The Role of Leptin-Melanocortin System Gene Variants in Antipsychotic-Induced Weight Gain

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
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Doctor of Philosophy
The Institute of Medical Sciences
University of Toronto
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Abstract

The use of second-generation antipsychotic medications can result in the development of severe side effects such as weight gain and metabolic syndrome. Previous studies have proposed that a genetic component contributes between 40% and 80% to the development of this side effect. However, genetic studies thus far have yielded mixed findings. The genetics of the melanocortin system has not yet been extensively examined in the context of antipsychotic-induced weight gain. Objectives: The first objective was to investigate whether melanocortinergic single nucleotide polymorphisms from genes that were associated with high weight and obesity were also associated with antipsychotic-induced weight gain. The second objective was to determine whether gene-gene interactions better predict the onset of antipsychotic-induced weight gain in psychiatric patients. Hypothesis: It was predicted that the melanocortin-3 receptor, melanocortin-4 receptor, pro-opiomelanocortin, cocaine-amphetamine regulated transcript, and agouti related protein would be associated with antipsychotic-induced weight gain, and that a combination of these genes would provide improved prediction of this phenotype. Results: The melanocortin-3 and melanocortin-4 receptors were associated with antipsychotic-induced weight...
gain. An additive model did not demonstrate that the combined effect in these genes explains increased variance of the weight gain phenotype. **Conclusions:** The melanocortin receptor system may have a more substantial role in predicting antipsychotic-induced weight gain, as compared to previously studied gene systems. Though further exploration and functional analyses are required, these findings contribute novel knowledge to the pharmacogenetics of antipsychotic-induced weight gain, and may help to enhance the development of clinical genetic tests to guide medication decision-making in the future.
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Finally, I would very much like to express my gratitude to my parents and younger brother for their unconditional love and unwavering support. Without them, I would not be where I am today.
Contributions

The research presented in this thesis consists of three original research studies. Chapter 3 was published in *The Pharmacogenomics Journal*, and Chapter 4 was published in *The World Journal of Biological Psychiatry*. The research described in Chapter 5 is currently in preparation for publication in a peer-reviewed journal.

The author conducted all of the experiments, with the guidance and expertise of the individuals listed below:

**Mr. Sajid Shaikh:** Supervised and helped Ms. Chowdhury to conduct polymerase chain reaction for experiments in Chapter 3.

**Dr. Arun Tiwari:** Conducted the electrophoretic mobility shift assay described in Chapter 3, and contributed to the experimental design and statistical analyses for genotypic analysis in Chapters 3, 4 & 5.

**Dr. Sheng Chen:** Provided guidance on quantities and reagents to be used in electrophoretic mobility shift assay in Chapter 3.

**Dr. Fang Liu:** Provided laboratory space and reagents for electrophoretic mobility shift assay experiment in Chapter 3.

