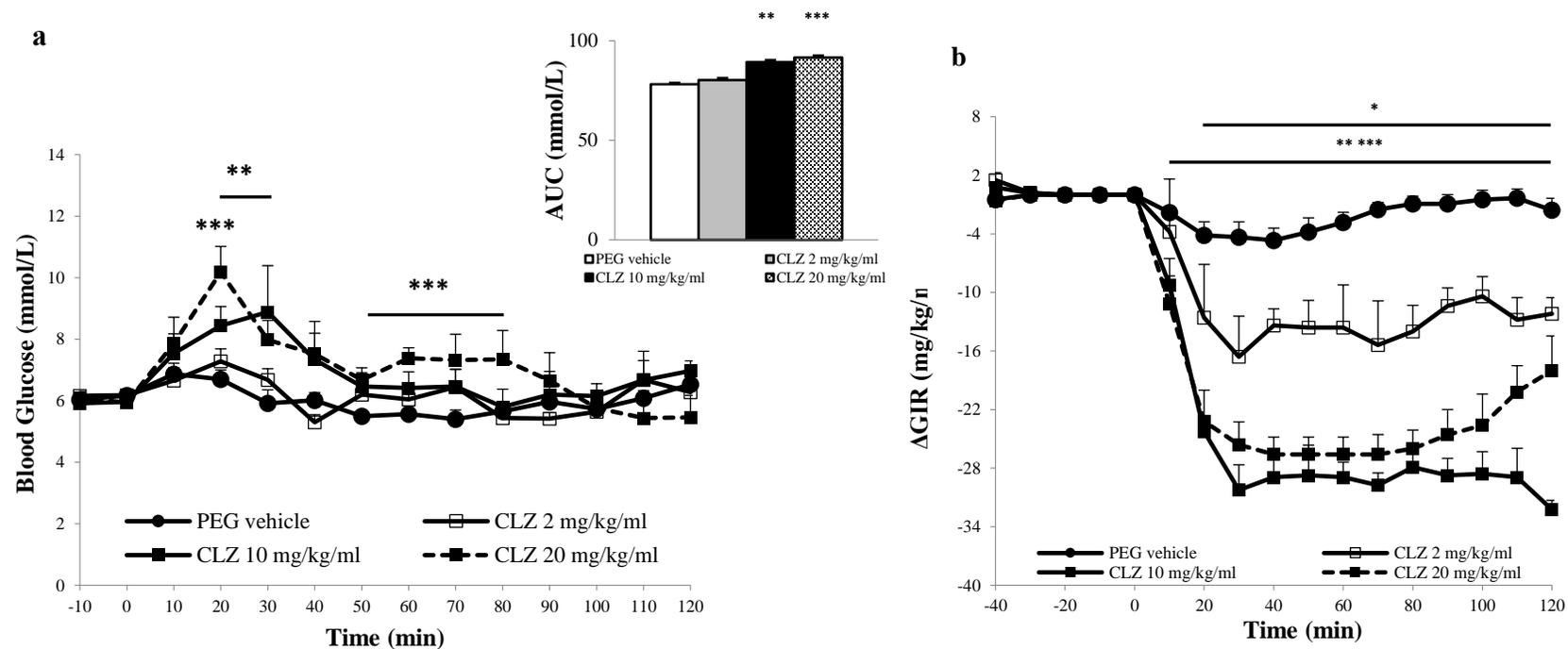


Figure 3.3: The acute dose-dependent effects of clozapine on blood glucose in female rats. The HIEC was performed to assess insulin sensitivity in freely moving, conscious rats. Female rats were fasted overnight and randomly administered PEG vehicle (n = 6), 2 (n = 5), 10 (n = 6) or 20 mg/kg/ml CLZ (n = 5) once euglycemia was reached. Blood samples from the arterial cannula were obtained every 10 minutes to determine blood glucose levels. **(a)** Fasting blood glucose measurements largely remained consistent throughout the experiment. CLZ administered at 10 mg/kg/ml and 20 mg/kg/ml had significant effects on total blood glucose (top right inset). ** Statistical significance for 10 mg/kg/ml CLZ compared to vehicle, $p < 0.05$. *** Statistical significance for 20 mg/kg/ml CLZ compared to vehicle, $p < 0.05$. **(b)** A reduction in GIR indicative of insulin resistance was observed for all doses of CLZ tested. The decrease in GIR is more prominent at higher doses, lasting from $t = 20$ mins until the end of the experiment. * Statistical significance for 2 mg/kg/ml CLZ compared to vehicle, $p < 0.05$. ** Statistical significance for 10 mg/kg/ml CLZ compared to vehicle, $p < 0.05$. *** Statistical significance for 20 mg/kg/ml CLZ compared to vehicle, $p < 0.05$.

Dose experiments were initially performed for the selected antagonists (Figures 3.4 – 3.8). The full analyses of these pilot studies are not included for the sake of brevity, but the graphic representations in Figures 3.4 – 3.8 demonstrate the extensive work that was performed to determine the optimal doses of each of the adrenoceptor antagonists, as well as key significant findings. This work is also informative for future research in this field. Blood glucose generally remained consistent among all the antagonists and doses tested throughout the experiment. The α -adrenoceptor blockers, IDA and PRA, when co-administrated with CLZ, lowered GIR significantly for the duration of the experiment regardless of the dose tested. Following CLZ administration, the β -adrenoceptor antagonists, ATEN and BUT, initially decreased the GIR until approximately $t = 80$ mins where glucose infusion was restored. The ganglionic blocker, MEC, induced intermittent suppression of GIR throughout the experiment except for the highest dose following CLZ treatment. At 5 mg/kg/ml, MEC significantly decreased GIR for the duration of the experiment.

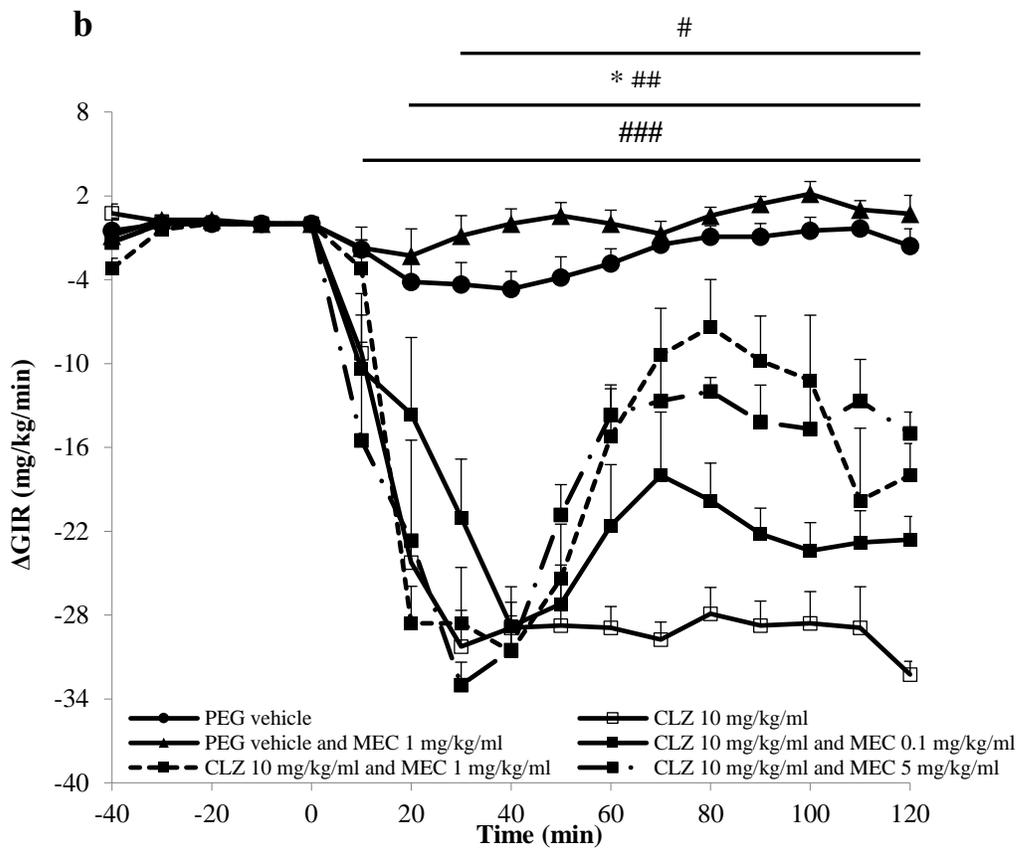
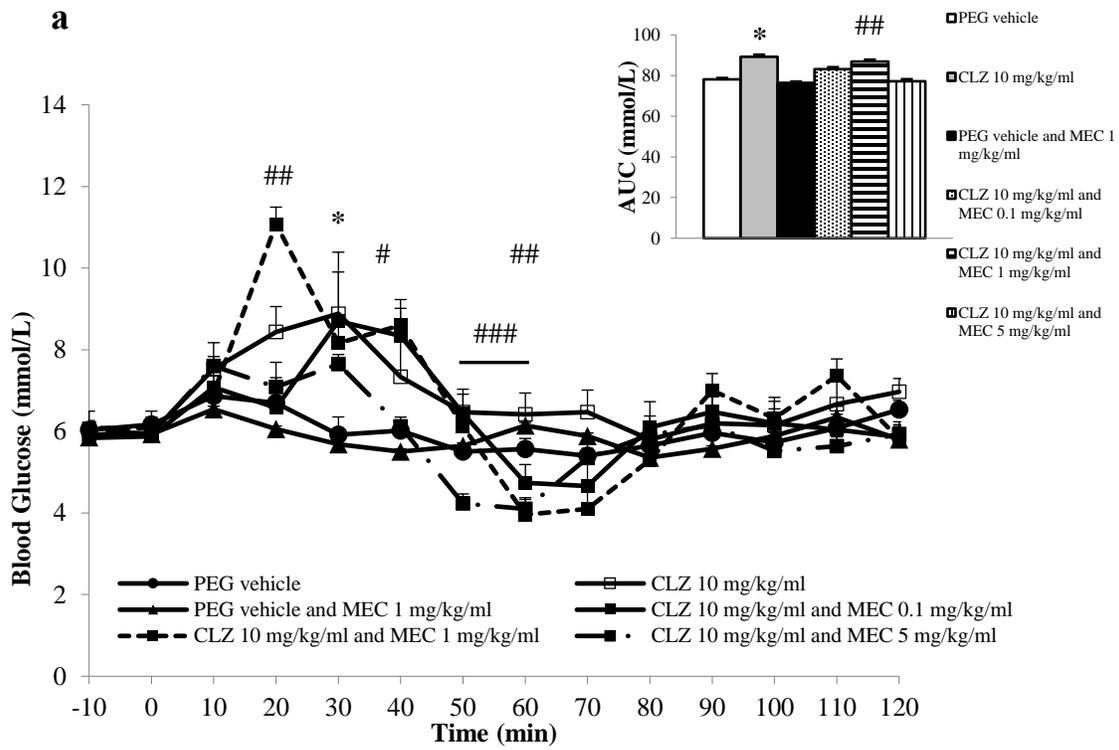


Figure 3.4: Acute dose-dependent effects of mecamlamine on insulin sensitivity in clozapine pre-treated animals. The HIEC was performed to assess insulin sensitivity in freely moving, conscious rats. Female rats were fasted overnight and randomly administered PEG vehicle or 10 mg/kg/ml CLZ once euglycemia was reached. Animals were then randomly administered 1 of 3 doses of MEC (0.1, 5, 1 mg/kg/ml, i.p.). Blood samples from the arterial cannula were obtained every 10 minutes to determine blood glucose levels. **(a)** Blood glucose measures and the resultant total blood glucose remained relatively constant for all treatment groups, apart from animals that received CLZ only. Values are expressed in means \pm SEM. *Significant difference for 10 mg/kg/ml CLZ group compared to vehicle, $p < 0.05$. **(b)** MEC was administered at $t = 30$ mins, where 5 mg/kg/ml caused a pronounced decrease in GIR compared to vehicle, ### $p < 0.05$. # Significant difference for 0.1 mg/kg/ml MEC compared to vehicle, $p < 0.05$. ## Significant difference for 1 mg/kg/ml MEC compared to vehicle, $p < 0.05$.

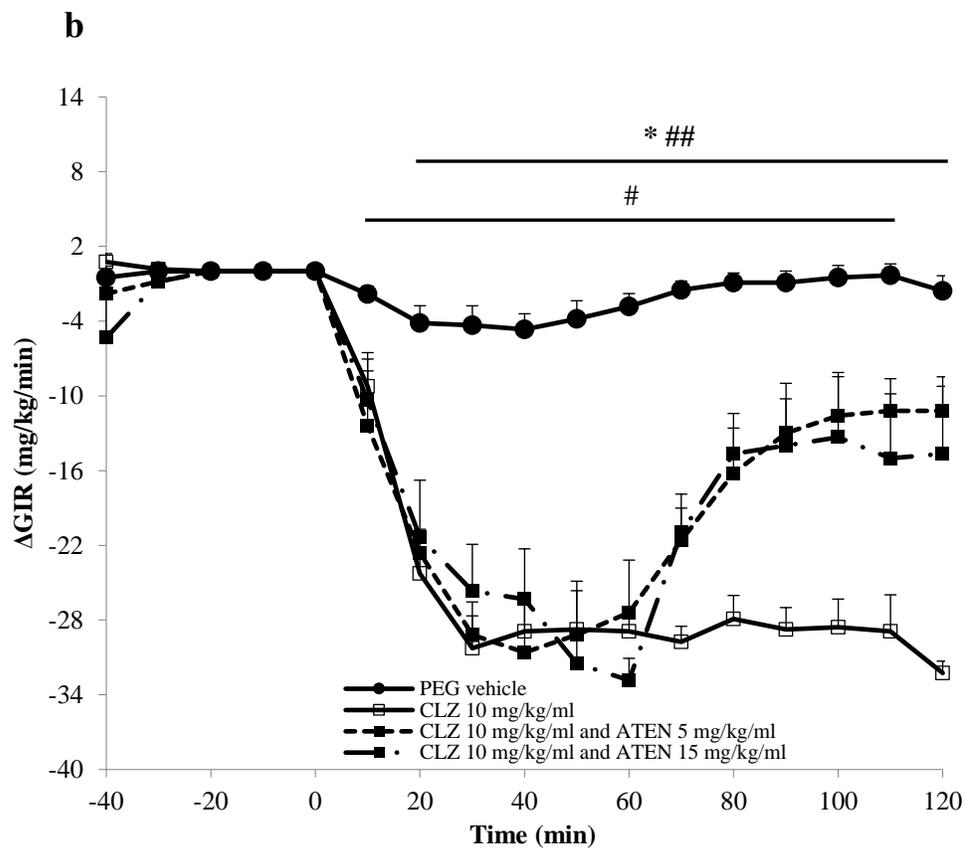
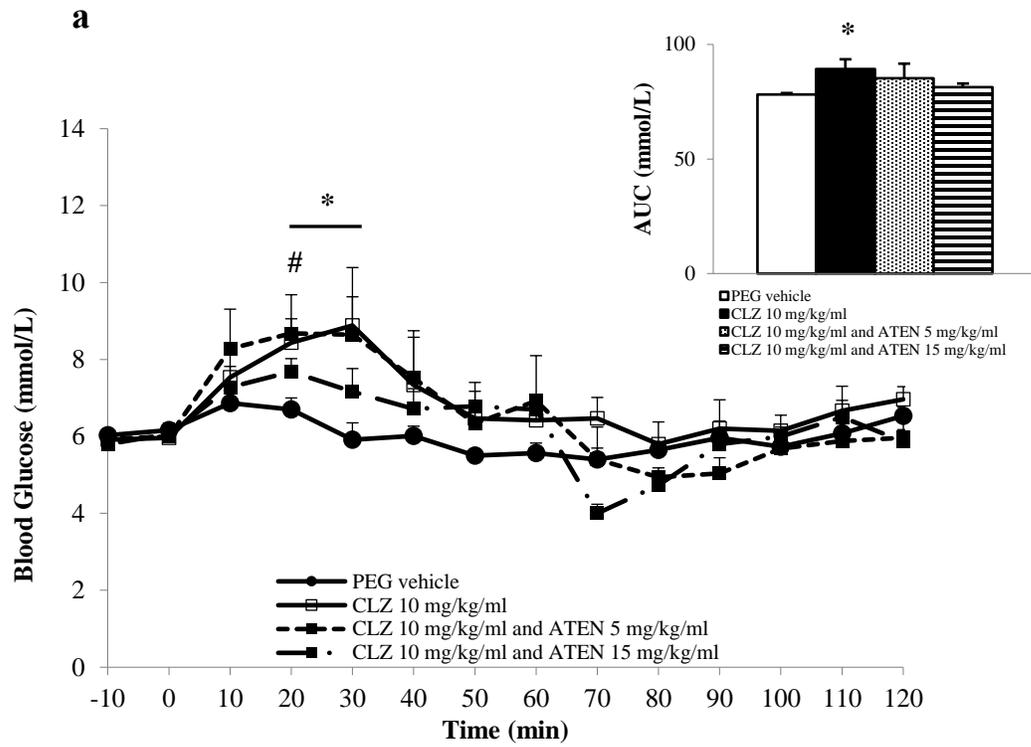


Figure 3.5: Acute dose-dependent effects of atenolol on insulin sensitivity in clozapine pre-treated animals. The HIEC was performed to assess insulin sensitivity in freely moving, conscious rats. Female rats were fasted overnight and randomly administered PEG vehicle or 10 mg/kg/ml CLZ once euglycemia was reached. Animals were then administered ATEN (5, 15 mg/kg/ml, i.p.) at t = 50 mins and blood glucose levels were measured every 10 minutes. **(a)** Blood glucose measures and the resultant total blood glucose remained relatively constant for all treatment groups, apart from animals that received CLZ only. Values are expressed in means \pm SEM. *Significant difference for 10 mg/kg/ml CLZ group compared to vehicle, $p < 0.05$. **(b)** ATEN at 5 mg/kg/ml rapidly increased GIR where it was comparable to the vehicle group by t = 120 mins, # $p < 0.05$. ATEN at 15 mg/kg/ml also increased GIR, although this remained significantly different from the vehicle group for the duration of the experiment, ## $p < 0.05$.