**Dr. Clement Zai:** Conducted statistical analysis of gene-gene interaction in Chapter 5. Guided haplotype analysis presented in Chapter 5.
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<td>ADRA1A</td>
<td>alpha-1 (α1) adrenergic receptor</td>
</tr>
<tr>
<td>ADRA2B</td>
<td>adrenoceptor alpha 2B</td>
</tr>
<tr>
<td>ADRA2A</td>
<td>α2A adrenoceptor</td>
</tr>
<tr>
<td>ADRB3</td>
<td>beta-3 adrenergic receptor</td>
</tr>
<tr>
<td>AGRP</td>
<td>agouti related protein</td>
</tr>
<tr>
<td>AIM</td>
<td>ancestry informative marker</td>
</tr>
<tr>
<td>AIWG</td>
<td>antipsychotic induced weight gain</td>
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<tr>
<td>AMPK</td>
<td>5' AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of co-variance</td>
</tr>
<tr>
<td>ASTN2</td>
<td>astrotactin 2</td>
</tr>
<tr>
<td>ATF7IP2</td>
<td>activating transcription factor 7 interacting protein 2</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CART</td>
<td>cocaine and amphetamine regulated transcript</td>
</tr>
<tr>
<td>CEPH</td>
<td>Centre d'Etude du Polymorphisme Humain</td>
</tr>
<tr>
<td>CTCF</td>
<td>CCCTC-binding factor</td>
</tr>
<tr>
<td>CNR1</td>
<td>cannabinoid receptor 1</td>
</tr>
<tr>
<td>CNV</td>
<td>copy number variation</td>
</tr>
<tr>
<td>DISC1</td>
<td>disrupted-in-schizophrenia 1</td>
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<tr>
<td>DRD1</td>
<td>dopamine receptor D1</td>
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<td>DRD4</td>
<td>dopamine receptor D4</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<tr>
<td>ENCODE</td>
<td>encyclopedia of DNA elements</td>
</tr>
<tr>
<td>eQTLs</td>
<td>expression quantitative trait loci</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FHOD3</td>
<td>formin homology 2 domain containing 3</td>
</tr>
<tr>
<td>FTO</td>
<td>fat mass and obesity</td>
</tr>
<tr>
<td>GABRA2</td>
<td>gamma-aminobutyric acid receptor subunit alpha-2</td>
</tr>
<tr>
<td>GCG</td>
<td>glicentin-related polypeptide -1 encoding gene</td>
</tr>
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<td>GLP-1</td>
<td>glucagon-like peptide</td>
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<td>GWAS</td>
<td>genome wide association study</td>
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<td>GHS-R</td>
<td>growth hormone secretagogue receptor</td>
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<td>GPR98</td>
<td>G protein-coupled receptor 98</td>
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<tr>
<td>HCRTR2</td>
<td>orexin receptor</td>
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<td>histamine 1 receptors</td>
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<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
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<td>LEP</td>
<td>leptin</td>
</tr>
<tr>
<td>LEPR</td>
<td>leptin receptor</td>
</tr>
<tr>
<td>mbmdr</td>
<td>model based multifactor dimensionality reduction</td>
</tr>
<tr>
<td>MCH</td>
<td>melanin concentrating hormone</td>
</tr>
<tr>
<td>MC3R</td>
<td>melanocortin-3 receptor</td>
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<tr>
<td>MC4R</td>
<td>melanocortin-4 receptor</td>
</tr>
<tr>
<td>MIR137</td>
<td>microRNA 137</td>
</tr>
<tr>
<td>MSX2</td>
<td>muscle segment homeobox</td>
</tr>
<tr>
<td>NDUFS1</td>
<td>NADH-ubiquinone oxidoreductase 75 kDa subunit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>NPY5R</td>
<td>neuropeptide Y receptor Y5</td>
</tr>
<tr>
<td>NRG1</td>
<td>neuregulin</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGC</td>
<td>psychiatric genetics consortium</td>
</tr>
<tr>
<td>PMCH</td>
<td>pro-melanin concentrating hormone</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PRKAA1</td>
<td>protein kinase, AMP-activated, alpha 1 catalytic subunit</td>
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<tr>
<td>PRKAA2</td>
<td>protein kinase, AMP-activated, alpha 2 catalytic subunit</td>
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<td>PRKAB1</td>
<td>protein kinase, AMP-activated, beta 1 non-catalytic subunit</td>
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<td>ring finger protein 144A</td>
</tr>
<tr>
<td>SCZ</td>
<td>schizophrenia</td>
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<tr>
<td>SGA</td>
<td>second-generation antipsychotic</td>
</tr>
<tr>
<td>SH2B1</td>
<td>SH2B adapter protein 1</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOX5</td>
<td>SRY-related HMG-box</td>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
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<tr>
<td>5-HT1A</td>
<td>5-hydroxytryptamine serotonin receptor 1A</td>
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<tr>
<td>5-HT6</td>
<td>5-hydroxytryptamine serotonin receptor 6</td>
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</tbody>
</table>
5-HT7    5-hydroxytryptamine serotonin receptor 7
VGAT     vesicular GABA transporter
TMEM18   transmembrane protein 18
NRXN3    neurexin 3
MC4R     melanocortin-4 receptor
MEIS2    meis homeobox 2
PRKAR2B  protein kinase cAMP-dependent, regulatory, type II, beta.
SEC16B   SEC16 homolog B
GNPDA2   glucosamine-6-phosphate deaminase 2
TNNI3K   TNNI3 interacting kinase
TSPO     translocator protein-18
QPCTL    glutaminyl-peptide cyclotransferase-like
Preface

Schizophrenia is a chronic debilitating psychiatric disease, wherein patients exhibit increased mortality rate compared to the general population (Brown et al., 2000). A subset of patients experience substantial weight gain when treated with second-generation antipsychotic medications. Schizophrenia patients may experience stigma due to the weight gain, and the use of these drugs has been associated with the onset of metabolic side effects such as type 2 diabetes, and cardiovascular diseases (reviewed in Lett et al., 2012). Genetic factors have been reported to contribute to the development of antipsychotic induced weight gain (AIWG) (Gebhardt et al., 2010), though no specific biological mechanism has been identified. Discovering key genetic determinants of AIWG could further understanding of the development of this serious side-effect, and eventually guide patient treatment. The melanocortinergic gene pathway has a critical role in weight regulation. However, this biological system has not yet been extensively investigated in the context of AIWG. We examined whether melanocortinergic gene variants were associated with increased weight in a psychiatric population.
Chapter 1 Literature Review
1.0 Introduction

1.1 Schizophrenia: Background

The exact nature of schizophrenia (SCZ) has long been an enigma and remains elusive in contemporary psychiatry (Brüne et al., 2004). To date, there have been over 40 definitions of SCZ. In the nineteenth century, descriptions of patients with SCZ were reported across European hospitals, namely Bethlem hospital in England and the Charite hospital in Berlin (Stone, 2006). The term SCZ has evolved over time from Dr. Benedict Morel initially describing a sickness affecting young adults, termed démence precoce. In the late 1800s, Emil Kraeplin, a German psychiatrist, termed the phrase dementia praecox or premature dementia, and the term SCZ meaning split mind was set by Eugene Bleuler (Kaplan et al., 2008; McNally et al., 2009).