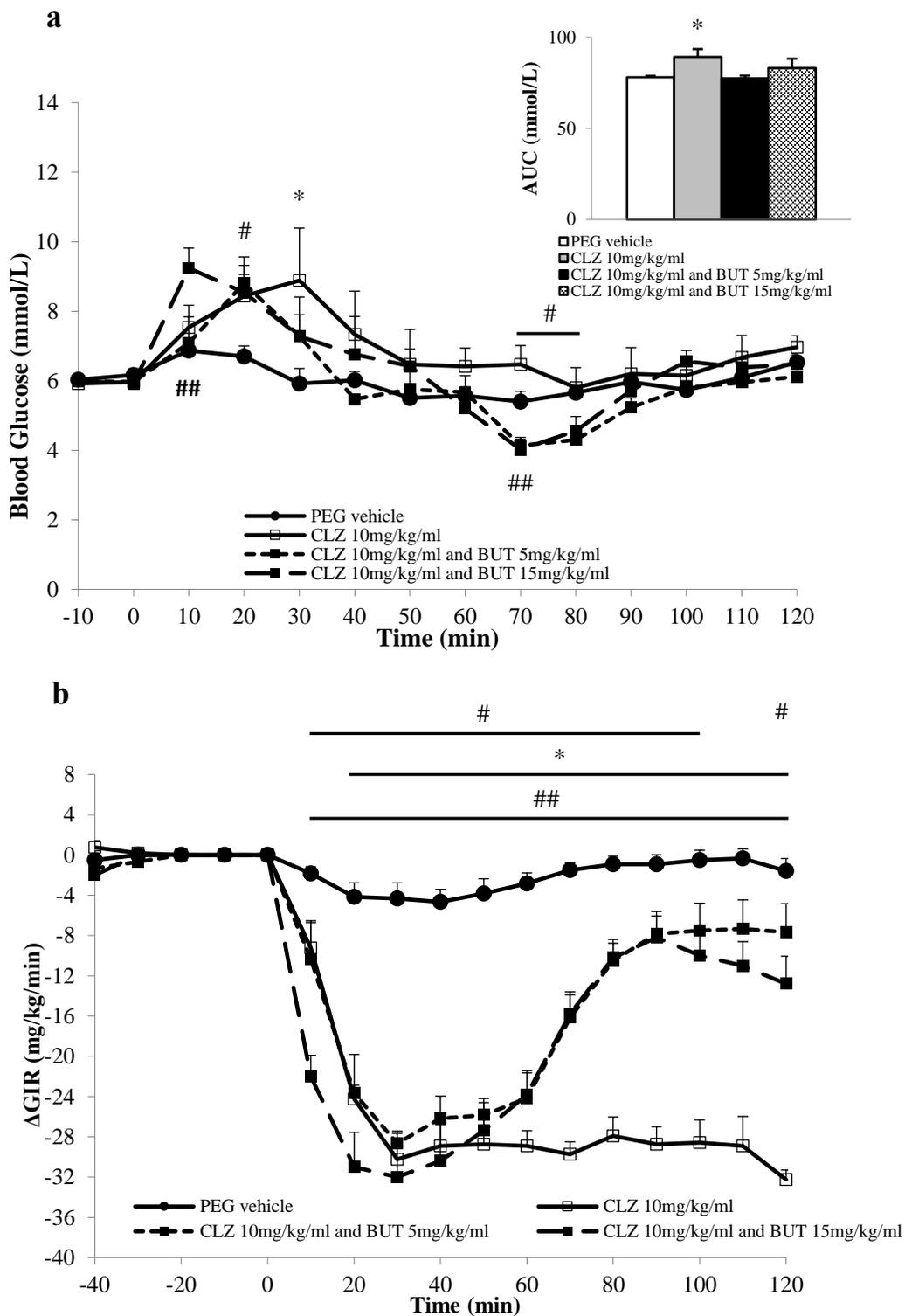


Figure 3.6: Acute dose-dependent effects of butoxamine on insulin sensitivity in clozapine pre-treated animals. The HIEC was performed to assess insulin sensitivity in

freely moving, conscious rats. Female rats were fasted overnight and randomly administered PEG vehicle or 10 mg/kg/ml CLZ once euglycemia was reached. Animals were then randomly assigned and treated with 1 of 2 doses of BUT (5, 15 mg/kg/ml, i.p.). Blood samples from the arterial cannula were obtained every 10 minutes to determine blood glucose levels. **(a)** Blood glucose measures and the resultant total blood glucose remained relatively constant for all treatment groups, apart from animals that received CLZ only. Values are expressed in means \pm SEM. *Significant difference for 10 mg/kg/ml CLZ group compared to vehicle, $p < 0.05$. **(b)** BUT treatment showed a similar pattern to ATEN, where both doses increased GIR albeit remaining statistically insignificantly from vehicle. # Significant difference for 5 mg/kg/ml BUT compared to vehicle, $p < 0.05$. ## Significant difference for 15 mg/kg/ml BUT compared to vehicle.

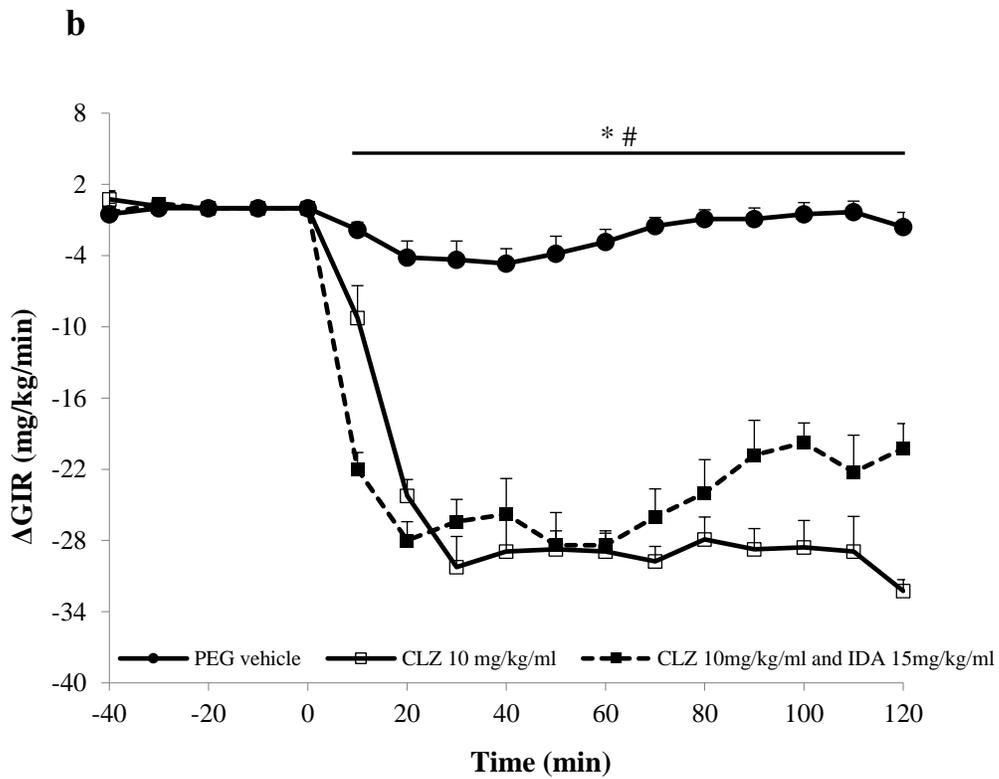
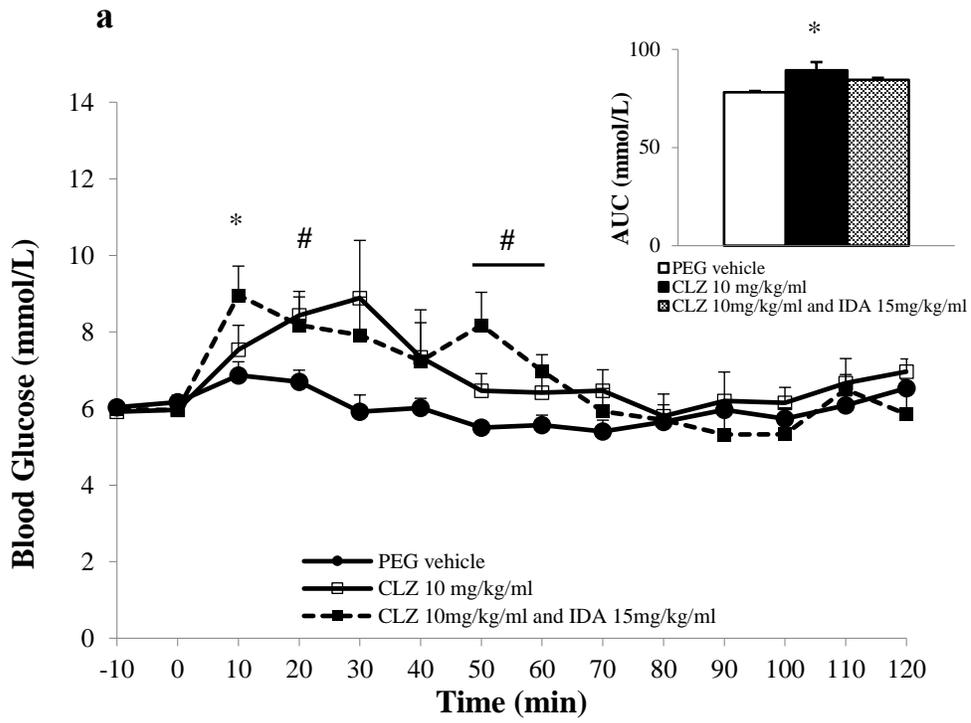


Figure 3.7: Acute dose-dependent effects of idazoxan on insulin sensitivity in clozapine pre-treated animals. The HIEC was performed to assess insulin sensitivity in freely moving, conscious rats. Female rats were fasted overnight and randomly administered PEG vehicle or 10 mg/kg/ml CLZ once euglycemia was reached. Animals were then treated with IDA at 15 mg/kg/ml, i.p. at t = 50 mins and blood was sampled every 10 minutes to determine blood glucose levels. **(a)** Blood glucose measures and the resultant total blood glucose remained relatively constant for all treatment groups, apart from animals that received CLZ only. Values are expressed in means \pm SEM. *Significant difference for 10 mg/kg/ml CLZ group compared to vehicle, $p < 0.05$. **(b)** IDA was tested at 15 mg/kg/ml, where it caused a significant decrease in GIR for the duration of the experiment, # $p < 0.05$.

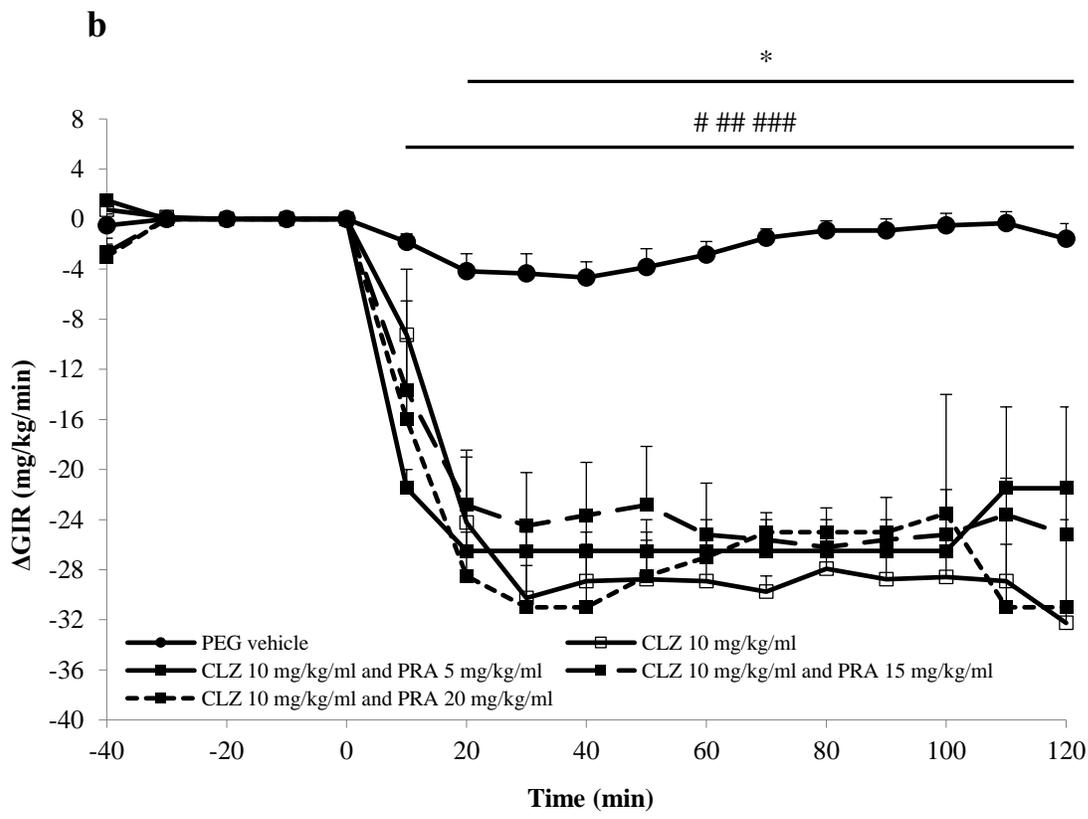
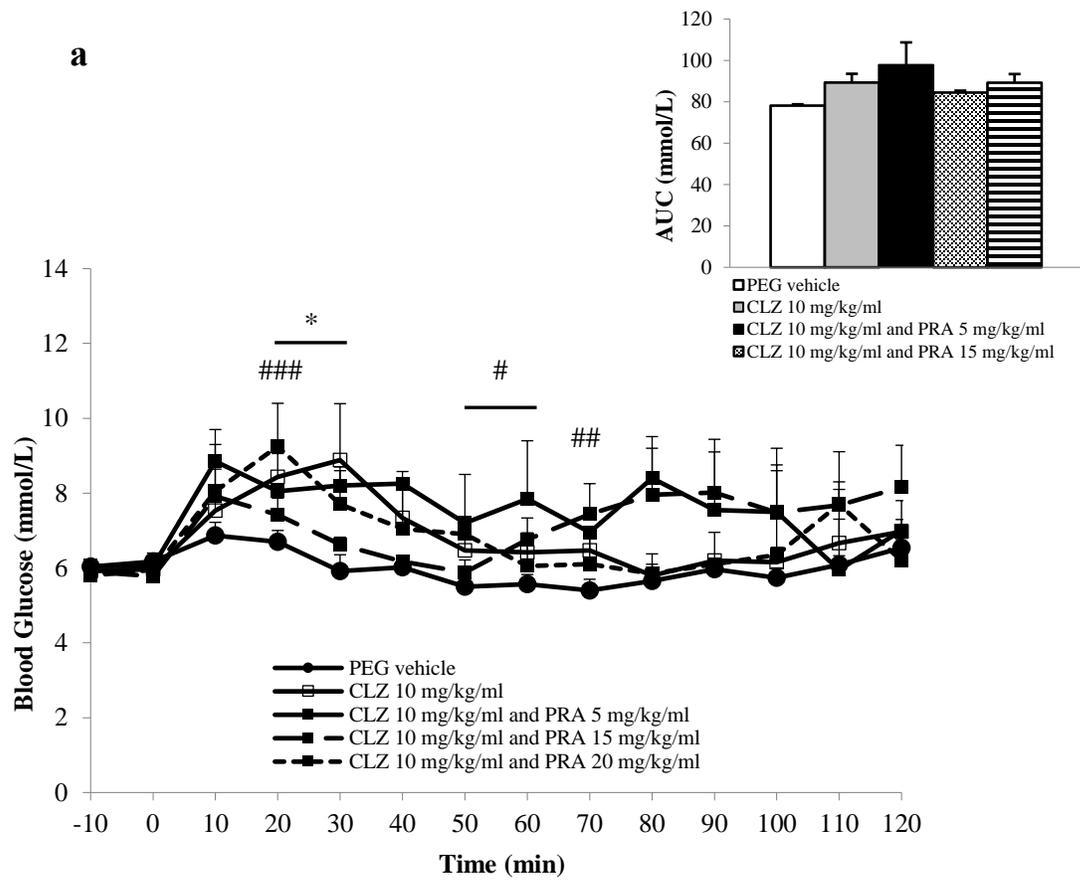
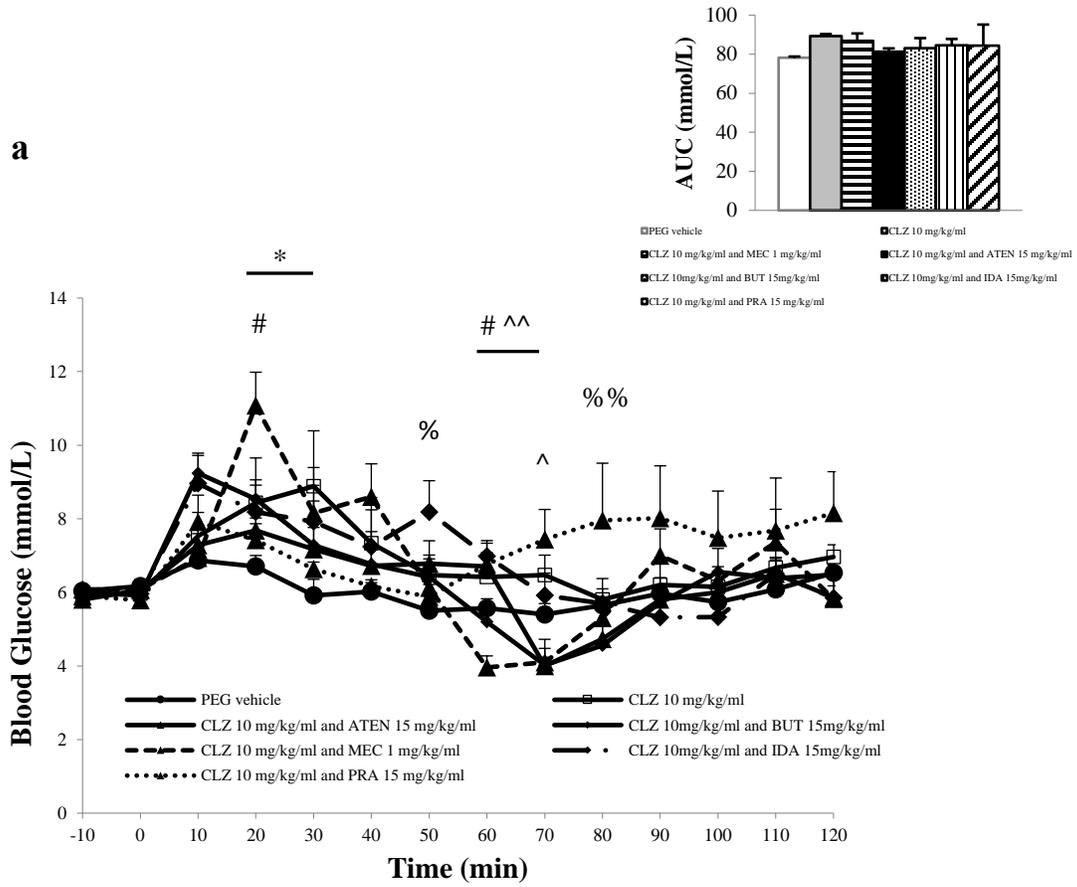


Figure 3.8: Acute dose-dependent effects of prazosin on insulin sensitivity in clozapine pre-treated animals. The HIEC was performed to assess insulin sensitivity in freely moving, conscious rats. Female rats were fasted overnight and randomly administered PEG vehicle or 10 mg/kg/ml CLZ once euglycemia was reached. Animals were then randomly administered 1 of 3 doses of PRA (5, 10, 15 mg/kg/ml, i.p.). Blood samples from the arterial cannula were obtained every 10 minutes to determine blood glucose levels. **(a)** Blood glucose measures and the resultant total blood glucose remained relatively constant for all treatment groups, apart from animals that received CLZ only. Values are expressed in means \pm SEM. *Significant difference for 10 mg/kg/ml CLZ group compared to vehicle, $p < 0.05$. **(b)** PRA caused a pronounced decrease in GIR at all doses tested that lasted for the entire experiment, 5 (# $p < 0.05$), 10 (## $p < 0.05$) and 15 mg/kg/ml (### $p < 0.05$).

Based on the results from the pilot studies above, the dose selected for the main study of MEC was 1 mg/kg/ml and 15 mg/kg/ml for the adrenoceptor antagonists (Figure 3.9).

a



b

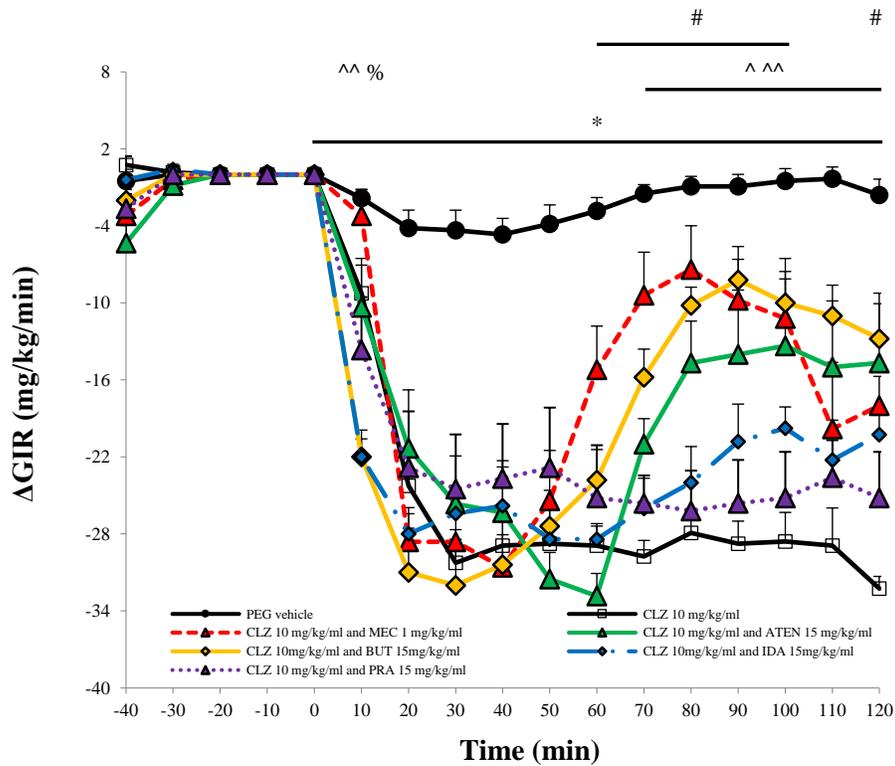


Figure 3.9: Acute effects of clozapine on insulin resistance and subsequent pharmacological intervention with adrenoceptor and ganglionic antagonists in adult female rats. Insulin sensitivity was evaluated using the HIEC in freely moving, conscious adult female rats. Animals were treated with CLZ (10 mg/kg/ml, i.p.) or vehicle (1 mg/kg/ml, i.p.) after reaching euglycemia. Following CLZ treatment, animals were randomly assigned 1 of 4 adrenoceptor antagonists, ATEN (15 mg/kg/ml, i.p.), BUT (15 mg/kg/ml, i.p.), IDA (15 mg/kg/ml, i.p.) or PRA (15 mg/kg/ml, i.p.), or the ganglionic blocker MEC (1 mg/kg/ml, i.p.). **(a)** Blood glucose measures were largely consistent for all treatment groups, apart from where indicated. **(b)** There was an initial decrease in GIR for all groups treated with CLZ, indicative of insulin resistance. Animals administered α -adrenoceptor antagonists in addition to CLZ (IDA and PRA) had no apparent effect on GIR. The CLZ-ATEN, CLZ-BUT and CLZ-MEC groups generally increased GIR, although these were statistically insignificant compared to PEG. Values are expressed in means \pm SEM. * Significant difference for the CLZ group compared to control, $p < 0.05$. # Significant difference for CLZ-MEC compared to CLZ treatment, $p < 0.05$. ^ Significant difference for CLZ-ATEN compared to vehicle, $p < 0.05$. ^^ Significant difference for CLZ-BUT compared to CLZ treatment, $p < 0.05$. % Significant difference for CLZ-IDA compared to CLZ treatment, $p < 0.05$. %% Significant difference for CLZ-PRA compared to CLZ treatment, $p < 0.05$.

In the main test of our hypotheses with the doses of drugs based on our pilot work, the findings were in general agreement with the results of the previous pilot tests. There was no difference in fasting glucose levels between groups in the final glucose measurement immediately prior to drug treatment [$F_{(6,38)} = 0.81$, NS]. Following treatment with the different adrenoceptor antagonists and ganglionic blocker, glucose levels showed an initial increase in some groups until restored to euglycemia following change of GIR. There was no main effect of drug treatment [$F_{(6,30)} = 1.67$ NS], but a main effect of time [$F_{(12,360)} = 13.16$, $p < 0.001$] and treatment \times time interaction [$F_{(72,360)} = 2.26$, $p < 0.001$] on glucose levels. Post-hoc analysis indicated that this effect was due to a general increase in glucose levels by all drugs, but this was significant only for MEC at a single time point.

For the main index of insulin resistance – i.e. the change in glucose infusion rate over time – the repeated measures ANOVA indicated potent main effects of drug treatment [$F_{(6,30)} = 16.90, p < 0.0001$], as well as a main effect of time [$F_{(12,360)} = 56.78, p < 0.001$] and a treatment \times time interaction [$F_{(72,360)} = 5.80, p < 0.001$] on the change in GIR. Following up these main effects and interaction with post-hoc tests, the analysis revealed that – as previously – CLZ treatment in all groups administered the drug resulted in a large decrease in the GIR, indicating whole body insulin resistance. This stayed significant in the CLZ-only group until the end of the experiment. Post-hoc analyses taken from the point of administration of adrenoceptor antagonists or MEC indicated that MEC increased the GIR in CLZ-treated rats at 60 minutes into the clamp, and this stayed significantly greater than the CLZ only group until the end of the HIEC (apart from at 110 mins, when the effect was only marginally significant, $p = 0.07$). Thus, MEC was able to reverse the insulin resistance caused by CLZ treatment. Similarly, both ATEN and BUT increased glucose infusion rates in CLZ-treated rats starting at 70 mins into the clamp, and remained significantly above the GIR of the CLZ-only rats until the end of the HIEC. The other 2 drugs, IDA and PRA, did not have any effect on the glucose infusion rates in CLZ-treated rats, apart from IDA at the final time point, where there was a small increase in the rate.

HPLC

Acute plasma levels of CATs following a single i.p. injection of CLZ were assessed using HPLC at $t = 0, 15, 60$ and 120 mins (Figure 3.10). In consideration of the rapid clearance of CLZ in rat plasma (Baldessarini et al., 1993), we included a 15 min time point in the present

pilot study. Unfortunately, the assay for NE did not work reliably and so the data are not included. The reasons for this are not entirely evident and will require further validation of the experimental protocol. However, levels of plasma E were consistently detected. Analysis with a repeated measures ANOVA indicated that there was a significant main effect of time [$F_{(3,33)} = 10.99$, $p < 0.0001$], a non-significant trend of an effect of drug treatment [$F_{(1,11)} = 4.49$, $p = 0.058$], and a significant interaction of drug treatment and time [$F_{(3,33)} = 4.03$, $p < 0.05$]. Post-hoc analysis indicated that the 2 groups did not differ at baseline in terms of their E levels. Both groups then also showed a large, highly significant increase in E levels following treatment with the 10 mg/kg/ml dose of CLZ (Figure 3.10). However, the subsequent treatment of 1 group with the MEC at 30 mins after CLZ treatment resulted in a large, significant reduction in E levels at 60 mins compared to the CLZ-only group. This effect was no longer significant at 120 mins after CLZ treatment.

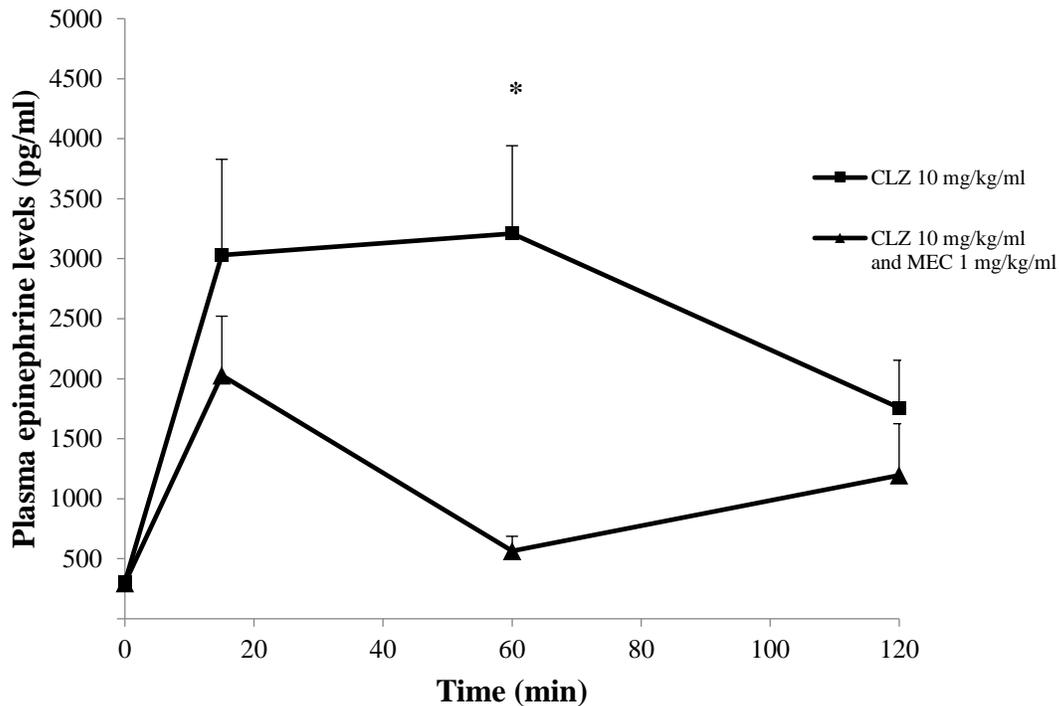


Figure 3.10: Acute changes in plasma CAT levels after CLZ treatment in adult female rats. Animals were given a single i.p.injection of CLZ, or a second MEC injection 30 mins following the initial CLZ injection. Values are expressed in means \pm SEM, * $p < 0.05$. Plasma E levels peaked at 15 mins post-injection of CLZ and remained elevated for the CLZ group. For the CLZ-MEC group, MEC decreased E levels to values similar to baseline.

3.4 Discussion

The present study assessed the acute metabolic changes of CLZ treatment and its reversal using selected blockers in conscious rats. The HIEC was used to evaluate whole-body insulin sensitivity, where a single dose of CLZ at 10 mg/kg/ml led to an immediate decrease in GIR. The results are indicative of profound insulin resistance from CLZ treatment, consistent with other similar animal studies in literature (Houseknecht et al., 2007; Chintoh et al., 2009). To assess ANS activity, we measured plasma CAT levels at $t = 0, 15, 60$ and 120 mins by

employing HPLC analysis. Plasma levels of E peaked at 15 mins following injection of CLZ, with E levels exceeding baseline values for the remaining 115 mins. The abovementioned glucose dysregulation and elevated E were diminished following the administration of MEC at t = 30 mins. As a pilot study to investigate the involvement of the ANS in CLZ-induced glucose dysregulation, we injected α - and β -adrenoceptor antagonists at t = 50 mins to reverse the insulin resistance in a separate cohort of animals. We found β -adrenoceptor blockers successfully increased GIR rates compared to CLZ-only treated rats, while treatment with α -adrenoceptors had minimal effect.

The finding that acute hyperglycemia from CLZ treatment can be reversed with the administration of β -blockers is of clinical significance. CLZ has known actions on multiple receptors, including α -adrenoceptors that contribute to insulin resistance, obesity and type 2 diabetes (Lindmark et al., 2005; Boyda et al., 2013a). On the contrary, CLZ is not known to have strong affinity for β -adrenoreceptors (Baldessarini et al., 1992). It is possible ATEN and BUT reversed the insulin resistance by binding to available β -adrenoceptors, whereas most α -adrenoceptors were already occupied by CLZ. The latter likely accounts for the unchanged insulin sensitivity from application of the α -adrenoceptor antagonists PRA and IDA. CATs are key in the regulation of lipolysis, where they exert inhibitory effects via α_2 -adrenoceptors and promotes lipolysis by activating β -adrenoceptors (Lafontan and Berlan, 1993; Arner, 1995). Specifically, the release of NE stimulates lipolysis by binding to β -adrenoceptors while E can inhibit or stimulate lipolysis depending on the ratio of β -adrenoceptors to α_2 -adrenoceptors and location of the adipocytes (Arner and Langin, 2014). In respect to β -adrenoceptors, CATs have demonstrated efficacy on stimulating lipolysis on 3 separate

subtypes of receptors— β_{1-3} -adrenoceptors (Arner, 1995; Jocken and Blaak, 2008; Morigny et al., 2016).

The resultant mobilization of fatty acids is normally inhibited by insulin, whose secretion is suppressed by α_2 -adrenoceptors that are in turn activated by E and NE (Koeppen and Stanton, 2008; Karpe et al., 2011). CLZ administration at 10 mg/kg resulted in a profound increase in plasma E levels as soon as 15 mins. The surge in E (and possibly NE, which we could not determine), resulting in hyperglycemia and significantly decreased GIR among all groups treated with CLZ. These observations are largely consistent with literature, where CLZ-induced hyperglycemia (Murashita et al., 2007; El-Seweidy et al., 2014) and insulin resistance (Yazici et al., 1998; Melkersson and Dahl, 2003) are well documented.

Due to the novelty of this pilot study, current evidence for using adrenoceptor antagonists towards reversal of CLZ-induced metabolic side effects is limited. It is possible the lipolytic effects and insulin resistance associated with CLZ were attenuated by displacement of NE from β -adrenoceptors following administration of ATEN and BUT. Conversely, CLZ's potent binding to $\alpha_{1,2}$ -adrenoceptors could not be as readily displaced by IDA and PRA. However, these propositions are purely speculative as evidence from binding studies is lacking. Furthermore, CLZ has actions on multiple receptors and it is possible the effects noted in our study are the result of antagonism of 5-HT and M receptors.

Under normal physiological conditions, pancreatic β -cells secrete compensatory insulin in response to glucose challenges (Reaven, 1988). However, as noted in our experiments, animals treated with CLZ remained in the hyperglycemic state. It is possible CLZ's interactions with multiple receptors on β -cells inhibited insulin secretion, thus explaining the decreased response to insulin while in the hyperglycemic state. Namely, the

α_2 -adrenoceptor, 5-HT_{1a} receptor and M₃ receptor (Starrenburg and Bogers, 2009). The α_2 -adrenoceptor has known inhibitory actions on insulin secretion, mediated through suppression of adenylate cyclase (Yamazaki et al., 1982). The plasma E levels after CLZ administration is in line with literature, where it is known 0.5 to 150 ng/ml of E is sufficient to suppress insulin secretion in islet cells.

Limitations of our study include the specificity of the pharmacological agents applied. PRA is also known to bind α_2 -adrenoceptors despite being classified as an α_1 -adrenoceptor antagonist (Ruffolo et al., 1993). Also, because this is a pilot study, a vehicle group was not included for the plasma collection experiments. Future studies measuring drug levels in combination with CATs may be of interest, as our results suggest involvement of the ANS in mediating metabolic dysregulation induced by CLZ. In particular, at the receptor level, as β -adrenoceptor blockers successfully reversed CLZ-induced glucose dysregulation in the current study.

Chapter 4: Evaluation of the acute cardiovascular side effects of clozapine in adult rats

4.1 Introduction

Cardiovascular complications of CLZ are well documented, ranging from the commonly reported tachycardia and orthostatic hypotension, to the rare but fatal myocarditis. As perturbances to autonomic nervous system (ANS) function may play a role in the cardiovascular side effects of CLZ, we developed a novel, acute model of CLZ-induced tachycardia in rodents. Pharmacological interventions targeting the ANS at the ganglionic and receptor levels were employed to determine whether the cardiovascular effects were reversible.

Mecamylamine (MEC) is a ganglionic blocker that readily traverses the blood-brain barrier and competes with nicotinic acetylcholine receptors (nAChRs) to inhibit sympathetic neuronal transmission, thereby acting as an antihypertensive (Shytle et al., 2002). At therapeutic doses, MEC also blocks the parasympathetic nervous system (PNS) in addition to the sympathetic nervous system (SNS) and elicits undesirable effects such as dryness of mouth and constipation, resulting in discontinuation of its use as an antihypertensive (Shytle et al., 2002). Yet at lower doses, autonomic side effects associated with MEC can be avoided, thereby enabling novel uses of the drug.

Propranolol (PRO) is a non-selective β -adrenoceptor blocker used to treat hypertension and cardiac arrhythmias, including supraventricular tachycardia (Stephenson et al., 1980; Moffett et al., 2015). PRO's efficacy in treating arrhythmias is due to prolonged refractory periods and delayed nodal conduction of the atrioventricular node (Seides et al.,

1974). Importantly, elevated CATs generate early afterdepolarizations and repolarizations that contribute to ventricular arrhythmias and sudden cardiac death (Haissaguerre et al., 2008), to which PRO has demonstrated efficacy in suppression of arrhythmias via β -adrenoceptor blockade (Lemberg et al., 1970; Woosley et al., 1979; Shimizu et al., 1995; Lampert et al., 2003).

As a novel approach, we attempted to induce tachycardia in adult, freely moving rats by administering CLZ at low, medium and high doses (5, 10, 15 mg/kg, i.p.). Tachycardia was defined as an increase in HR 50 bpm at or above baseline HR. Cardiovascular parameters including HR, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), left ventricular peak systolic pressure (LVP) and maximal rates of increase and decrease of left ventricular pressure ($dP/dt_{\max/\min}$) were averaged every 15 mins for a total of 150 mins. LVP and its derivatives are used as indicators of left ventricular contractility, where dP/dt_{\max} reflects contractility and dP/dt_{\min} reflects relaxation (Prinzen and Peschar, 2002; Vanagt et al., 2005). Depressed LVP and $dP/dt_{\max/\min}$ are indicative of myocardial dysfunction (Horton et al., 1988; Tang et al., 1993; Leung et al., 2014). Our laboratory has previously shown acute treatment with olanzapine reduced LVP and dP/dt_{\max} in rats, suggesting contractile dysfunction may contribute to the development of antipsychotic-related arrhythmias (Leung et al., 2014).

We then attempted to reverse the tachycardia by administering MEC or PRO at 3 different doses, 15 mins following the injection of CLZ. Finally, plasma samples from rats randomly administered CLZ, CLZ and MEC, or vehicle were analyzed for CAT levels, as an indication of ANS activity.

4.2 Materials and methods

Animals

Male Sprague Dawley rats (Charles River Laboratories, Montreal, Canada) weighing 300 – 350 g were habituated in the UBC Animal Research Unit facility for at least 7 days prior to experimentation and kept under a 12-hour light/dark cycle. Animals were pair-housed in a temperature ($22 \pm 1^\circ\text{C}$) and humidity controlled environment with access to food and water *ad libitum*. All experimental procedures involving animals were approved by UBC's Animal Care Committee and in strict compliance with Canadian Council on Animal Care guidelines.

Pharmacological agents and solutions

CLZ was purchased from Toronto Research Chemicals Inc. (Toronto, Canada) and was dissolved in a vehicle PEG solution consisting of 50% PEG, 40% distilled water and 10% ethanol. MEC hydrochloride and PRO hydrochloride were purchased from Sigma-Aldrich (St. Louis, Missouri) and dissolved in 0.9% saline before use.

Experimental Design

Rats were randomly assigned to 1 of 6 treatments: control, CLZ, CLZ and saline (SAL), CLZ and MEC, CLZ and PRO. CLZ was administered at dosages of 5 mg/kg (n = 10), 10 mg/kg

(n = 12) and 15 mg/kg (n = 17), to reflect low, medium and high doses. SAL was administered at 1 ml/kg (n = 9), MEC was administered at 0.01 mg/kg (n = 10), 1 mg/kg (n = 10) and 5 mg/kg (n = 8) and PRO was administered at 1 mg/kg (n = 9), 5 mg/kg (n = 7) and 10 mg/kg (n = 8). Dosages were determined after preliminary dosing experiments. After collecting cardiovascular parameters over a baseline period of 15 mins, CLZ or PEG was immediately administered. Saline, MEC or PRO was administered 15 mins following the injection of CLZ. Rats injected with PEG vehicle served as the control group (n = 8). All pharmacological agents were administered via i.p. injections at volumes of 1 ml/kg.