In the present day, SCZ is diagnosed with criteria set by the “Fifth Edition of the Diagnostic and Statistical Manual of Mental Disorders – DSM-5,” or the “Tenth Revision of the International Classification of Diseases” (ICD-10) (definitions comprehensively reviewed in Hyman, 2007). SCZ symptoms are typically divided into a set of broad categories, including positive and negative symptoms (van Os & Kapur, 2009). Positive symptoms include hallucinations, delusions, thought disorders and disorganized behaviour, while negative symptoms consist of impaired executive functioning — the ability of higher thought processes, lack of focus or attention, and impaired working memory.

Currently, no given treatment or medication is efficacious for the entire spectrum of SCZ symptoms. For example, a medication may reduce the prevalence of positive symptoms, but have little to no effect on negative or cognitive symptoms. Furthermore, some of the most effective
pharmaceutical therapies can result in debilitating side effects, and may even result in the onset of a co-morbid disorder or disease. For instance, the use of a subset of psychiatric medications may result in the development of tardive dyskinesia (reviewed in Chowdhury et al., 2011), a disorder associated with dysregulated motor abilities. The alternative class of psychiatric medications can result in a substantial increase in weight gain and develop into morbid obesity. Therefore, further understanding of the etiology of how these side effects develop could benefit the understanding of drug action at receptors, enhance personalized treatment and improve outcome of patient quality of life.

1.2 Schizophrenia: Epidemiology

The prevalence and incidence of SCZ has been reported to be variable across geographical regions. The prevalence, or the proportion of patients diagnosed with SCZ in a given time period, has been estimated to be between 2.7/1000 and 8.3/1000 persons (Messias et al., 2007). The approximate lifetime prevalence estimate in the U.S. and Canada is one per one hundred (Eaton & Chen, 2006). The incidence rate, or the likelihood of developing the disease over a lifetime, is estimated to be between 0.11/1000/year and 0.70/1000/year individuals (Messias et al., 2007). SCZ has been reported to affect males and females at a different rate, with a male-to-female ratio of 1.4:1 (McGrath et al., 2008).

1.3 Schizophrenia: Etiology

The underlying pathophysiology or specific biological cause of SCZ is currently unknown. No established biological markers or tests exist that can conclusively identify whether a patient is
afflicted with the SCZ. Overall, the literature demonstrates that familial factors are important contributory elements to the development of SCZ, though the exact genetic mechanisms remain elusive (van Os & Kapur, 2009; Kennedy et al., 2003). As such, both genetic and environmental factors have been proposed to contribute to the development of SCZ (Sullivan et al., 2003).

1.3.1 Environmental Risk Factors: Schizophrenia

A number of environmental factors have been reported to contribute to the onset of SCZ, and these may occur at different developmental periods throughout an individual’s lifetime. Factors that may leave an individual vulnerable to SCZ during the prenatal period include exposure to influenza (Brown et al., 2006), maternal rubella and respiratory infections (Brown et al., 2006), low socioeconomic status (Dohrenwend et al., 1992), obstetric complications (Murray & Lewis, 1987), urban residence (Marcelis et al., 1999), prenatal famine (Susser et al., 1996), season of birth (Torrey et al., 1997) and low folate intake (Marzullo & Fraser, 2005). Post-natal environmental risk factors for SCZ include living in an urban environment (Krabbendam & van Os, 2005; McGrath et al. 2008) and cannabis use (van Os, et al., 2002; De Sousa et al., 2013).

1.3.2 Genetic Risk Factors: Schizophrenia

Schizophrenia has a well-established genetic component, though the likelihood of a single major gene for risk of the disease is highly unlikely. Schizophrenia is a complex polygenic disease, and almost certainly has several, if not many, contributing genetic factors (Kennedy et al., 2003; Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011). Individuals with a family member afflicted with SCZ are more likely to develop the disease
(Gottesman & Wolfgramm, 1991), and if both biological parents suffer from SCZ, an individual has a 44% risk of developing the disease as well. The concordance rate for SCZ development between siblings is relatively high for dizygotic twins (17%), and for monozygotic twins (48%) (Gottesman & Wolfgramm, 1991). Based on a number of twin studies, SCZ has also been reported to have a heritability rate as high as 80% (Gottesman and Shields, 1991; Sullivan et al., 2003; Owen et al., 2004).

Though it appears that a genetic component contributes to the predisposition of developing SCZ, concrete genetic mechanisms have not yet been determined. Over the past decades, a large number of small genetic effects have been associated with SCZ. Taken together, these genetic variants have been proposed to explain 23% of the variance in SCZ risk (Lee et al., 2012). Recently, the Psychiatric Genetics Consortium (PGC) reported a five-fold increase for risk for SCZ based on a polygenic model incorporating several thousand genetic variants from GWAS studies (Sullivan et al., World Congress of Psychiatric Genetics, Oct. 2012, Hamburg, Germany).

A subset of individuals diagnosed with SCZ has more distinct genetic abnormalities, including 22q11.2 deletion syndrome (Murphy et al., 2002) and 1q21.1 microdeletion syndrome (Stefanson et al., 2008).