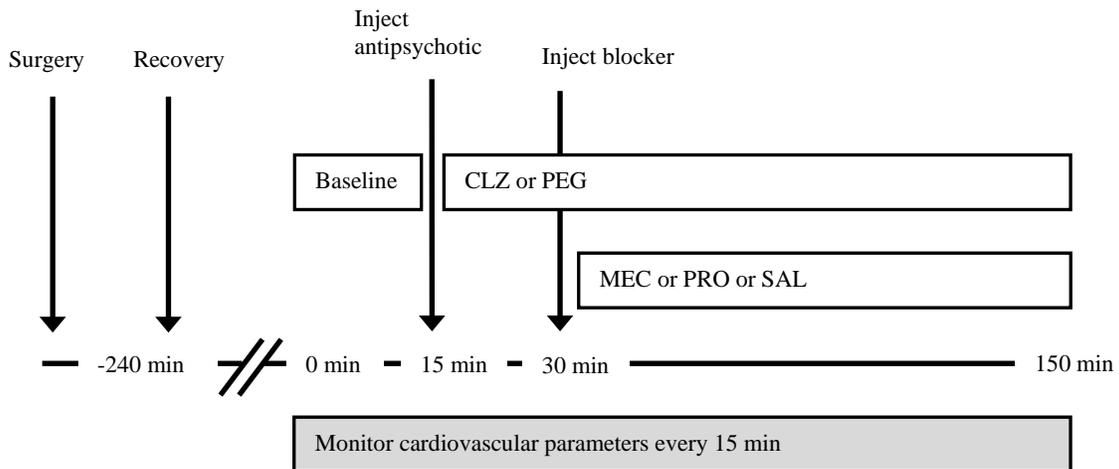


Figure 4.1: Overview of the HIEC procedure for acute administration of CLZ and reversal with selected adrenoceptor and ganglionic blockers.

Surgical procedures

Animals were anesthetized with isoflurane (Baxter, Deerfield, Illinois) and given pre-operative ketoprofen (5 mg/kg, s.c.). Bupivacaine was topically applied to incision sites.

PE50 cannulae filled with heparinized saline were inserted into the left iliac artery for blood

pressure and HR measurements and into the LV (via the right carotid artery) for LVP readings. The cannulae were tunneled subcutaneously from the ventral neck to the dorsal neck, exteriorized and sealed. Animals were allowed to recover for a minimum of 4 hours before beginning experiments.

Heart rate and blood pressure monitoring

BP, HR and LVP were continuously monitored by connecting the arterial and ventricular cannulae to P23DB pressure transducers (Gould Statham, California). Measurements were recorded using a BIOPAC system and analyzed with AcqKnowledge software (Biopac Systems Canada Inc, Montreal, Canada). Data was extracted for analysis at 15 minute intervals.

Plasma preparation for High Performance Liquid Chromatography (HPLC)

Catecholamine (NE, E, DA) levels were determined in plasma samples collected from rats treated with i.p. injections of PEG vehicle, 15 mg/kg CLZ or 15 mg/kg CLZ and 1 mg/kg MEC (n = 7 per group). Blood was collected from the saphenous vein in heparinized collecting tubes at 0, 60, 120 and 240 minutes, where CLZ or PEG was administered immediately following the baseline blood collection at t = 0 min. The CLZ and MEC treatment group received an additional injection of MEC at t = 30 mins. Blood samples were centrifuged (10,000 rpm, 10 min, 4°C) and the plasma was extracted and stored at -80°C. A

catecholamine standard curve was derived from diluting a stock 1 mg/kg catecholamine solution with catecholamine standard diluent, both purchased from Thermo Fisher Scientific Inc. (Chelmsford, Massachusetts). The internal standard was diluted from a 1.0 mg/ml 3,4-Dihydroxybenzylamine (DHBA) stock solution (Thermo Fisher Scientific Inc., Chelmsford, Massachusetts). Electroactive species were removed using a preliminary acid-washed alumina extraction procedure (Lucot et al., 2005). Eppendorf tubes containing 5 mg of alumina, 50 µl plasma, 90 µl 3 M Tris-5% ethylene-diamine-tetraacetic acid (EDTA) buffer and 10 µl 20 ng/ml DHBA were vortex mixed, centrifuged (10,000 rpm, 5 min, 4°C), and the supernatant discarded. Samples were washed with 400 µl double-distilled water, centrifuged (5000x g, 1 min, 4°C) and aspirated for a total of 3 washes. After the third wash, 50 µl of 0.1 M perchloric acid was added to the samples. These were vortex mixed, centrifuged and 40 µl of the acidic aqueous layer was transferred into individual vials for analysis.

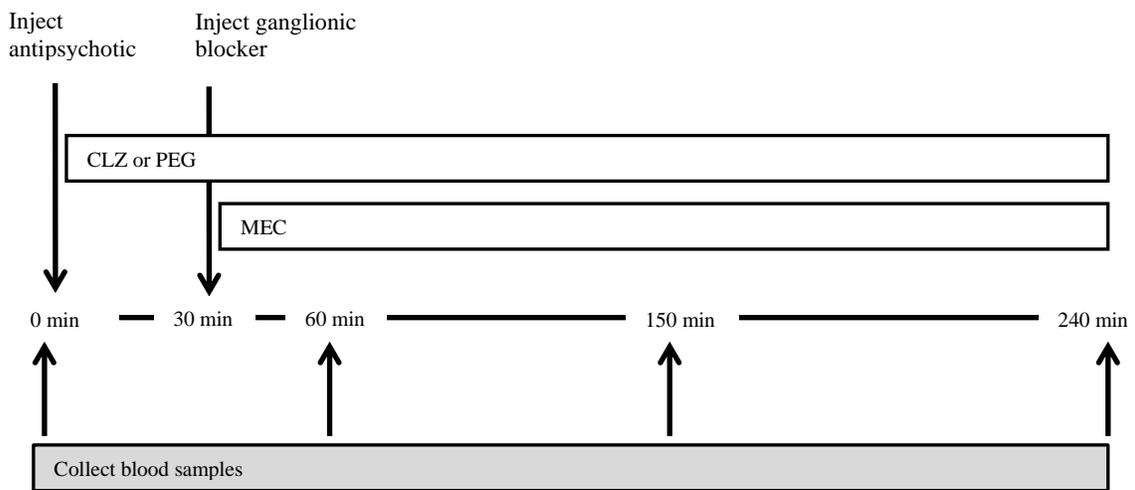


Figure 4.2: Overview of the experimental procedure for blood sample collection.

Detection of plasma catecholamine levels

A Shimadzu series HPLC system (Shimadzu Corporation, Kyoto, Japan) coupled to a Coulochem III Electrochemical Detector (ESA Biosciences, Inc., Chelmsford, Massachusetts) was used for determining plasma catecholamine levels. Mobile phase (3 L double-distilled water, 20.7 g sodium phosphate, 88.2 g sodium citrate, 0.6 g EDTA, 6.6 g diethylamine HCl, 2.94 g 1-octanesulfonic acid, 144 ml acetonitrile, 66 ml dimethylacetamide, pH 3.1) was delivered at a flow rate of 0.4 ml/min, sample injection volumes were set at 20 μ l and analytes were separated on an ESA 80x4.6 mm column packed with 3 μ m C18 resin. The resultant peaks were analyzed using LCSolution software (Shimadzu Corporation, Kyoto, Japan).

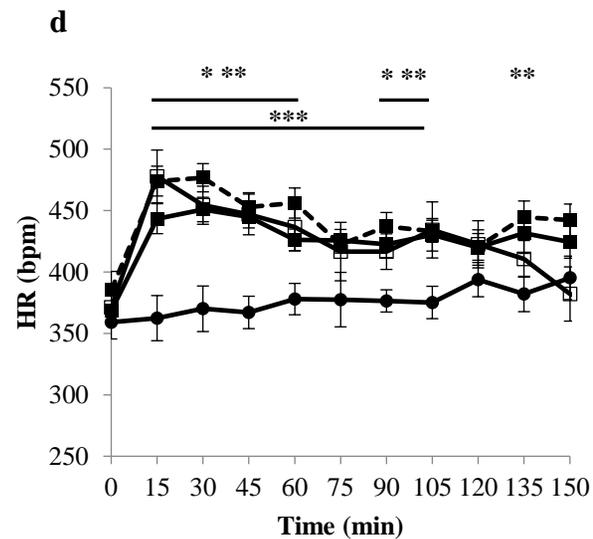
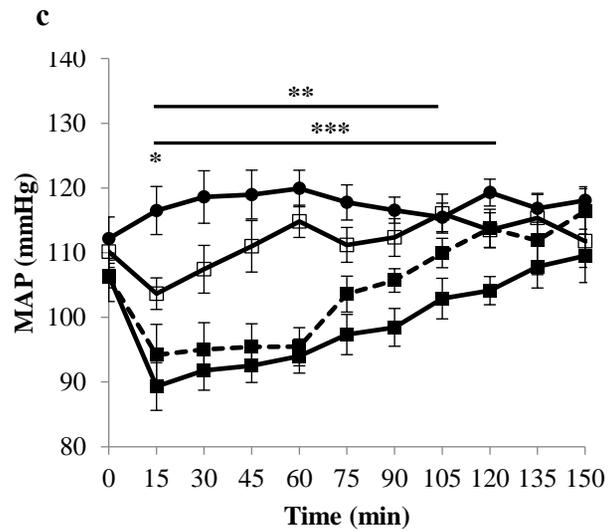
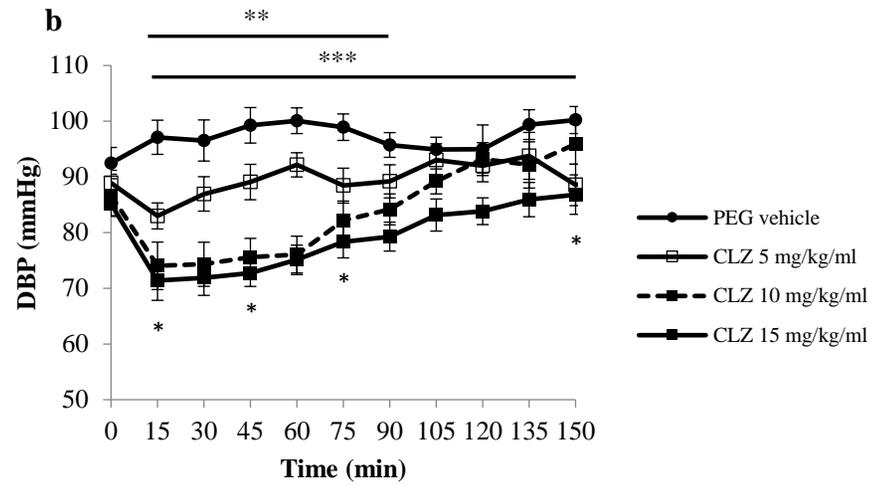
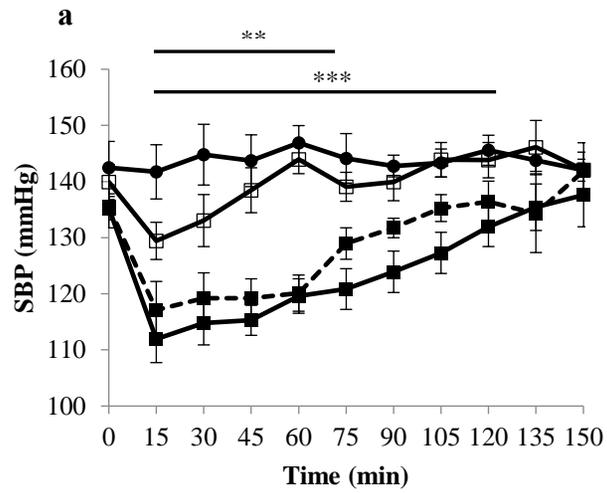
Statistical Analysis

Cardiovascular data were analyzed using two-factor ANOVA with drug treatment set as the between subjects factor and time as the within subjects factor. Significant effects were followed up with LSD post-hoc tests. HPLC results were analyzed using ANOVA and follow up analysis was done with LSD post-hoc tests when significant main effects were found. Alpha values were set at $p < 0.05$ and statistical analysis was performed using SPSS software (v21), Chicago, Illinois.

4.3 Results

Preliminary dosing experiments

Dosing experiments were performed prior to the main experiment to choose the optimum dose for CLZ, CLZ-MEC and CLZ-PRO. CLZ's effect on cardiovascular indices was evaluated at 3 doses, 5, 10 and 15 mg/kg/ml, using methods as described in the previous section. Similarly, 3 doses were used for MEC (0.01, 1 and 5 mg/kg/ml) and PRO (1, 5 and 10 mg/kg/ml). For brevity, the detailed statistical analyses will not be discussed and is instead outlined in Figures 4.3, 4.4. and 4.5. In general, CLZ elicited depressor effects and elevated HR in comparison to vehicle. PRO administered at the highest dose consistently reversed the tachycardia and depressor effects from CLZ treatment, whereas MEC at 1 mg/kg/ml lowered HR but also exacerbated depressor effects. The dose chosen for CLZ is 15 mg/kg/ml, MEC is co-administered with CLZ at 1 mg/kg/ml and PRO is co-administered at 10 mg/kg/ml. The following sections will provide further discussion of the effects of CLZ and the antagonists on individual cardiovascular parameters.



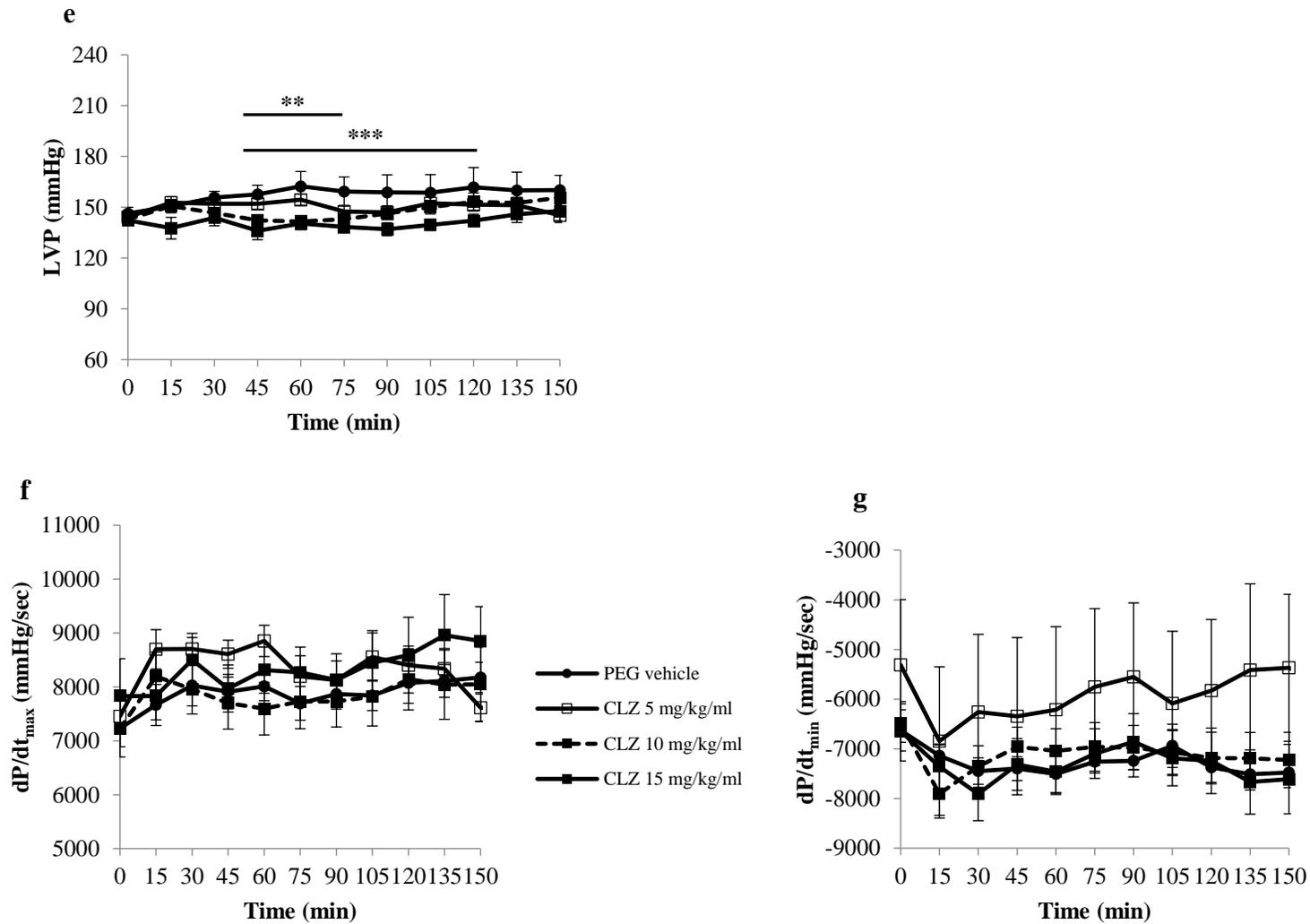
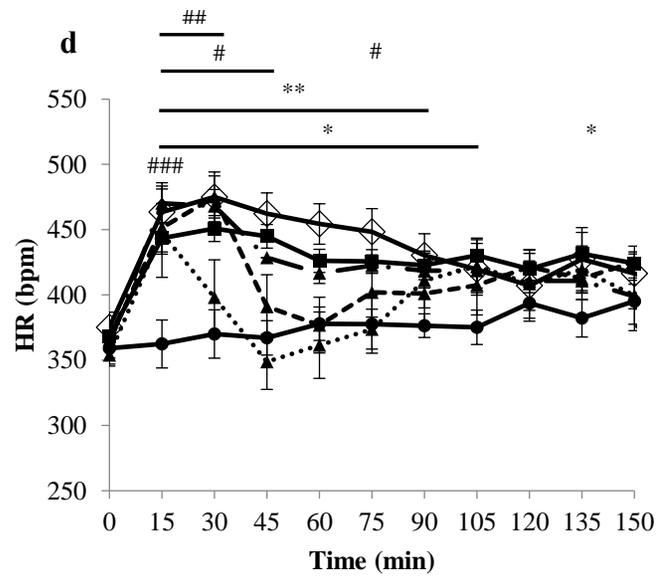
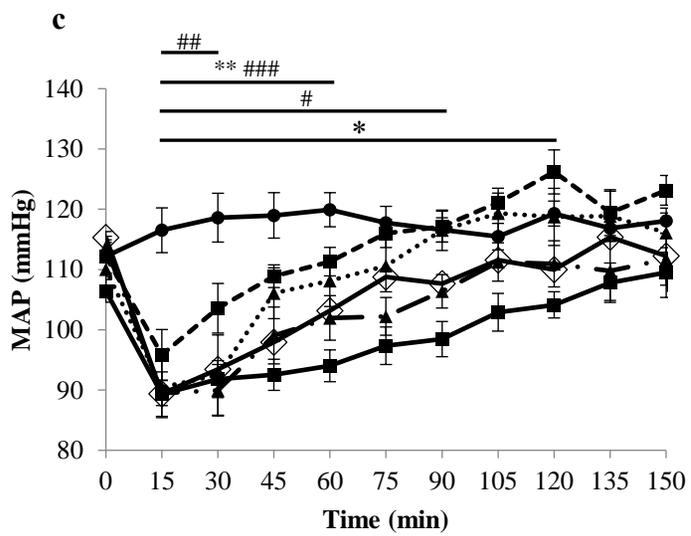
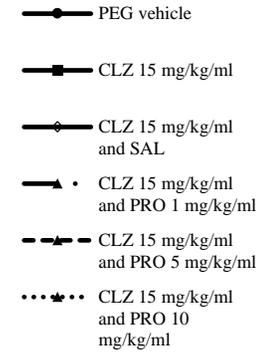
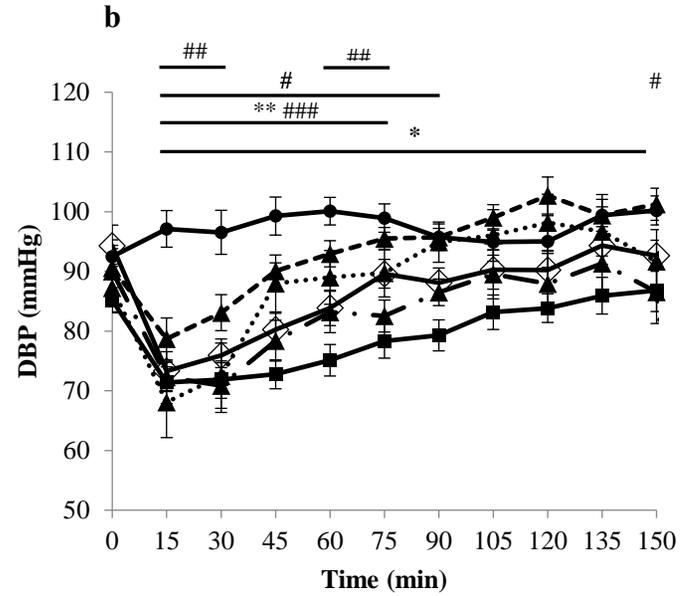
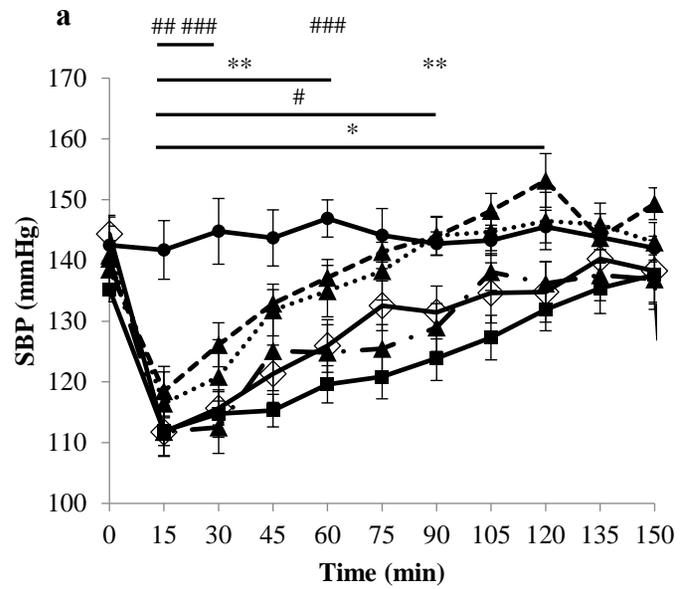


Figure 4.3: Acute dose-dependent effects of clozapine on cardiovascular parameters. The beat-to-beat cardiovascular parameters were recorded in 15 minute intervals for animals treated with a single injection of CLZ at varying doses (5, 10 or 15 mg/kg/ml, i.p.) or PEG vehicle at t = 15 mins. (a) SBP generally decreased in all CLZ-treated groups and was dependent on dose as the highest dose had

the most pronounced effects, lasting until $t = 120$ mins. The intermediate dose's depressor effect on SBP was statistically significant until $t = 75$ mins whereas the lowest dose had no effect. **(b)** High dose CLZ suppressed DBP to a greater degree than lower doses which lasted for the duration of the experiment. A depressor response was observed with the intermediate dose, lasting for 75 mins post-injection. Low dose CLZ lowered DBP at 4 separate time points ($t = 15, 45, 75$ and 150 mins). **(c)** MAP was suppressed by CLZ treatment, with the most pronounced effects observed in the 15 mg/kg/ml CLZ group that persisted for 105 mins. Intermediate dose CLZ also significantly lowered MAP, lasting for 90 mins. **(d)** High dose CLZ induced a profound increase in HR until $t = 105$ mins. The low and intermediate doses of CLZ only induced transient increases in HR, most notable for the initial 45 mins post-injection. **(e)** LVP was significantly suppressed by CLZ in a dose dependent manner. CLZ at 10 mg/kg/ml caused a depressor response lasting 30 mins, whereas high dose CLZ induced a similar yet prolonged response from $t = 45 - 120$ mins. **(f), (g)** No significant effects were observed for all CLZ treatment groups on $dP/dt_{\text{max/min}}$. * Statistical significance for 5 mg/kg/ml CLZ as determined by post-hoc tests compared to vehicle, $p < 0.05$. ** Statistical significance for 10 mg/kg/ml CLZ $p < 0.05$. *** Statistical significance for 15 mg/kg/ml CLZ, $p < 0.05$.



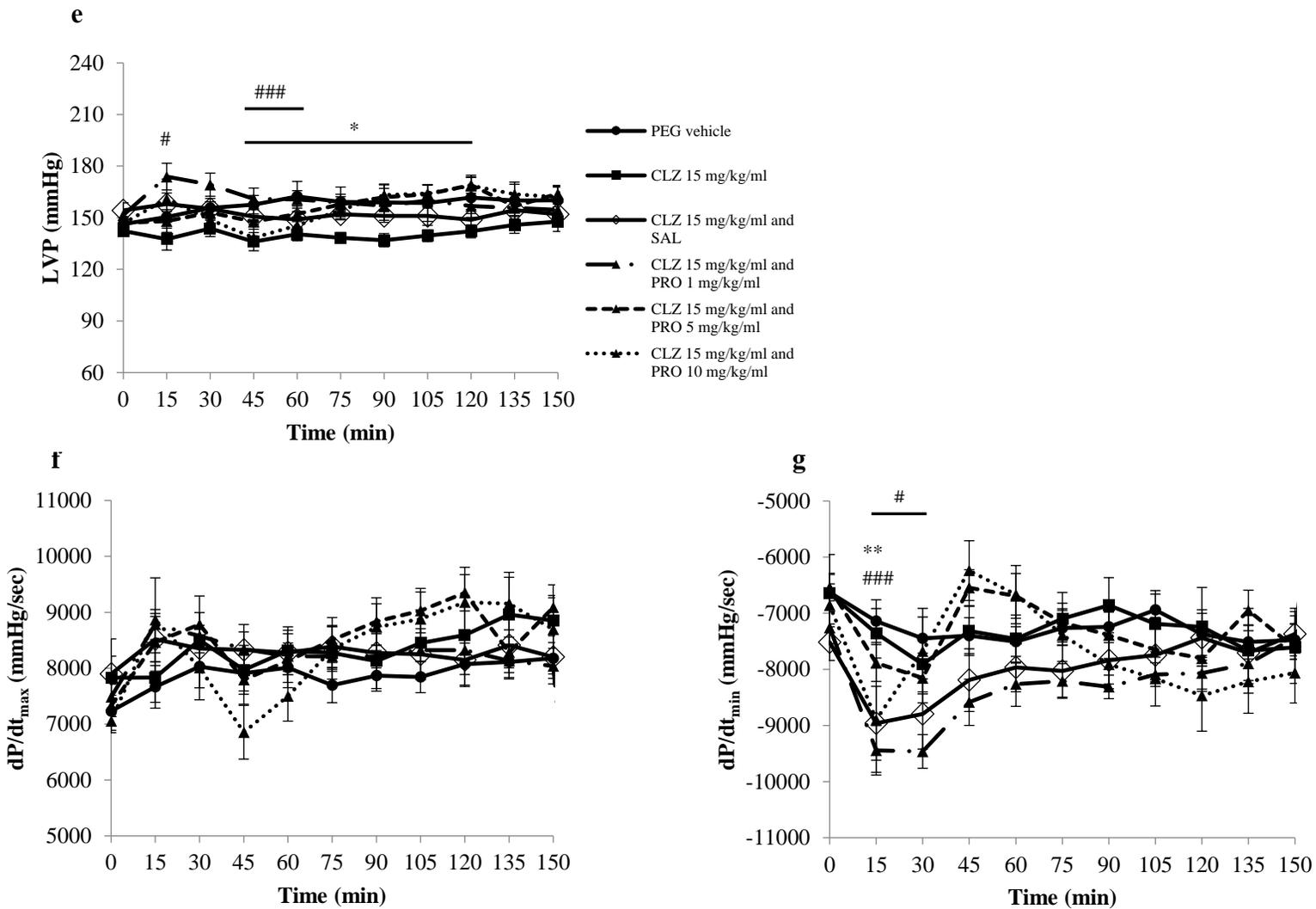
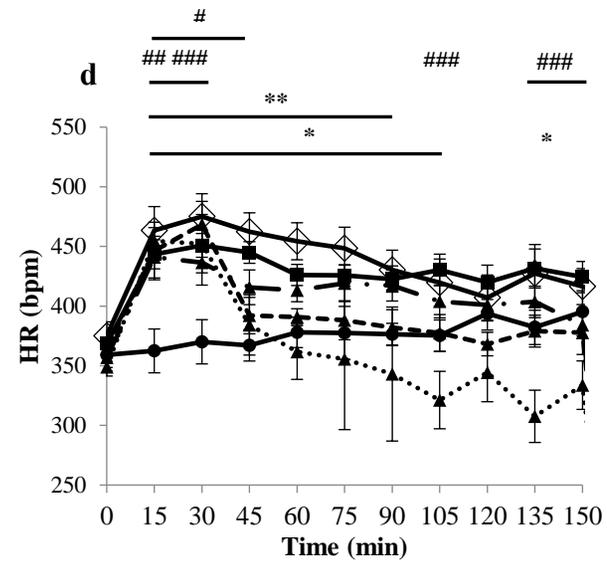
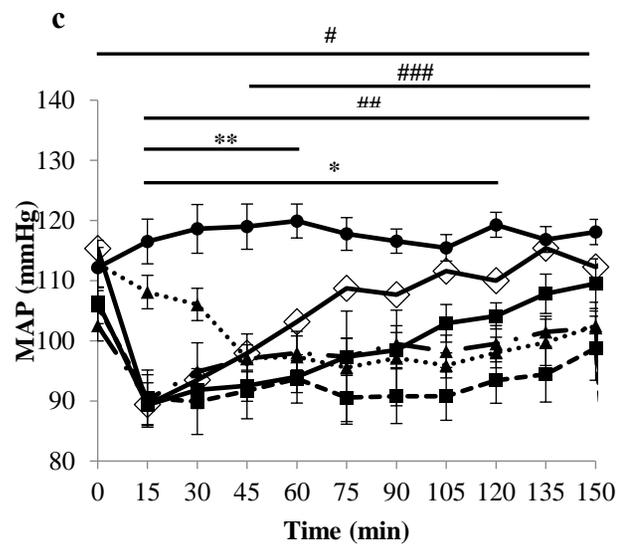
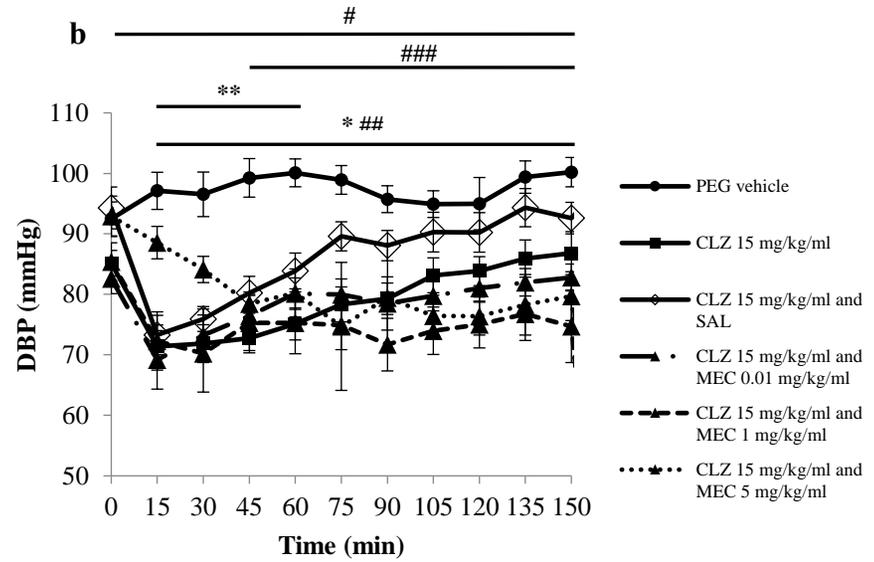
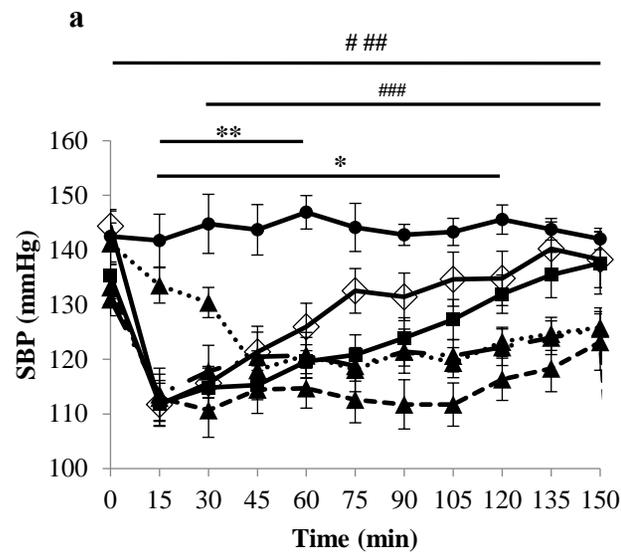


Figure 4.4: Acute effects of propranolol on cardiovascular parameters in adult rats treated with clozapine. The beat-to-beat cardiovascular parameters were recorded for animals treated with a single injection of CLZ at 15 mg/kg/ml or PEG vehicle at t = 15

mins. The CLZ-treated rats were then randomly administered SAL or PRO (1, 5 or 10 mg/kg/ml) at $t = 30$ mins. **(a)** The depressor effects of CLZ was reversed by administering PRO, where the 2 higher doses of PRO demonstrated immediate reversal of SBP. **(b)** Administration of PRO effectively reversed depressor effects caused by CLZ treatment. The 5 and 10 mg/kg/ml doses significantly raised DBP approximately 45 mins post-injection whereas low dose PRO's effects started at $t = 90$ mins. **(c)** Decreased MAP induced by CLZ lasted until $t = 120$ mins, which was reversed by all 3 doses of PRO. The MAP of 15 mg/kg/ml PRO became statistically comparable to PEG within 30 mins of injection. Mid-dose PRO's effects were immediate, whereas there was a delay of 60 mins in the low dose PRO group. **(d)** CLZ-induced tachycardia was reversed by PRO almost immediately following injection for all doses. Where elevated HR was seen in the CLZ and CLZ-SAL groups until $t = 90 - 105$ mins, PRO effectively reversed CLZ-induced tachycardia immediately and the effects persisted until the end of the experiment. **(e - g)** CLZ-PRO largely had no pronounced effects on LVP and $dP/dt_{\max/\min}$ unless otherwise indicated at individual time points. * Statistical significance for 15 mg/kg/ml CLZ as determined by LSD post-hoc tests $p < 0.05$. ** Statistical significance for 15 mg/kg/ml CLZ and SAL compared to vehicle, $p < 0.05$. # Statistical significance for 15 mg/kg/ml CLZ and 1 mg/kg/ml PRO, $p < 0.05$. ## Statistical significance for 15 mg/kg/ml CLZ and 5 mg/kg/ml PRO, $p < 0.05$. ### Statistical significance for CLZ 15 mg/kg/ml and PRO 10 mg/kg/ml, $p < 0.05$.



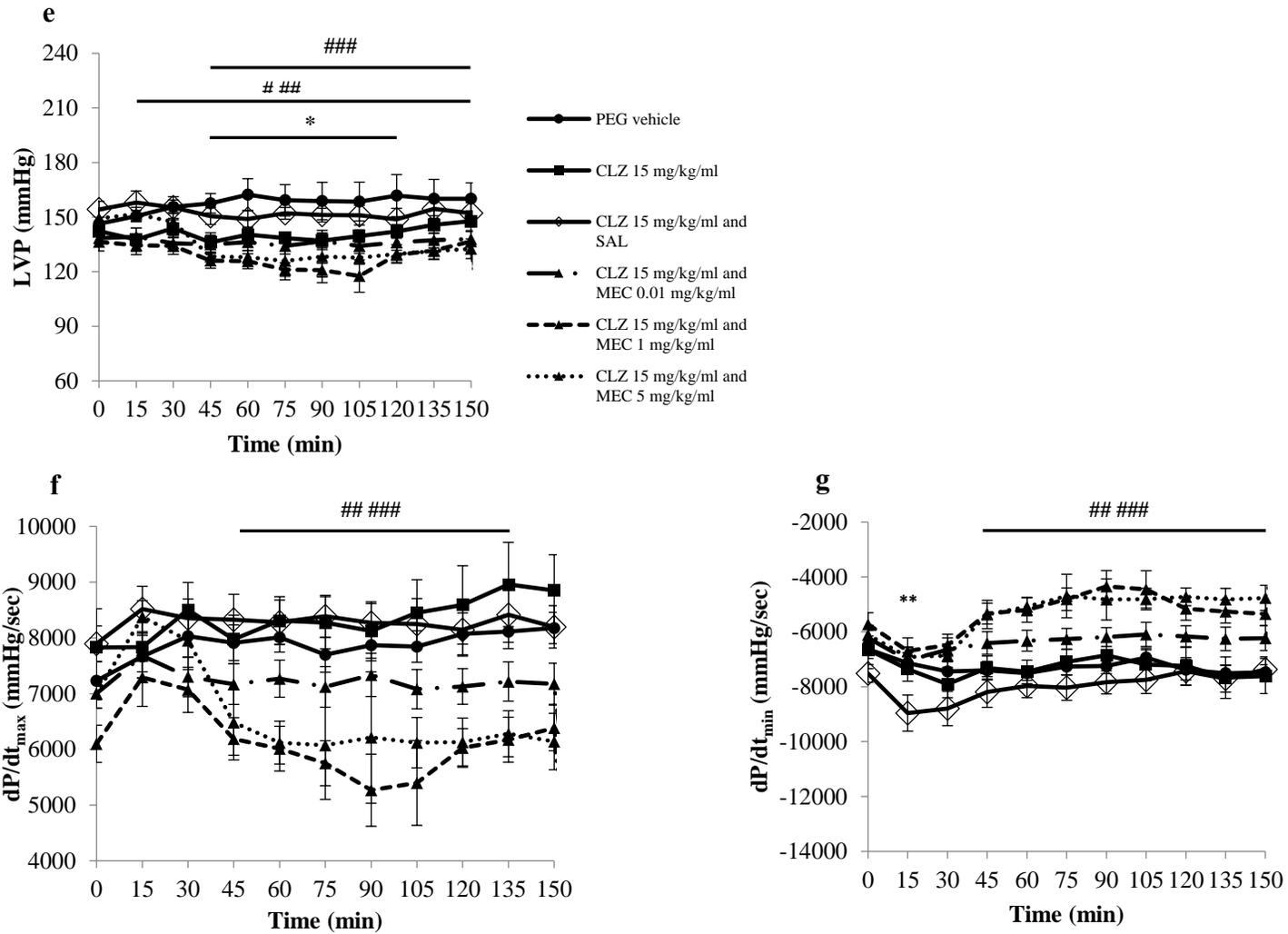


Figure 4.5: Acute effects of mecamlamine on cardiovascular parameters in adult rats treated with clozapine. The beat-to-beat cardiovascular parameters were recorded for animals treated with a single i.p. injection of CLZ at 15 mg/kg/ml or PEG vehicle at t =

15 mins. The CLZ-treated rats were then randomly administered SAL or MEC (0.01, 1 or 5 mg/kg/ml) at t = 30 mins. **(a – c)** The depressor effects of CLZ was exacerbated with the administration of MEC, most notable at the 1 mg/kg/ml dose, where SBP, DBP and MAP were decreased significantly for the majority of the experiment. **(d)** MEC effectively reversed CLZ-induced tachycardia for all doses tested. **(e)** CLZ suppressed LVP transiently from t = 45 – 120 mins. Administration of MEC at t = 30 mins prolonged CLZ's depressor effects that lasted for the duration of the experiment. **(f–g)** MEC at 1 and 5 mg/kg/ml significantly decreased $dP/dt_{\max/\min}$ immediately following injection. CLZ and the lowest dose of MEC had no significant effect on $dP/dt_{\max/\min}$. * Statistical significance for CLZ 15 mg/kg/ml as determined by post-hoc tests $p < 0.05$. ** Statistical significance for CLZ 15 mg/kg/ml and SAL compared to vehicle, $p < 0.05$. # Statistical significance for CLZ 15 mg/kg/ml and MEC 0.01 mg/kg/ml, $p < 0.05$. ## Statistical significance for CLZ 15 mg/kg/ml and MEC 1 mg/kg/ml, $p < 0.05$. ### Statistical significance for CLZ 15 mg/kg/ml and MEC 5 mg/kg/ml, $p < 0.05$.