Several candidate gene studies for risk of SCZ have been reported (Ripke et al., 2011). The serotonin system, particularly the serotonin 2A receptor (5HTR2A), has been implicated as being associated with SCZ (Inayama et al., 1996; Polesskaya & Sokolov, 2002). The growth and differentiation factor, neuregulin 1 (NRG1), has been reported to be among the top validated risk genes for SCZ, psychotic and bipolar disorders (Douet et al., 2014). The neuregulin 1 gene was first reported to be associated with SCZ in a sample consisting of Icelandic and Scottish patients (Stefansson et al., 2002), and this finding has been consistently replicated (Stefansson et al.,
2003; Williams et al., 2003; Yang et al., 2003; Bakker et al., 2004; Tang et al., 2004; Corvin et al., 2004; Munafò et al., 2006; Georgieva et al., 2006; Walker et al., 2010). The promoter SNP NRG1 rs6994992 has also been of high interest, given that this variant has been reported to predict psychosis (Hall et al., 2006) and can alter promoter activity (Tan et al., 2007).

The disrupted-in-Schizophrenia 1 gene (DISC 1) is also of high interest in the context of studies examining risk for SCZ. An examination of a large family with high incidence of SCZ, identified genes and chromosomal translocation that resulted in the truncation of the DISC 1 protein (Millar et al., 2000; Morris et al., 2003). The DISC1 gene has been implicated to be contributory to SCZ, as this gene affects neuronal functions dependent on proper cytoskeletal regulation, neuronal migration, neurite architecture and intracellular transport (Morris et al., 2003).

Recently, the rs1625579 SNP, an intronic putative primary transcript that encodes for the microRNA 137 (MIR137), was the top hit for a genome-wide association study assessing common variants for SCZ risk (Ripke et al., 2011). Follow-up work to the association between the MIR137 and SCZ has been reported in several studies. The MIR137 risk genotype has been reported to strongly predict earlier age-at-onset of psychosis, and SCZ patients with the MIR137 risk genotype were also reported to have reduced white matter integrity (Lett et al., 2013). A study by van Erp et al., (2014) reported that the MIR137 rs1625579 TT genotype was associated with the SCZ risk phenotype dorsolateral of prefrontal cortex hyperactivation, which is commonly considered a measure of brain inefficiency relevant to SCZ. However, the exact role of the MIR137 rs1625579 SNP and its effect on white matter microstructure in SCZ patients requires further study. It is still unknown exactly how the MIR137 rs1625579 SNP mediates risk for SCZ, though this finding appears to be promising and warrants further investigation.
Of interest, one of the classic candidate genes for SCZ, namely the dopamine D2 receptor (DRD2), has now emerged as significant in the most recent genome-wide association study (Ripke et al., 2014). The D2 is the only receptor that is a target of all antipsychotic drugs (Seeman et al., 1976). Dopamine is generally well known to have a role in modulating antipsychotic response, and is associated with a reduction in positive symptoms. The dopamine system is an integral component in neural reward circuitry, and thereby may affect appetitive behaviours. If the dopamine system is altered by D2-blocking medication (i.e., antipsychotics), this may result in a blunted reward perception, and in turn result in subsequent altered behaviours such as over-eating. Specifically, patients may not feel satiated after ingesting food content, and continue to eat in order to feel a sense of reward. Currently, classical first- and second-generation antipsychotic medications are the main treatment for SCZ, and the detrimental side effects, for example, weight gain, that occur with the use of these medications remain a troubling issue.

1.4 Actions of Antipsychotics at Receptor Targets

Overall, the majority of antipsychotic medications represent a ‘pharmacological shotgun’ approach to receptor binding, in that they bind to several different targets (Meltzer et al., 1989; Kapur et al., 2001; Lett et al., 2012). Many antipsychotic medications have a high affinity for the serotonin 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, as well as an affinity toward histaminergic, dopaminergic and many other receptors (see Figure 1). The first antipsychotic medication used to treat SCZ was chlorpromazine, which was introduced in the 1950s (Meyer & Simpson, 1997). Chlorpromazine has been proposed to act as an antagonist on dopamine D2 receptors (DRD2), an action that has been suggested to reduce positive symptoms of SCZ. In past decades, first-
**generation** antipsychotic medications, also called typical antipsychotics, have been used for SCZ treatment. These medications have been reported to act primarily on DRD2 (Seeman et al., 1976; Remington et al., 2006). **Second-generation** antipsychotic medications have a high affinity for 5-HT2 receptors, though their atypical qualities (e.g., reduced extrapyramidal symptoms) have been attributed to rapid disassociation from the DRD2 (Kapur et al., 2001). Though second-generation antipsychotic medications are effective in treating positive symptoms as well as reducing the risk of developing extra pyramidal side effects, a major health risk remains when these medications are used for treatment. With the use of second-generation antipsychotic medications comes the risk of substantial weight gain, or antipsychotic induced weight gain (AIWG) as well as a number of metabolic abnormalities including impaired glucose and lipid levels (Müller & Kennedy, 2006). The risk of weight gain is variable across individuals and among different second-generation antipsychotic medications. The exact biological processes by which weight gain and metabolic abnormalities occur are elusive not yet well understood. However, differing receptor-binding profiles have been proposed as a contributing reason to explain drug-induced weight gain. Those medications that have a relatively high blockade of 5-hydroxytryptamine (serotonin) receptor 2C (5-HT2C) and histamine 1 receptors (HR1) have been reported to be associated with higher weight gain, whereas medications that primarily block DRD2 and dopamine receptor D3 (DRD3) receptors are associated with lower weight gain. Among the drugs that have the highest propensity for weight gain are clozapine, where the action at the dopamine receptor D4 (DRD4) receptor was initially proposed to partially explain its weight-causing properties (Wong & Van Tol, 2003), and olanzapine, where its action at muscarinic 3 (M3) receptor has been suggested for its diabetes risk characteristic (Weston-Green et al., 2013; Johnson et al., 2005; Silvestre & Prous, 2005). However, it is unknown which
exact specific pharmacological actions are responsible for weight gain and associated metabolic disturbances. A recent meta-analysis conducted by Lett et al. (2012) maintains that both olanzapine and clozapine result in more weight gain in comparison to other commonly prescribed medications, and labeled the two drugs as “high risk.”