Clozapine's effect on heart rate

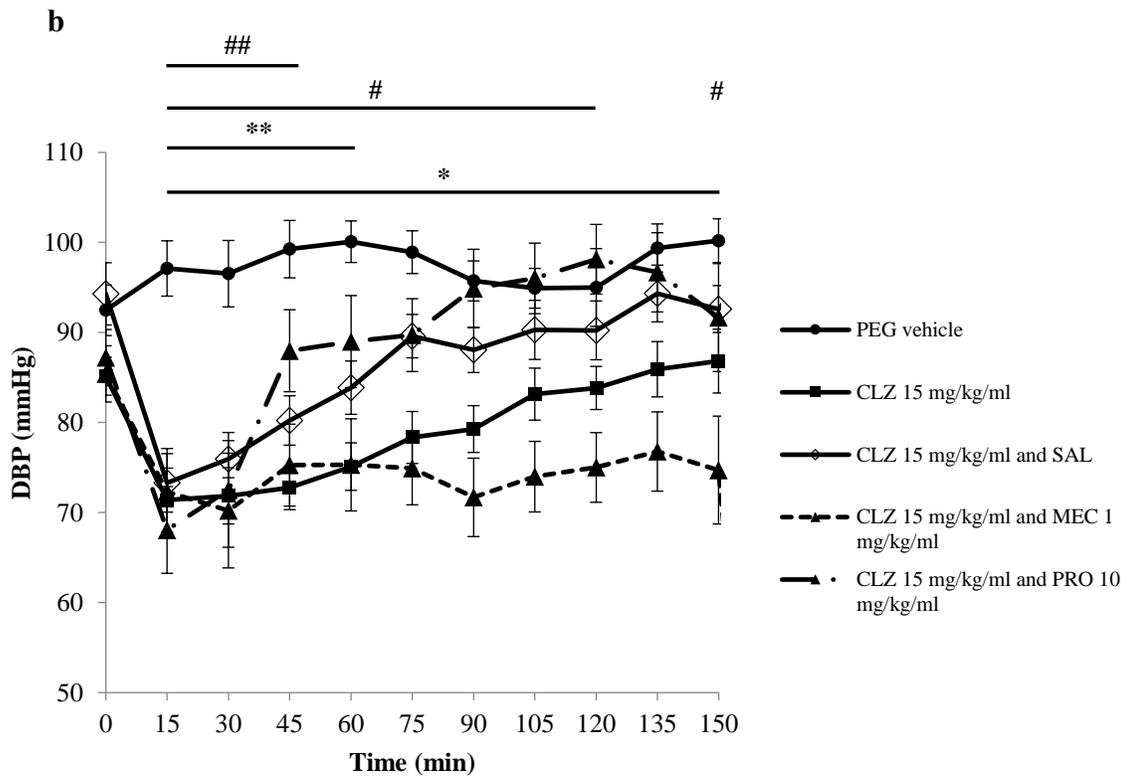
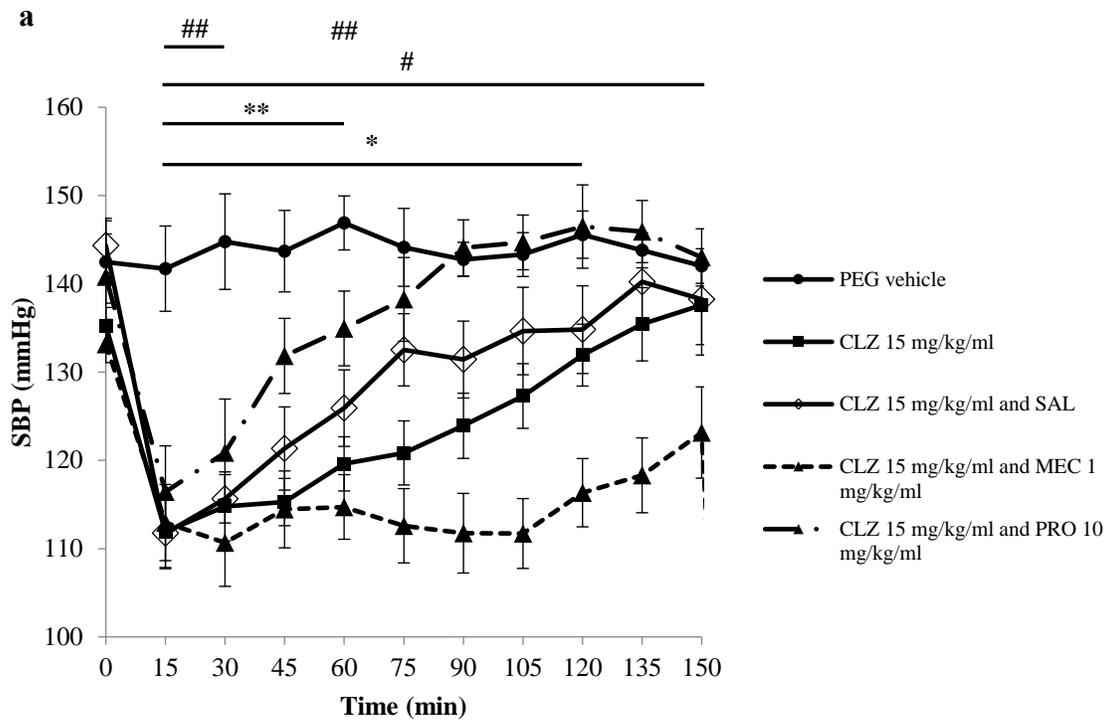
The immediate cardiovascular effects of administering CLZ in comparison to vehicle are listed in Table 4.1. Prior to CLZ treatment, there was no difference in HR between the 3 doses at baseline [$F_{(2,36)} = 0.62$, NS]. Immediately following treatment, HR was elevated for all 3 doses tested. The effects were longer in high dose CLZ, persisting until $t = 105$ mins. A main effect for drug treatment was absent [$F_{(2,28)} = 1.30$, NS], yet there was a strong main effect of time [$F_{(9,252)} = 6.07$, $p < 0.001$] and no effect of treatment \times time [$F_{(18,252)} = 1.13$, $p < NS$]. Post-hoc analysis revealed the effect was due to high dose CLZ, as the other 2 doses had only transient effects on HR (Figure 4.3d).

Likewise, when analyzing the effects of antagonists in combination with CLZ, the HR did not differ significantly among all groups at baseline [$F_{(2,32)} = 0.61$, NS]. Following administration of the antagonists, a main effect of drug treatment was observed [$F_{(2,28)} = 3.35$, $p < 0.05$], as well as a strong main effect of time [$F_{(9,252)} = 6.1$, $p < 0.001$] and treatment \times time [$F_{(18,252)} = 3.03$, $p < 0.001$]. Post-hoc tests indicated that CLZ-MEC induced a significant decrease in HR until $t = 135$ mins. On the contrary, CLZ-PRO's effects were shorter in duration, lasting until $t = 75$ mins.

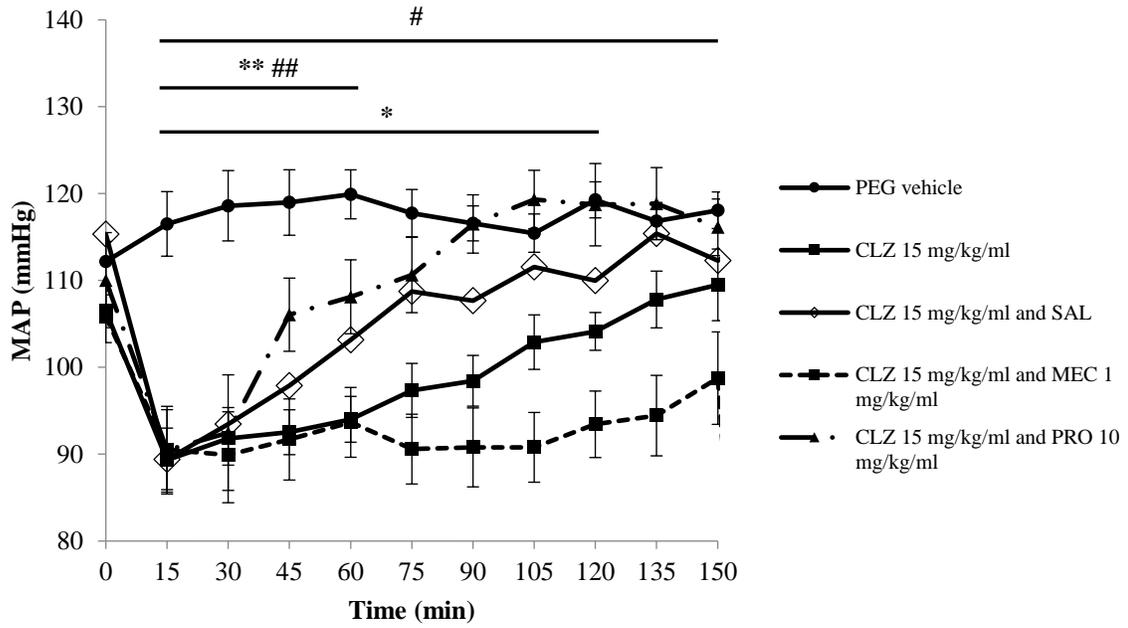
Table 4.1: Changes in cardiovascular parameters immediately following clozapine administration.

Variable	Vehicle		CLZ (mg/kg/ml)					
			5		10		15	
	t = 0 min	t = 30 min	t = 0 min	t = 30 min	t = 0 min	t = 30 min	t = 0 min	t = 30 min
HR (bpm)	359 ± 13	370 ± 18	371 ± 12	454 ± 15*	385 ± 15	477 ± 11*	368 ± 8	451 ± 10*
MAP (mmHg)	112 ± 3	119 ± 4	110 ± 2	107 ± 4	106 ± 4	95 ± 4*	106 ± 2	92 ± 3*
SBP (mmHg)	142 ± 5	145 ± 5	140 ± 3	133 ± 5	136 ± 4	119 ± 5*	135 ± 2	115 ± 4*
DBP (mmHg)	92 ± 3	97 ± 4	89 ± 2	87 ± 3	87 ± 4	74 ± 4*	85 ± 2	72 ± 3*
LVP (mmHg)	146 ± 4	156 ± 4	144 ± 4	152 ± 3	143 ± 4	147 ± 3	142 ± 3	144 ± 5
dP/dT _{max} (mmHg/sec)	7231 ± 342	8030 ± 381	7456 ± 294	8706 ± 211	7223 ± 523	7956 ± 457	7829 ± 695	8501 ± 493
dP/dT _{min} (mmHg/sec)	-6630 ± 413	-7448 ± 266	-5312 ± 1316	-6261 ± 1565	-6482 ± 386	-7357 ± 419	-6648 ± 597	-7909 ± 539

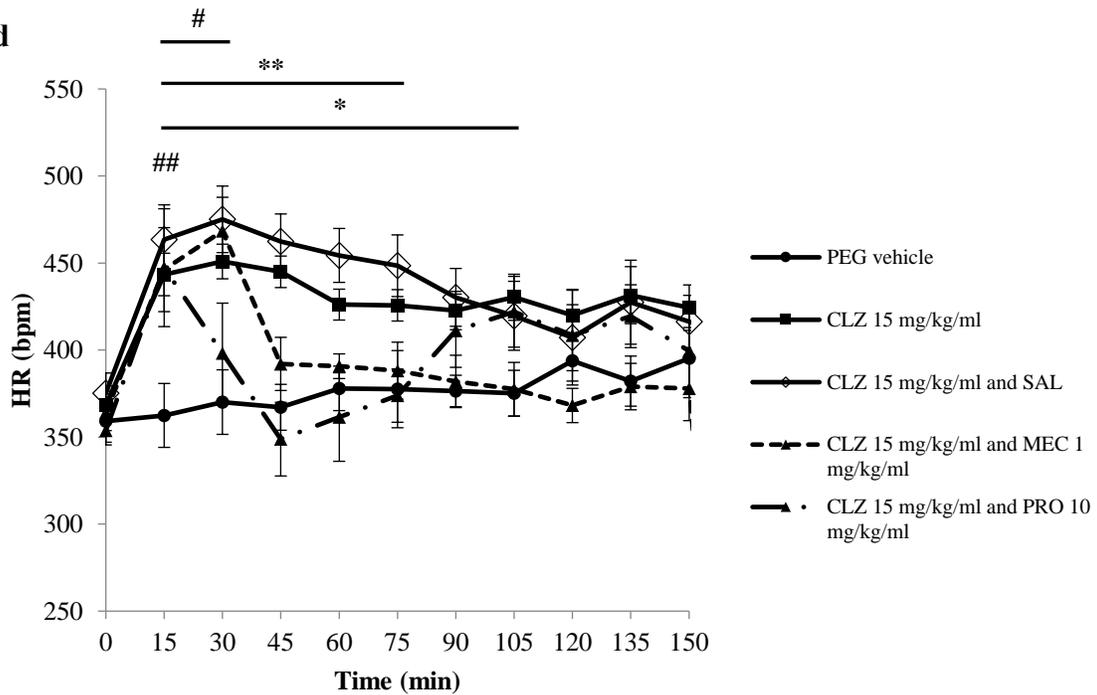
The beat-to-beat cardiovascular parameters at baseline t = 0 min and immediately following CLZ treatment at t = 30 mins are presented. There were no significant differences between groups at baseline t = 0 min. At t = 30 mins, all 3 doses of CLZ induced tachycardia and significantly. Only CLZ at 10 and 15 mg/kg/ml also significantly suppressed SBP, DBP and MAP. CLZ had no effect on LVP and dP/dT_{max/min}. Values are expressed as group mean ± SEM. * Statistically significant difference compared to the vehicle group, p < 0.05

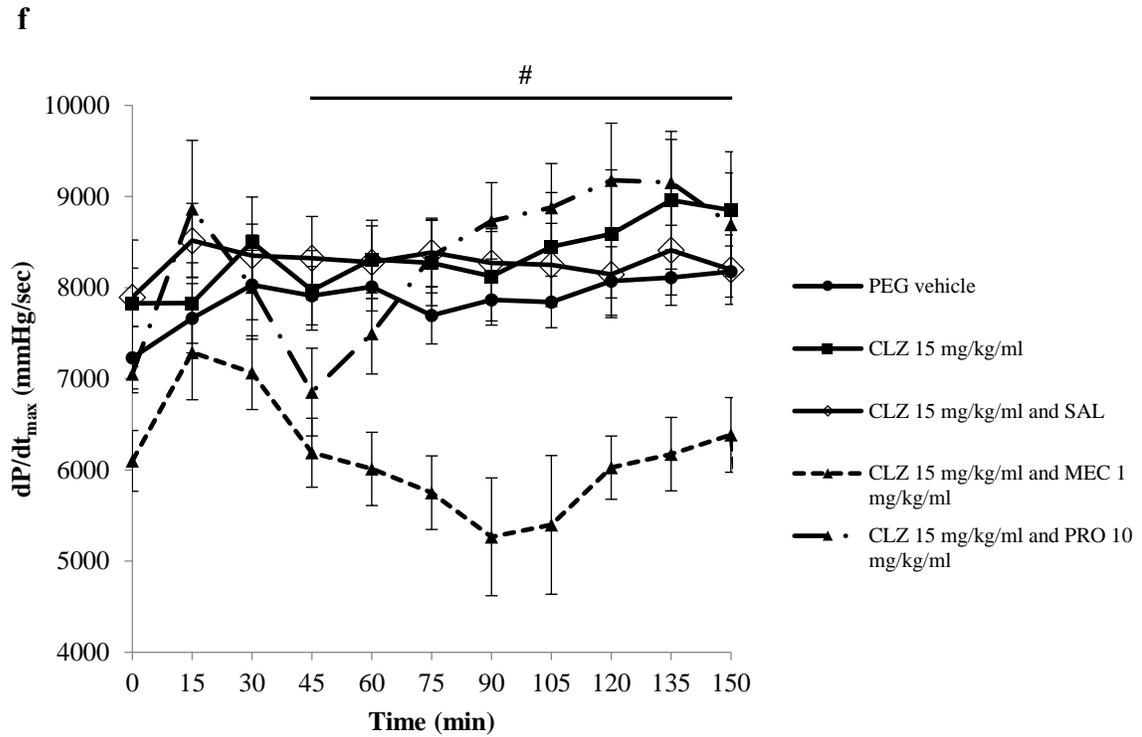
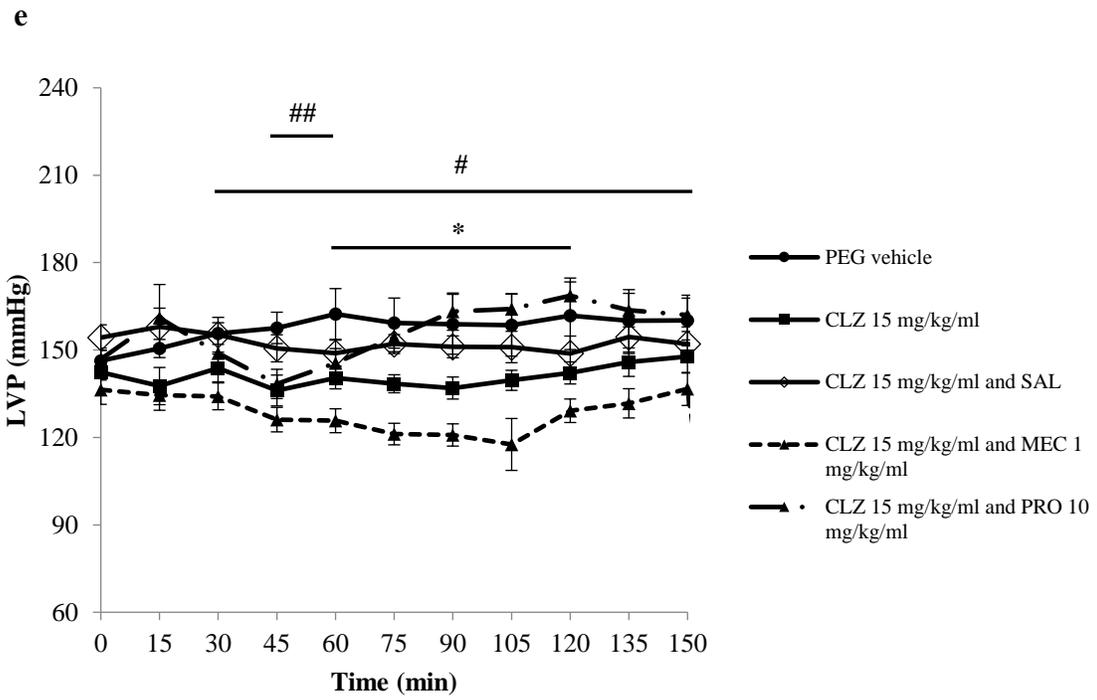