The atypical antipsychotic drugs have been characterized by their affinities for serotonergic receptors and lower affinities for the D_2 receptors (Kapur & Seeman, 2001). Both the typical and atypical antipsychotic drugs interact with a various number of receptors including the serotonergic 5-hydroxytryptamine serotonin receptor 1A (5-HT1A), 5-hydroxytryptamine serotonin receptor 2A (5-HT2A), 5-hydroxytryptamine serotonin receptor 2C (5-HT2C), 5-hydroxytryptamine serotonin receptor 6 (5-HT6), and 5-hydroxytryptamine serotonin receptor 7 (5-HT7) (Roth et al., 1992, 1994, 1998), dopaminergic (DRD2, DRD3, and DRD4) (Van Tol et al., 1991; Seeman et al., 1997), histaminergic receptors (HR1) (Kroeze et al., 2003), and adrenergic receptors adrenoceptor alpha 1A (ADRA1A), and adrenoceptor alpha 2B (ADRA2B) (Nutt, 1994; Hertel et al., 1997, 1999a).

The receptor targets of antipsychotic medications provide a starting point to understand AIWG, but may not necessarily provide all of the answers. Second-generation antipsychotic medications and their affinities for different receptor targets are summarized below.
### Table 1-1. Receptor Affinities of Commonly Prescribed Second-Generation Antipsychotic Medications

<table>
<thead>
<tr>
<th>Medication</th>
<th>D₁</th>
<th>D₂</th>
<th>D₃</th>
<th>D₄</th>
<th>5HT₂C</th>
<th>5HT₂A</th>
<th>α</th>
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**D**: dopamine, **α**: alpha-adrenergic, **H**: histamine, **M**: muscarinic **5-HT**: serotonin

**** very high affinity, *** high affinity, ** moderate affinity, * low affinity, (--) negligible affinity

Adapted from: Ananth et al., 2004; Onrust et al., 2001; Davis et al., 2002; Leucht, et al., 2009; Komossa et al., 2010; Brunton et al., 2010; Komossa et al., 2010; Taylor et al., 2012; Leucht et al., 2013; Kishi et al., 2013.
1.5. Schizophrenia and Pharmacogenetics of Antipsychotic Induced Weight Gain

1.5.1 Contributing Factors to Antipsychotic-Induced Weight Gain

To date, there are no predictive biomarkers with high enough sensitivity and specificity to determine, with clinical usefulness, whether an individual is susceptible to AIWG. Some known predictors of AIWG include baseline weight, antipsychotic treatment and duration (Jones et al., 2001; McIntyre et al., 2001). One of the strongest predictors for AIWG includes family history in a first-degree relative (Gebhardt et al., 2010). AIWG is without doubt a polygenic phenotype, in that multiple polymorphisms contribute to this phenotype, and each variant contributes a relatively small amount to the overall variance. Overall, genetic factors are among the leading predictors for whether individuals are susceptible to developing this side effect (Gebhardt et al., 2010; Lett et al., 2012). Further understanding of genetic determinants of AIWG could lead to greatly improved and more personalized patient treatment regimes.

1.5.2 Gene Systems and Antipsychotic-Induced Weight Gain

Both dopamine and serotonin systems are key regulators of appetite, satiety and food reward. Second-generation antipsychotic medications have been shown to act upon several receptor types including serotonergic and dopaminergic receptors. Thus, dysfunction within the serotonergic and dopaminergic systems has been proposed as potential mechanisms for the onset of AIWG.
To date, serotonergic gene variants have been the most extensively explored system in the field of AIWG. The 5-HT receptors are generally known to regulate weight, appetite and satiety in healthy populations. The HTR2C promoter polymorphism rs3813929 (−759 C/T) has been consistently reported to be associated with AIWG, and a meta-analysis showed an association between this single nucleotide polymorphism (SNP) and AIWG (Sicard et al., 2010). Altered functioning of dopamine system gene variants has also been proposed as a potential mechanism for AIWG. Dopamine D2 receptor (DRD2) SNPs have been a primary interest for studies in AIWG. A recent meta-analysis by Müller et al., (2012) reported that the three DRD2 SNPs, rs6277 (C957T), rs1079598 and rs1800497 (TaqIA), were associated with AIWG. A functional promoter region polymorphism in DRD2, the -141C Ins/Del (rs1799732), has been associated with antipsychotic-induced weight gain SCZ patients treated with either olanzapine or risperidone (Lencz et al., 2010). This study was conducted in a small sample set (n=58), and further work in larger samples is required.

Both serotonin and dopamine system genes have been in the spotlight as potential genetic mechanisms in AIWG, due to their role as primary targets for antipsychotic medications as well as their critical roles in weight regulation in the general population. Thus far, the 5-HT2C promoter polymorphism rs3813929 (−759 C/T) has been the most consistently replicated SNP with AIWG (Lett et al., 2012). However, the associations between serotonergic genes and this phenotype do not necessarily completely explain the substantial weight gain observed in a number of psychiatric patients. For instance, the second-generation antipsychotic medication, aripiprazole, has a high affinity for the 5-HT2C receptor, yet has low weight gain liability (Nguyen et al., 2012). Thus, it is very likely that serotonergic genes act in tandem with other genetic factors to regulate the development of AIWG. In addition to dopamine and serotonin,
other candidate mechanisms for association with AIWG include the histamine system variants, as well as the melanocortin system.

1.5.3 Dopamine and Antipsychotic-Induced Weight Gain

The dopamine system, specifically the dopamine D2 receptor (DRD2), has been extensively studied in its role as a primary target of antipsychotic action. Some antipsychotic medications have been proposed to alter central reward circuitries, leading to impaired reward responses, which may be associated with addictive behaviours. The DRD2 Taq1A allele has been implicated in addictions including cocaine (Noble et al., 1993), alcohol (Blum et al., 1990) and nicotine use (Munafò et al., 2009), and to a lesser extent, general obesity (Carpenter et al., 2013). Antipsychotic medication use may result in alteration of binge eating symptomatology, which may lead to increased food intake (Theisen et al., 2002). It has been postulated that, in a subset of individuals, atypical antipsychotic medications may alter brain reward circuitry to make food appear more palatable, which leads to weight gain.

The DRD2 gene encodes the dopamine D2 receptor, and is the most commonly examined gene in the context of AIWG. In addition, polymorphisms across the other dopamine receptor genes dopamine receptor D1 (DRD1), dopamine receptor D2 (DRD2), dopamine receptor D3 (DRD3), dopamine receptor D4 (DRD4), dopamine receptor D5 (DRD5), have been investigated with mixed implications for AIWG. Lane et al. (2006) reported no significant association between the DRD1, DRD2, and DRD3 SNPs with weight gain in a patient sample treated with risperidone. A recent analysis conducted by Müller et al. (2012), examined polymorphisms across the DRD1-DRD5 receptors using tagSNP software. In this study, a nominally significant association was reported between the DRD2 rs1079598 SNP and the categorical variable of
AIWG with a 7% weight gain cut-off (P = 0.03). In a sub-sample stratified for ethnicity, and medications with the highest propensity for weight gain (clozapine & olanzapine), associations with weight gain and three DRD2 SNPs, rs6277 (C957T), rs1079598 and rs1800497 (TaqIA), were reported. The rs6277 SNP is of particular interest given that it has been reported to have a functional role in vitro and in vivo. The rs6277T allele has been reported to be associated with decreased mRNA stability and half-life, which in turn was associated with decreased D2 affinity in the striatum (Duan et al., 2003; Hirvonen et al., 2009). The −141C Ins/Del (rs1799732) DRD2 SNP has been reported to be associated with decreased efficacy of antipsychotic treatment (Lencz et al., 2010). In addition, this SNP was associated with increased weight in a sample consisting of first-episode SCZ patients (Lencz et al., 2010). The ‘C’-allele of the rs4436578 SNP has been associated with weight gain in a Chinese SCZ sample treated with clozapine, olanzapine or risperidone (Hong et al., 2010). The DRD3 receptor gene has not yet been widely investigated in AIWG, though a recent study reported a non-significant trend between the DRD3 9Gly allele and risperidone-induced weight gain in a population of Spanish SCZ patients (Almoguera et al., 2013).

The DRD4 has been a receptor of interest in SCZ studies, as it has been reported to have a stronger affinity for clozapine, compared to DRD2 (Van Tol et al., 1991). However, DRD4 has been proposed to have a minor role in antipsychotic response and regulation (Wong & Van Tol, 2003). The DRD4 hypofunctional 7-repeat (7R) allele of the exon 3 VNTR has been reported to confer greater risk for weight gain (p = 0.003) (Popp et al., 2009). However, a study conducted by Müller et al. (2012) reported no significant association between DRD4 7R allele and AIWG. Both studies consist of relatively low sample sizes and this variant needs further exploration in this phenotype.
Overall, the role of dopamine in AIWG remains very compelling. A number of postulated mechanisms of the involvement of dopamine in AIWG have been described in the literature. Antipsychotic action may mediate hyperphagia via blockade of D2 receptors expressed in the hypothalamus (Baptista et al., 1987; Baptista, 1990; Parada et al., 1988). The blockade of dopamine in the anterior pituitary can result in hyperprolactinaemia, which has been correlated to body mass index in patients being treated with first-generation antipsychotics (Kalinichev et al., 2005). Finally, arguably the most common proposed DRD2-AIWG mechanism is that the neural reward circuitry is affected by antipsychotic administration. Striatal D2 receptor availability is inversely associated with weight gain (Wang et al., 2006), and it has been suggested that decreased levels of dopamine D2 receptors result in obese individuals to seek reward or reinforcement in the form of food intake (Roerig et al., 2011).