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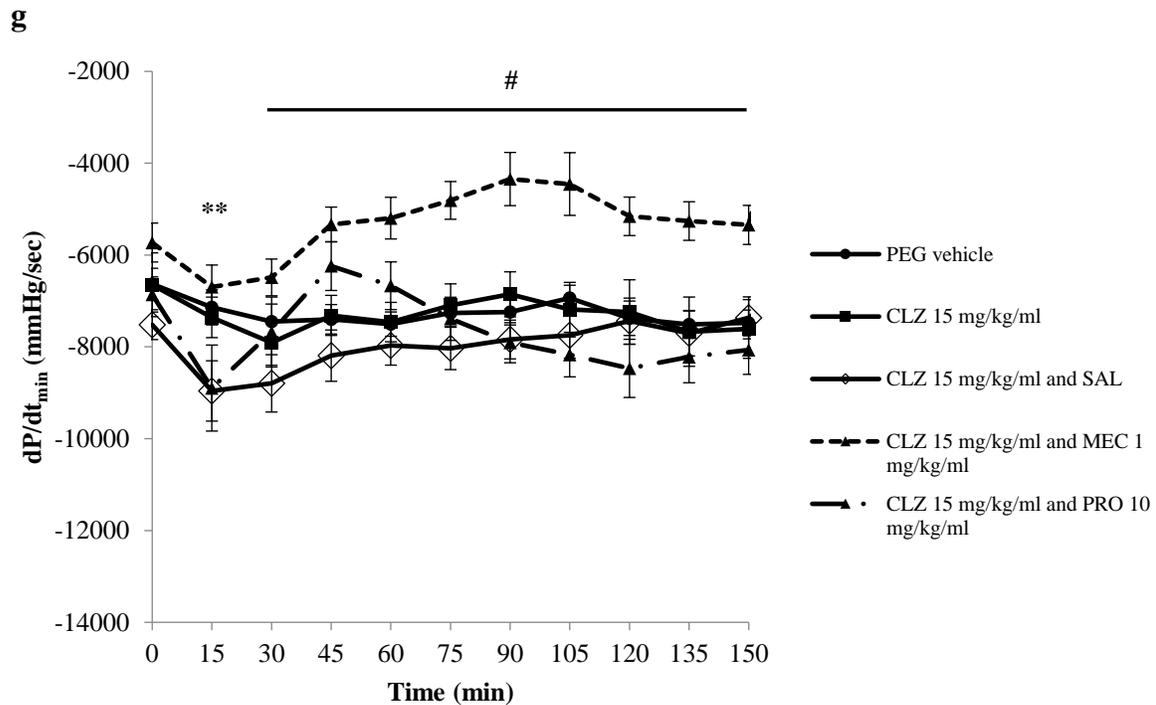


Figure 4.6: The effect of acute clozapine and antagonist treatment on cardiovascular parameters in adult female rats. The beat-to-beat cardiovascular parameters for animals treated with a single injection of CLZ (15 mg/kg/ml, i.p.), CLZ (15 mg/kg/ml, i.p.) and saline, PEG vehicle, CLZ (15 mg/kg/ml, i.p.) and MEC (1 mg/kg/ml, i.p.), or CLZ (15 mg/kg/ml, i.p.) and PRO (10 ml/kg, i.p.), are presented in this figure. CLZ or PEG was administered at $t = 15$ mins, and antagonists or SAL were administered at $t = 30$ mins. **(a)** CLZ suppressed SBP for most of the experiment, which was readily reversed with the administration of PRO. On the contrary, MEC prolonged the depressor effects of CLZ, while the CLZ-SAL group was comparable to vehicle starting at $t = 60$ mins. **(b)** CLZ had depressor effects on DBP for the duration of the experiment. PRO reversed these effects immediately following injection. The CLZ-MEC group displayed similar depressor effects to CLZ treatment alone, apart from a single time point at $t = 135$ mins. The CLZ-SAL group was comparable to control from $t = 60$ mins. **(c)** MAP was suppressed with CLZ treatment until $t = 120$ mins. PRO and SAL had similar trends on MAP following CLZ treatment, where MAP was statistically insignificant from control starting at $t = 60$ mins. MEC prolonged the depressor effects of CLZ to the end of the experiment. **(d)** HR was significantly increased immediately following CLZ treatment, persisting until $t = 105$ mins. Both PRO and MEC reversed CLZ-induced tachycardia. The SAL treated group had significantly elevated HR that lasted for 45 mins. **(e)** CLZ induced a reduction in LVP beginning at $t = 60$ mins that lasted for 60 mins in total. MEC prolonged CLZ's effects to the end of the experiment. CLZ-PRO and CLZ-SAL treatment groups did not display pronounced changes in LVP. Other treatment groups had no effect on LVP. **(f–g)** Co-administration of MEC significantly decreased $dP/dT_{\max/\min}$ immediately following injection

and its effects lasted until the end of the experiment. None of the other treatment groups had significant effects on $dP/dT_{\max/\min}$. Values shown are group mean \pm SEM. * Statistical significance for 15 mg/kg/ml CLZ compared to vehicle as determined by LSD post-hoc tests, $p < 0.05$. ** Statistical significance for 15 mg/kg/ml CLZ and SAL, $p < 0.05$. # Statistical significance for 15 mg/kg/ml CLZ and 1 mg/kg/ml MEC, $p < 0.05$. ## Statistical significance for 15 mg/kg/ml CLZ and 10 mg/kg/ml PRO, $p < 0.05$.

Clozapine's effect on systolic and diastolic blood pressure, and mean arterial pressure

Acute administration of CLZ generally lowered SBP in a dose-dependent manner (Figure 4.3a). High dose CLZ's effects persisted until $t = 120$ mins. Statistical analysis of SBP at baseline showed there was no difference among the 3 doses of CLZ tested [$F_{(2,35)} = 0.68$, NS]. Further analysis of the effects of CLZ treatment revealed a main effect of drug treatment [$F_{(2,27)} = 5.23$, $p < 0.01$], a main effect of time [$F_{(9,243)} = 18.57$, $p < 0.001$] and treatment \times time interaction [$F_{(18,243)} = 2.22$, $p < 0.01$]. The significant effects were primarily from the 10 and 15 mg/kg/ml doses of CLZ.

No significant differences were noted for the treatment groups prior to administration of CLZ and antagonists, [$F_{(2,31)} = 1.54$, NS]. There was a main effect of drug treatment [$F_{(2,27)} = 8.52$, $p < 0.01$], main effect of time [$F_{(9,243)} = 17.09$, $p < 0.001$] and treatment \times time interaction [$F_{(18,243)} = 3.27$, $p < 0.001$]. These effects were mainly due to the potent depressor effects of MEC on SBP that lasted for the duration of the experiment (Figure 4.6a). Post-hoc analysis indicated CLZ-PRO raised SBP immediately following injection, with its effects dissipating at $t = 120$ mins, as compared to CLZ. CLZ-MEC displayed a comparable depressor response to CLZ for the initial 45 mins, after which its effects outlasted CLZ and persisted until the end of the experiment.

DBP was immediately suppressed following CLZ treatment until the end of the experiment (Figure 4.6b). There was no statistical difference among the groups prior to CLZ and antagonist treatment [$F_{(2,31)} = 0.14$, NS]. Repeated measures analysis indicated a significant main effect of drug treatment [$F_{(2,27)} = 4.05$, $p < 0.05$], a strong main effect of both time [$F_{(9,243)} = 12.66$, $p < 0.001$] and treatment \times time [$F_{(18,243)} = 3.50$, $p < 0.001$]. Further analysis with post-hoc tests revealed the effect was due to the CLZ-PRO group, where there was a rapid and profound increase in DBP.

CLZ demonstrated similar depressor effects on MAP as with SBP and DBP (Figure 4.6c). MAP for all groups did not differ at baseline [$F_{(2,31)} = 0.50$, NS]. A main effect of drug treatment was noted [$F_{(2,27)} = 6.0$, $p < 0.01$], as with time [$F_{(9,243)} = 16.24$, $p < 0.001$] and treatment \times time [$F_{(18,243)} = 3.71$, $p < 0.001$]. This was followed up with post-hoc tests, where the results indicate CLZ-PRO's actions on MAP contributed to these significant effects. On the contrary, CLZ-MEC further decreased MAP from $t = 105 - 135$ mins as compared to CLZ.

Clozapine's effect on left ventricular pressure

LVP, dP/dt_{\max} and dP/dt_{\min} were used as indicators of LV contractile function. LVP did not differ significantly among treatment groups at baseline [$F_{(2,32)} = 1.62$, NS]. Repeated measures analysis of LVP revealed highly significant main effects of drug treatment [$F_{(2,24)} = 10.72$, $p < 0.001$] and time [$F_{(9,216)} = 5.48$, $p < 0.001$] on this measure. The interaction of time and drug treatment was also significant [$F_{(18,216)} = 1.93$, $p < 0.05$]. CLZ-MEC and CLZ-PRO treatment had opposite effects on LVP. In comparison to the CLZ group, CLZ-MEC lowered

LVP from 30 to 90 mins post-injection while CLZ-PRO increased LVP from $t = 75 - 135$ mins (Figure 4.6e).

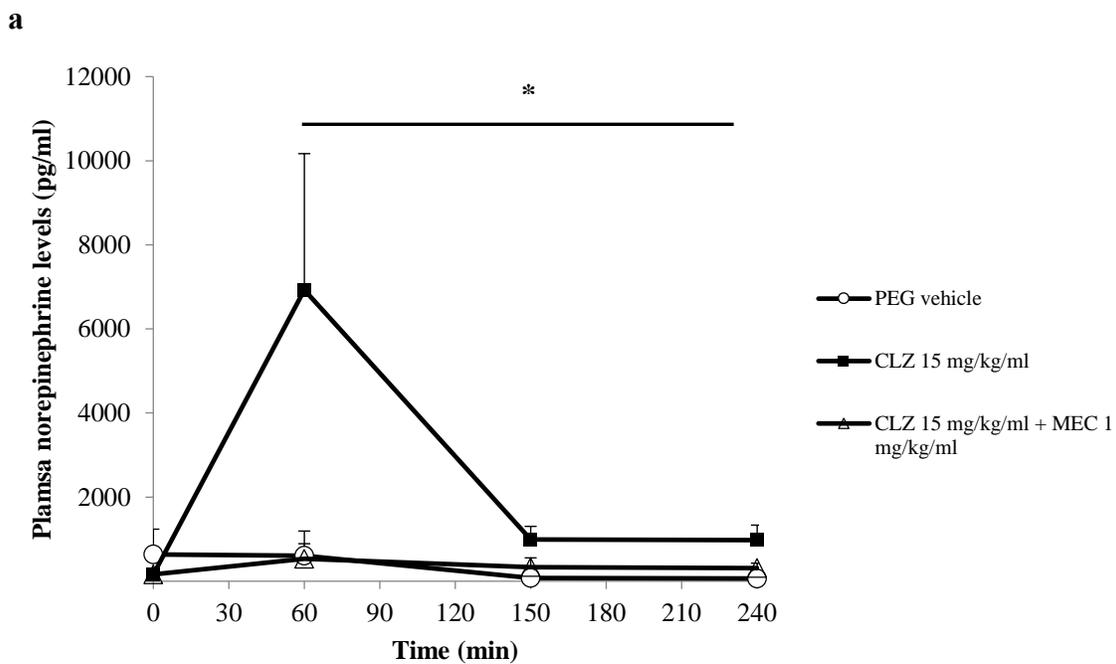
Clozapine's effect on maximal rate of increase and decrease of left ventricular peak systolic pressure

The maximum rate of rise in LVP, dP/dt_{max} , did not differ among groups at baseline [$F_{(2,32)} = 2.11$, NS]. After treatment with antagonists, a main effect of drug treatment was noted [$F_{(2,24)} = 4.08$, $p < 0.0001$]. There was a main effect of time [$F_{(9,216)} = 4.08$, $p < 0.001$] and treatment \times time [$F_{(18,216)} = 2.59$, $p < 0.001$]. Subsequent post-hoc analysis showed the effect was due to the pronounced depressor effects of MEC on dP/dt_{max} (Figure 4.6f). Neither CLZ nor CLZ-PRO had significant effects on dP/dt_{max} .

Drug treatment had a significant effect on dP/dt_{min} [$F_{(2,24)} = 7.32$, $p < 0.01$]. At baseline, the treatment groups did not differ significantly [$F_{(2,32)} = 0.95$, NS]. There was a highly significant effect of time on dP/dt_{min} [$F_{(9,216)} = 9.98$, $p < 0.001$]. The interaction of dP/dt_{min} and drug treatment was also highly significant [$F_{(18,216)} = 2.72$, $p < 0.001$]. Of the treatment groups, only CLZ-MEC had significant effects on dP/dt_{min} . Specifically, there was an immediate elevation of dP/dt_{min} that persisted until the end of the experiment (Figure 4.6g).

Clozapine's effect on plasma catecholamine levels

CLZ raised plasma NE to levels consistently higher than vehicle-treated animals from t = 60 mins to the end of the experiment (Figure 4.7a). Prior to drug treatment, all groups had similar NE levels [$F_{(2,18)} = 0.58$, NS]. There was a main effect of drug treatment [$F_{(2,17)} = 5.73$, $p < 0.05$], a main effect of time [$F_{(2,34)} = 3.87$, $p < 0.05$] and treatment \times time interaction [$F_{(4,34)} = 2.61$, $p < 0.05$]. At its peak, the mean plasma NE level of the CLZ group at t = 60 mins was 6926.76 ± 3240.72 pg/ml compared to 602.64 ± 590.4 pg/ml of the vehicle group, where post-hoc analysis revealed a significant difference between the 2 groups ($p < 0.05$). At the remaining 2 time points, the mean plasma NE levels of the CLZ group were also significantly higher than vehicle ($p < 0.01$). In contrast, MEC treatment effectively suppressed CLZ-induced elevation in NE levels, where the NE levels were statistically indifferent from PEG vehicle ($p > 0.05$). No significant changes in E levels were noted for all treatment groups (Figure 4.7b).



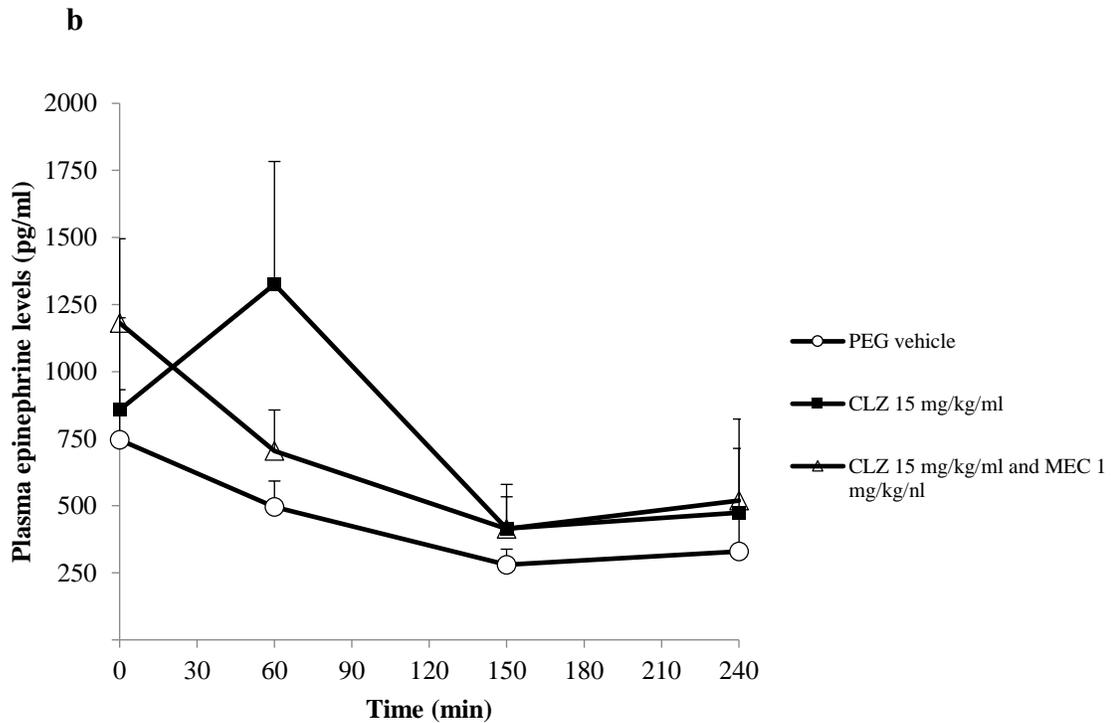


Figure 4.7: Acute effects of clozapine on plasma catecholamine levels. The change in plasma NE and E levels in response to CLZ treatment over time is shown. CLZ or PEG was administered at $t = 0$ min. **(a)** There was a significant rise in plasma NE levels following CLZ treatment, peaking at $t = 60$ mins and reached a plateau within 90 mins post-injection. Plasma NE levels in CLZ treated animals remained significantly higher than that of control animals. Treatment of MEC effectively suppressed the surge in NE levels. **(b)** CLZ treatment did not have any significant effects on the plasma levels of E. Values shown are group mean \pm SEM. *Statistical significance for the 15 mg/kg/ml CLZ group compared to vehicle, as determined by LSD post-hoc tests $p < 0.05$.

4.4 Discussion

The present study demonstrated acute CLZ administration can induce tachycardia and this is reversible with the use of the conventional antihypertensive, PRO, and through nAChR blockade using MEC. Acute administration of CLZ alone raised the baseline HR by more than 50 bpm, with effects peaking at 15 mins post-injection and remaining elevated up to 105 mins. PRO at a dose of 10 mg/kg/ml and MEC at 1 mg/kg/ml reversed CLZ-induced

tachycardia, where MEC had a more pronounced effect. There were significant depressor effects on MAP, SBP and DBP from CLZ treatment, which was prolonged by MEC but not PRO. LVP was also significantly decreased by CLZ, albeit lasting for only 60 mins. The depressor effects of MEC was more pronounced in comparison, decreasing LVP immediately following injection and continuing for the duration of the experiment. PRO surprisingly caused pressor responses as BP steadily rose to levels comparable to that of PEG vehicle animals immediately following injection. No significant effects were noted with PRO on LVP (apart from 2 time points) and $dP/dt_{\max/\min}$. CLZ did not induce any changes in $dP/dT_{\max/\min}$, whereas CLZ-MEC treatment decreased dP/dT_{\max} in general, reaching statistical significance at $t = 75$ mins. There was also a general trend of elevating dP/dT_{\min} with CLZ-MEC treatment, that persisted for the entire experiment. Plasma NE levels were significantly raised with CLZ treatment, peaking at $t = 60$ mins, and remained elevated for the remainder of the experiment. Administration of MEC at 30 mins effectively prevented the surge in plasma NE levels. Collectively, our results suggest CLZ induces tachycardia via a central sympathetic mechanism, namely stimulation of cardiac β_1 -adrenoceptors by increased NE spillover.

Acute tachycardia (> 50 bpm above baseline HR) was evident with the administration of CLZ in our animal model. Accelerated HR is believed to result from CLZ's anticholinergic properties (Buchanan, 1995), in combination with antagonism of α -adrenoceptors and elevated circulating NE levels. (Lieberman and Safferman, 1992; Breier et al., 1994) The surge in NE is attributable to CLZ's blockade of both postjunctional α_1 -adrenoceptors and prejunctional α_2 -adrenoceptors, which collectively increases sympathetic outflow via reflexive action (Breier et al., 1994). Furthermore, blockade of α_2 -adrenoceptors

on sympathetic nerve terminals inhibits the α_2 -adrenoceptor mediated negative regulation of NE release. In agreement with literature (Sarafoff et al., 1979; Breier et al., 1990; Davidson et al., 1993; Green et al., 1993a; Elman et al., 1999), we observed a significant elevation of plasma NE levels following CLZ administration in our animal model. The combination of increased NE presence and blockade of peripheral α -adrenoceptors by CLZ is expected to have primarily hypotensive effects, as demonstrated in our animal model. Although NE preferably binds to α_1 -adrenoceptors (Langer and Hicks, 1984), NE can also bind β -adrenoceptors of vasculature and cardiac tissue (Taylor, 2007). Positive chronotropy observed in our experiments is possibly the result of stimulation of cardiac β_1 -adrenoceptors by NE, which in turn increases the firing rate of the sinoatrial node. This is supported by the immediate decrease in HR when PRO was administered. In vasculature, NE binds β_2 -adrenoceptors to cause vasodilation, possibly exacerbating the hypotension caused by CLZ blockade of α_1 -adrenoceptors.

Interestingly, we noted a gradual rise in BP following PRO injection that reached pressures comparable to vehicle-injected animals. This is contrary to the well-known but poorly understood antihypertensive effects of PRO, which are possibly due to suppressed release of renin by β_1 -adrenoceptor blockade and reduced central sympathetic outflow (Buhler et al., 1972; Buhler et al., 1973). One explanation is when CLZ is administered alone, vasodilation is initially caused by vascular α_1 -adrenoceptor blockade, followed by increased plasma NE spillover resulting from the inhibition of α_2 -adrenoceptors on sympathetic nerve terminals. Vascular β_2 -adrenoceptors are then targeted by the excess NE in circulation, thereby exacerbating hypotension. When PRO was administered, vascular β_2 -adrenoceptors are inhibited, and BP gradually rises over time as CLZ is cleared away from

circulation and NE binds the α_1 -adrenoceptors vacated by CLZ. Further studies are warranted as there is currently a lack of studies investigating pharmacological treatments for CLZ-induced tachycardia (Lally et al., 2016; Yuen et al., 2018).