Recent proposed mechanisms include the peripheral dopamine signalling being altered in response to antipsychotic use. Melanocortin expression within DRD1 expressing neurons has recently been explored in terms of body energy homeostasis. The melanocortin-4 receptor (MC4R) signalling in DRD1 neurons has been reported to suppress food intake and weight gain in mice (Cui & Lutter et al., 2013). Though preliminary, this finding illustrates a potential interactive mechanism between the melanocortin and dopamine system that could further promote understanding of AIWG.
1.5.4 Serotonin and Antipsychotic-Induced Weight Gain

Second-generation antipsychotic medications have been reported to have the highest propensity for weight gain among patients, and are also potent inverse agonists of serotonin (5-HT) receptors. In terms of weight regulation, the 5-HT receptors expressed within the brain are highly integrated and influential in hunger and satiety circuitries. Evidence of the role of the 5-HT in weight regulation includes the finding that d-fenfluramine, a 5-HT agonist, functions as a strong appetite suppressant (Leibowitz & Alexander, 1998). Among the serotonin receptor genes, the 5-HT2C rs3813929 promoter SNP (-759C/T) has been the most widely investigated variant that has been associated with AIWG (Ellingrod et al., 2005; Miller et al., 2005). To a lesser extent, the 5-HT1RA, 5HTR2A, 5HTR2C and HTR6 have been reported to have a role in AIWG (reviewed in Mueller & Kennedy, 2006; Lett et al., 2012).

The X-linked 5HT2C rs3813929 (C/T) promoter SNP (-759 C/T) has been reported to be associated with AIWG across several studies (Reynolds et al., 2002; Templeman et al., 2005; Ellingrod et al., 2005; Miller et al., 2005; Godlewska et al., 2009; Opgen-Rhein et al., 2010; McCarthy et al., 2005; Kuzman et al., 2008; De Luca et al., 2007; Sicard et al., 2010; Mulder et al., 2009). A meta-analysis conducted by De Luca et al. reported no association between AIWG and the rs3812929 C-allele. However, another meta-analysis conducted by Sicard et al. (2010) reported the C-759T-G-697C-S ser23Cys haplotype was over-represented in SCZ patients with weight gain. To date, the 5-HT2C rs3813929 (C/T) SNP remains among the well-studied candidates in AIWG.

The 5HT2C rs3813929 ‘T’-allele has been associated with significantly less weight gain compared to the ‘C’-allele in antipsychotic-treated patients (Reynolds et al., 2002). Some
subsequent studies determined no association between the rs3813929 SNP and body weight (Basile et al., 2002; Tsai et al., 2002). Nonetheless, the number of positive associations and meta-analyses (Sicard et al., 2010) seem to suggest a role for the 5-HT2C rs3813929 in AIWG. Furthermore, the ‘T’-allele has been reported to reduce promoter activity (Hill et al., 2011), while the ‘C’-allele has been reported to reduce transcriptional activity (McCarthy et al., 2005; Buckland et al., 2005; Yuan et al., 2000). The exact action of the 5HT2C rs3813929 SNP is currently unknown, though the disruption of DNA-protein interaction due to antipsychotic treatment may be influential in relation to the onset of AIWG. Finally, the rs3812929 SNP has been reported to be more strongly associated with AIWG in individuals who have been treated with medications for less than three months, or a relatively short duration (Reynolds et al., 2002; Ellingrod et al., 2005; Templeman et al., 2005; Opgen-Rhein et al., 2010).

Aside from the HTR2C gene, the 5-hydroxytryptamine serotonin receptor 2A (5-HT2A) gene has also been reported to be associated with AIWG. The rs6313 (102 T/C) has been associated with weight gain in a study assessing SCZ patients treated with olanzapine (Gunes et al., 2009). Furthermore, carriers of the -1438A-102T- 452His haplotype had significantly higher C peptide levels, a peptide found in insulin, compared to haplotype 3 (-1438A, 102T, and 452Tyr) carriers. However, no association among 5-HT2A SNPs and serum triglyceride, insulin or cholesterol levels was reported (Gunes et al., 2009). The 5-HT2A genrs6313 TT genotype has also been associated with weight gain in a Han Chinese sample of SCZ patients treated with risperidone (Lane et al., 2006). The 102T allele of 5-HT2A has been associated with olanzapine-induced weight gain in a Japanese patient population (Ujike et al., 2008). Overall, the 5-HT2A gene is relatively under-studied in terms of AIWG, but warrants replication and further exploration in independent sample sets.
The overall findings indicate that the 5-HTR2C rs3812929 SNP is a genetic determinant for AIWG in patients of European descent treated with second-generation antipsychotics for a relatively short duration. The exact functional role of the 5-HTR2C rs3812929 SNP is unknown, and the mechanism by which this SNP may contribute to the regulation of AIWG is difficult to determine. Other genetic mechanisms may act with the serotonin system or independently to mediate the AIWG effect observed in psychiatric patients.