Our results provide support for the use of PRO for the acute management of CLZ-induced tachycardia. The use of β -adrenoceptor blockers such as PRO and ATEN are usually prescribed when tachycardia is indicated in CLZ-treated patients (Stryjer et al., 2009). A retrospective chart review showed 75% of patients responded to ATEN or PRO treatment for CLZ-induced tachycardia, suggesting therapeutic effects are achieved by suppression of sympathetic hyperactivity (Stryjer et al., 2009). However, it is important to note that not all patients respond to β -blockers and under certain circumstances these agents can exacerbate existing complications and side effects associated with CLZ. In a study comparing CLZ treatment alone and with the use of PRO or ATEN, both agents had additive effects on lipid measures (Baymiller et al., 2003). In comparison to ATEN, PRO had a more pronounced effect on the lipid measures including total cholesterol and serum triglycerides (Baymiller et al., 2003). Additional complications include increased risk for diabetes (Popp et al., 1984; Bangalore et al., 2007a), stroke (Lindholm et al., 2005; Bangalore et al., 2007b) and weight gain (Rossner et al., 1990; Sharma et al., 2001; Lainscak et al., 2006). Furthermore, β -adrenoceptor blockers potentially exacerbate bronchospasm and therefore poses a risk in asthmatic patients (Lin et al., 1999). In these situations, patients would benefit by switching to another antiarrhythmic agent such as the newer ivabradine which targets I_f or funny currents to inhibit erratic depolarization of the sinus node (Lally et al., 2014). The lack of alternatives for managing tachycardia, combined with intolerability and ineffectiveness issues associated with conventional β blockers (Lally et al., 2014), can have grave

consequences. For instance, the initial increases in HR are often mistaken as normal responses to CLZ titration (Ronaldson et al., 2011). Tachycardia associated with CLZ is normally dose related and asymptomatic, and patients can develop tolerance over time (Lieberman and Safferman, 1992; Sagy et al., 2014). Yet if left untreated, tachycardia can lead to fatal myocarditis (Shinbane et al., 1997).

In light of the need for alternative treatments and the grave consequences of CLZ-induced tachycardia, we evaluated the novel use of MEC as an antiarrhythmic. Although traditionally used as an antihypertensive and discontinued due to autonomic side effects, there is now renewed interest for the use of MEC in areas such as Alzheimer's disease, Tourette's syndrome and nicotine cessation (George et al., 1995; Young et al., 2001). Adverse autonomic events are likely avoided when MEC is administered below the therapeutic dose for hypertension (<30 mg/kg) (Shytle et al., 2002; Nickell et al., 2013). The use of MEC is advantageous over other ganglionic blockers such as hexamethonium since it readily crosses the blood brain barrier to act on central nAChRs, is easily absorbed by the gastrointestinal track, and has effects that are both rapid and long lasting (Blomqvist et al., 1993; Shytle et al., 2002; Bacher et al., 2009; Nickell et al., 2013). Based on these favorable properties, we studied the effect of MEC on HR and BP at a low dose of 1 mg/kg/ml in conscious rats treated with CLZ. MEC is expected to suppress sympathetic outflow by antagonism of brain and ganglionic nAChRs, leading to vasodilation and increased peripheral blood flow, reduced cardiac output and a decrease in BP (Young et al., 2001; Bacher et al., 2009). Indeed, our results show MEC at 1 mg/kg/ml following CLZ treatment rapidly suppressed HR to levels comparable to control animals and the effects lasted for the duration of the experiment. Hypotension was observed immediately following MEC injection, with

SBP, DBP and MAP remaining low even when CLZ's depressor effects began to subside at $t = 120$ mins. It remains unknown whether the hypotension can be avoided with lower doses of MEC, while preserving its HR lowering properties. MEC also abolished the rise in plasma NE levels present in CLZ-treated animals. Plasma E levels were statistically insignificant among all treatment groups. This suggests stress-induced release of E from the adrenal medulla is unlikely a factor as we did not observe a corresponding elevation of circulating E. It has been shown baroreflexes have little influence over secretion of E from the adrenal medulla while NE secretion is largely barosensitive (Natarajan and Morrison, 1999; Verberne et al., 2016). Thus, the mechanism by which MEC lowers NE spillover is likely associated with inhibition of baroreflexes that are activated by the combination of vasodilation and α -adrenoceptor blockade by CLZ (Breier et al., 1994). Of importance, it has been shown MEC can reduce myocardial damage caused by CAT infusion (Raab et al., 1961). CATs are known contributors to heart and blood vessel damage (Haft, 1974) and there is evidence of CLZ-induced myocarditis associated with raised catecholamine levels (Wang et al., 2008a). Further studies involving the use of MEC in managing myocarditis associated with CLZ use are therefore warranted.

Lastly, there was no evidence of LV contractile dysfunction following CLZ treatment in our study. Administration of PRO tended to increase dP/dt_{max} and reduce dP/dt_{min} , yet these changes were statistically insignificant. MEC had the opposite effects as PRO, decreasing dP/dt_{max} and increasing dP/dt_{min} . CLZ had no effect on $dP/dt_{max/min}$, implying contractility is not altered with the acute administration of CLZ. Our results contradict those in literature, where myocardial dysfunction is a known side effect of CLZ (Leo et al., 1996a; Merrill et al., 2005). This discrepancy can be explained by the acute nature of our

experiments, which may not have fully captured the time course of LV dysfunction. There is evidence that impairments in LV function appear after several weeks of CLZ initiation as demonstrated in both preclinical and clinical studies. In rats, LV dysfunction has been shown to emerge after 21 days of consecutive CLZ treatment (Abdel-Wahab et al., 2014). In humans treated with CLZ, emergence of LV dysfunction commences 4 weeks from the start of treatment (Curto et al., 2015). Others have reported CLZ-induced myocarditis can occur beginning from week 3 of treatment (Ronaldson et al., 2010; Ronaldson et al., 2011). As irregularities in LV function can precede fatal cardiomyopathy (Kilian et al., 1999), it is imperative to investigate the chronic effects of CLZ in our future studies.

Of note, there are limitations to the current study. Firstly, a control group was not used to independently examine the cardiovascular effects of MEC and PRO. As the effects of MEC and PRO have already been well documented in numerous peer-reviewed articles (Perry and Schroeder, 1957; Bengtsson, 1976; Hollifield et al., 1976; Smits et al., 1982; Young et al., 2001; Shytle et al., 2002; Nickell et al., 2013; Kiriyama et al., 2016), we sought to minimize the amount of animals used and avoid inducing unnecessary stress by focusing on co-administration of MEC and PRO with CLZ. Future studies can include control groups of these antagonists based on the results of our dosing experiments. Another limitation is the lack of a CLZ-PRO treatment group in our HPLC study. The HPLC study was the last of the present series of experiments. Time and personnel constraints, as well as technical difficulties in obtaining animals resulted in a choice between MEC or PRO to be co-administered with CLZ. Since MEC co-administration with olanzapine produced reliable results in previous catecholamine experiments in our laboratory, we chose to prioritize MEC in the present study. A CLZ-PRO treatment group will be included in follow-up studies.

In summary, we have shown several clinical signs of CLZ treatment including tachycardia, significant elevation of plasma NE levels and hypotension can be replicated in rodents. Increases in HR and circulating catecholamines associated with CLZ are reversible by administering MEC, while hypotension was surprisingly reversed with PRO. The results suggest CLZ elicits these cardiovascular side effects via a combination of baroreflexes and peripheral adrenoceptor blockade. It remains to be determined whether the observed tachycardia precedes cardiomyopathy and myocarditis in our animal model, which warrants further chronic studies given the grave consequences of myocarditis. Nevertheless, it is evident the ANS plays an important role in mediating adverse cardiovascular effects of CLZ. In particular, further investigation in the use of low dose MEC to suppress central sympathetic outflow may prove fruitful, especially for patients who have tolerability issues with PRO.

Chapter 5: General discussion

5.1 Summary

The present series of experiments were performed to assess the cardiometabolic effects of CLZ in a rodent model. Our first study investigated NOR's propensity to induce metabolic side effects in comparison to its parent compound, CLZ. The justification for this study stemmed from the finding that alteration of the ratio between NOR and CLZ can alleviate toxic side effects commonly associated with CLZ. Our results indicate NOR does not induce glucose dysregulation at the same severity as CLZ. NOR induced insulin resistance at high dose but not glucose intolerance. On the contrary, although CLZ was rapidly cleared away from plasma, the resultant insulin resistance and glucose intolerance were pronounced in comparison to vehicle.

The second study investigated the possibility of introducing adrenoceptor blockers as a means of countering the metabolic effects of CLZ. The main finding from this pilot study was β -adrenoceptor blockers effectively reversed the insulin resistance associated with CLZ use whereas α -adrenoceptor antagonists had no effect. Both BUT and ATEN displayed efficacy towards increasing GIR, suggesting β_1 - and β_2 -adrenoceptor blockade contribute to insulin sensitivity.

Our final study investigated CLZ's effects on cardiovascular parameters, where we noted pronounced tachycardia accompanied by depressor effects immediately following CLZ treatment. We subsequently introduced MEC and PRO as interventions for the increased HR.

While both drugs successfully reversed CLZ-induced tachycardia, MEC was associated with significant depressor effects. The effects were not noted with PRO use. Measurement of CAT levels in plasma revealed a significant elevation in NE levels, that was reversible upon administration of MEC.

5.2 Clinical significance

The aforementioned experiments were performed on freely moving, conscious adult rats, allowing for the replication of the clinical features of CLZ treatment and investigation of potential interventions. Our results suggest NOR has minimal involvement in inducing glucose intolerance and insulin resistance when administered alone (Yuen et al., 2019). NOR reportedly accumulates in plasma and contributes to the toxicity of CLZ (Gerson et al., 1994; Deliliers et al., 1998; Nooijen et al., 2011; Legare et al., 2013). The correlation between plasma NOR levels and therapeutic efficacy or toxicity is less established in comparison to its parent compound, yet there is evidence that the ratio of CLZ:NOR is a better predictor of efficacy and adverse effects than CLZ or NOR alone (Legare et al., 2013). Interindividual variability possibly explains the lack of predictability of CLZ levels when considered alone, as low levels of the drug can still induce a response and vice versa (Lee et al., 2009).

Whether interindividual variability and enzymatic saturation explains NOR's minimal metabolic liability requires further studies. In comparison, the metabolic ratio of CLZ:NOR has been extensively investigated in relation to cognitive function and adverse effects.

Clinically, therapeutic drug monitoring of CLZ and NOR can be used to assess drug compliance, risk for toxicity and to determine dosage (Couchman et al., 2010). The current

finding of NOR's low metabolic liability relative to its parent compound is potentially beneficial in terms of balancing side effects and drug efficacy, thus warranting further investigation of NOR's therapeutic potential.

The second study involved the effects of administration of adrenoceptor blockers on CLZ-induced metabolic dysregulation. The major finding from this pilot study is that β -blockers administered after acute CLZ treatment effectively improved insulin sensitivity whereas inhibition of α -adrenoceptors have no effect. As CLZ has limited affinity for β -adrenoceptors, we postulate the administration of ATEN and BUT blocked NE from stimulating β -adrenoceptors, thus inhibiting lipolysis and decreasing the amount of plasma free fatty acids. Acute elevations of plasma fatty acids are known to induce insulin resistance (Boden, 2003), hence the decline in free fatty acids potentially improved insulin sensitivity as noted by the increase in GIR in the ATEN and BUT treatment groups. Indeed, acute CLZ administration in rodents lead to significantly elevated serum free fatty acids as early as 15 minutes post-injection (Jassim et al., 2012). The rapid increase in free fatty acids was attributed to the immediate upregulation of lipase genes, SNS activation and CAT release, accompanied by CLZ's potent antagonism of α_2 -adrenoceptors that regulate lipolysis (Jassim et al., 2012). The combined blockade of α - and β -adrenoceptors may thus contribute to the improved insulin sensitivity of the CLZ-ATEN and CLZ-BUT treatment groups in our study, with the acknowledgement that results may differ in the chronic setting which will require further experimentation.

There is growing evidence that use of β -adrenergic antagonists can exacerbate undesired metabolic side effects such as dyslipidemia, insulin resistance and type II diabetes (Jacob et al., 1998), and their concurrent use with CLZ intensifies these side effects

(Baymiller et al., 2003). Although the actual dosages of ATEN, PRO and the other β -adrenergic antagonists were not mentioned in Baymiller et al.'s review (2003a), the reported effective dose of these agents are 50 – 100 mg/day for ATEN and 160 mg/day for PRO (Lithell, 1991; Wadworth et al., 1991; Viskin et al., 1995; Bolon et al., 2019). ATEN used in animal experiments range from 1 to 10 mg/kg (Yoshimoto et al., 2011). Conversely, BUT is rarely prescribed and is instead used as a β_2 -adrenoceptor blocker in animal experiments (Hunninghake et al., 1966). Of the few studies administering BUT in humans, the dosage given to subjects orally was 5 mg/kg (Hunninghake et al., 1966). In rats, dosages of 25 – 50 mg/kg have been used (Salvador et al., 1967, 1968). In comparison to that in literature, the dosages of BUT and ATEN used for our experiments are lower (15 mg/kg/ml) and thus potentially has less influence on the metabolic side effects of CLZ. Whether we can balance the risk of worsening CLZ's adverse metabolic effects with improving insulin sensitivity using lower dosages of ATEN and BUT requires further testing. In addition, future experiments can include newer β -adrenergic antagonists that can potentially alleviate the risk of exacerbation of metabolic side effects.

The final series of experiments investigated cardiovascular parameters in response to acute CLZ treatment were measured in real time. Administration of CLZ at 15 mg/kg/ml readily induced tachycardia that was accompanied by a significant increase in circulating NE and hypotension. Of interest, these clinical signs are also present in patients treated with CLZ. We administered MEC and PRO with the goal of lowering HR, to which we noted a reversal of the hypotension following PRO but not with MEC. The reversal of hypotension following PRO administration was a surprise finding, as PRO's uses in treating hypertension are well documented (Prichard and Gillam, 1969; Zacharias et al., 1972; Bangalore et al.,

2007b). The rise in BP is possibly due to compensatory actions resulting from CLZ's blockade of α -adrenoceptors in combination with PRO's hypotensive effects. It is important to acknowledge that PRO can exacerbate metabolic complications commonly associated with CLZ, including weight gain, insulin resistance and diabetes (Bangalore et al., 2007b). In this scenario, the usage of MEC may prove beneficial when there is a high risk of metabolic side effects with CLZ use. MEC itself is associated with autonomic side effects such as hypersalivation at therapeutic dosing and we have noted depressor effects with 1 mg/kg/ml in rodents in our study. When the dose was lowered to 0.5 mg/kg/ml, MEC had comparable HR lowering effects to 1 mg/kg/ml, yet the accompanying depressor effects were still present. It should be noted that the 0.5 mg/kg/ml dose will need to be repeated on a larger sample size as the current sample size is small ($n = 3$). Furthermore, testing at doses between 0.5 – 1 mg/kg/ml can possibly identify an optimal dose that can achieve HR lowering effects without the ensuing depressor effects. It may prove beneficial to administer MEC at low doses, as undesired autonomic side effects can be avoided.

5.3 Limitations and future directions

There are several limitations to the present series of experiments. Firstly, plasma NE levels are not always representative of sympathetic activity. Plasma NE concentrations are determined by a complex function of uptake, clearance and spillover (Goldstein, 2010). For example, high plasma NE levels can be the result of increased sympathetic activity, decreased uptake by sympathetic terminals and/or decreased removal of NE from plasma. Often underappreciated, vesicular leakage of CATs also plays an important role in NE levels

(Eisenhofer et al., 2004a). A common misconception of CAT turnover is that it is mainly determined by release from sympathetic nerve terminals. Yet this does not account for the sustained NE release during stressful situations, as NE synthesis from tyrosine hydroxylase alone cannot keep pace with exocytosis and will only deplete neurotransmitter stores and decrease release (Eisenhofer et al., 2004b). Notably during exercise, there is a 10-fold increase in NE release, greatly exceeding that of the maximal 4-fold increase in NE synthesis from increased sympathetic nerve activity (Eisenhofer et al., 2004a). Thus, the relationship between sympathetic activity and NE spillover should be interpreted with caution.

Secondly, our experiments are acute in nature whereas CLZ is known to induce side effects such as cardiomyopathy beyond 12 months (Iqbal et al., 2003). It will be of interest to conduct chronic studies to account for time dependent changes in cardiometabolic parameters resulting from CLZ treatment. It should also be noted CLZ and NOR metabolism in rats are rapid as compared to humans, hence it is possible our data did not reflect crucial changes in plasma drug levels and metabolic indices in the IGTT study, as the earliest time point was $t = 60$ mins (Chapter 2). Whereas because the HIEC experiments recorded metabolic changes within 10 mins post-injection of CLZ or NOR, changes in insulin sensitivity due to these agents can be monitored in relation to their peak drug levels. Future studies should consider adjusting the time points for drug level and IGTT experiments to be inclusive of the rapid elimination time of CLZ and NOR, namely the first 10 minutes of drug treatment (Baldessarini et al., 1993).

Clinical representation is another concern, as preclinical models do not encompass all clinical features of CLZ and schizophrenia. Furthermore, dosing regimens used in animals do not necessarily reflect clinical efficacy. CLZ has binding actions on multiple receptors, thus

adjusting the dose will result in changes in CLZ's affinities for the receptors (Kapur et al., 2003). One method to standardize the dosing is to determine the dose at which D₂ receptors occupancy in animals is comparable to that of humans. Kapur and colleagues have previously determined the dose of CLZ that produces clinically relevant D₂ receptor occupancy ranges between 5 – 15 mg/kg via subcutaneous injection (2003). Route of administration and the strain of the animals also affect the therapeutically relevant dosage. Yet these factors are often overlooked and authors tend to use the most commonly reported doses from previous articles (Kapur et al., 2000). Nevertheless, for the purposes of our present studies, our objective is to reliably reproduce CLZ-induced cardiometabolic side effects in an animal model and we determined the dose of CLZ accordingly. Previous experience of CLZ dosing in our laboratory as well of those in literature were also taken into account when determining the dosing range (Marx et al., 2003; Chintoh et al., 2009; Boyda et al., 2010b). Future studies can consider using histological measures to determine D₂ receptor occupancy at these dosages.

Despite demonstrating increased visceral adiposity associated with CLZ, animal models have failed to consistently reproduce weight gain comparable to human studies, suggesting CLZ promotes visceral adiposity independently of weight gain (Cooper et al., 2008). The conflicting results are perhaps due to the highly sedative effects of CLZ in rodents, which would affect ingestion if used at a clinical dose (Cooper et al., 2008). Despite this disadvantage, preclinical studies are valuable for studying the mechanisms leading to CLZ-induced weight gain.

Finally, several additional experiments can be added to the current thesis. As extensively reviewed elsewhere (Eisenhofer et al., 1988; Goldstein et al., 1988; Goldstein et

al., 2003; Eisenhofer et al., 2004a), CAT metabolites can be useful indicators of SNS and PNS activity. Metabolites such as DHPG, MHPG and NMN are of interest, as they reflect neuronal NE turnover, peripheral NE turnover and non-neuronal NE metabolism, respectively (Goldstein et al., 2003). By incorporating the measurement of these metabolites in our current HPLC protocol, we can increase specificity of our sympathetic measures while minimizing invasiveness to the animals and the amount of resources required. These measurements can also be combined with assessments of plasma drug levels at the relevant time points (Chapter 2) for a more comprehensive study on CAT changes in relation to drug levels. The combination of assessing drug levels, CAT levels and metabolic indices will be essential in understanding the etiology and treatment of cardiometabolic side effects induced by CLZ.

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