1.5.5 Histamine System

The histamine system, namely the histamine 1 receptor (H1R), has a well-described role in both general obesity and AIWG (Reynolds, 2012). Histamine neurotransmitters bind to the postsynaptic H1Rs, which result in the activation of downstream effectors, resulting in decreased food intake. Administration of antipsychotics such as olanzapine and clozapine, which are inverse agonists of the H1 receptors (H1R), result in increased food intake in animal models (Humbert-Claude et al., 2012). Furthermore, Kroeze et al. (2003) reported that both clozapine and olanzapine have a high affinity for the H1R, relative to eleven other receptor targets. Thus, it appears that the effect of these drugs at the H1R is worthy of investigation in order to determine if antipsychotic action at this histamine receptor has a role in AIWG.

In order to demonstrate specifically which receptors were associated with weight gain, Kroeze et al. (2003) assessed typical (chlorpromazine, perphenazine, trifluoperazine, thioridazine, thiothixene, fluphenazine, haloperidol, molindone) and atypical antipsychotics (clozapine, olanzapine, loxapine, sertindole, risperidone, ziprasidone, quietapine, and aripiprazole) for their affinities to 12 cloned biogenic amine receptors. It was reported that the high H1R affinities were associated with antipsychotics that were highly correlated with gain, and the medications
associated with low weight gain were found to have low H1R affinities. The H1R affinity was the single variable that reliably predicted weight gain across all 17 tested antipsychotic medications. Two histamine 2 receptor polymorphisms (−1018 G/A) and H3R (332Ser/Ser) were also investigated in an Asian sample for AIWG, and not associated with weight gain (Ujike et al., 2008). A more recent study examined an association for H1R SNPs with body mass index as well as glycolated hemoglobin (Hb1Ac) in 430 patients treated with second-generation antipsychotics (Vehof et al., 2011). Two H1R SNPs (rs346074 and rs346070) were examined, and a significant interaction was found between the haplotype rs346074-rs346070 and high body mass index (BMI) (p=0.025) in patients treated with antipsychotics that have a high affinity for the histamine receptor. The investigation of the histamine system is relatively under-explored in AIWG, especially in comparison to the dopamine and serotonin system genes. Nonetheless, the findings described above assessing histamine receptors have been further examined in combination with the enzyme 5′ AMP-activated protein kinase (AMPK) in order to determine how treatment with antipsychotic medications may result in substantial weight gain.

**AMP-kinase mediation through histamine receptors**

The 5′ AMP-activated protein kinase (AMPK) is a heterotrimer enzyme comprised of catalytic α-subunits and regulatory β- and γ subunits. The AMPK functions as a gage for monitoring cellular energy status (Minokishi et al., 2004). The AMPK has a critical role in regulating energy balance and metabolism, and is mediated by the cellular AMP: ATP ratio (Minokishi et al., 2004). Clozapine treatment has been shown to result in a four-fold increase in AMPK phosphorylation in wild-type mice, but has no effect in mice lacking the H1R (Kim et al., 2007). The findings from the study by Kim et al. (2007) provide evidence that antipsychotic medications such as clozapine activate hypothalamic AMPK activity, and that this action is depleted when mice lack
the H1R. Olanzapine administered to female rats has also been shown to elevate AMPK levels of phosphorylation (Sejima et al., 2011). Total body weight and food intake was also significantly elevated after a two-week period (Sejima et al., 2011). Acute administration of olanzapine in rats has recently been shown to result in hepatic insulin resistance in addition to increased levels of phosphorylation of AMPK (Girault et al., 2012).

The AMPK and its subunits (subunits that are coded by the protein kinase, AMP-activated, alpha 1 catalytic subunit (PRKAA1), protein kinase, AMP-activated, alpha 2 catalytic subunit (PRKAA2), protein kinase, AMP-activated, beta 1 non-catalytic subunit (PRKAB1), protein kinase, AMP-activated, beta 2 non-catalytic subunit (PRKAB2), protein kinase, AMP-activated, beta 1 non-catalytic subunit (PRKAG1), protein kinase, AMP-activated, gamma 2 non-catalytic subunit (PRKAG2), protein kinase, AMP-activated, gamma 3 non-catalytic subunit (PRKAG3) genes have been examined in the context of genetic association studies. The intronic PRKAA1 rs10074991 polymorphism was significantly associated with change in BMI, in a German SCZ patient sample treated with antipsychotic medications (Jassim et al., 2011). A study conducted by Souza et al. (2012) investigated schizoaffective patients and evaluated ten AMPK subunit polymorphisms across the regulatory sub-units. A nominally significant association between rs3766522 in PRKAB2 (AA vs. AT + TT; p = 0.022) and rs10789038 in PRKAA2 (GG + GA vs. AA, p = 0.023) with weight change (%) in patients of European ancestry following treatment with clozapine or olanzapine was found.

The AMPK gene variants have also been reported to have a role in Type II Diabetes susceptibility. Variants in the PRKAA2 gene have been associated with increased cholesterol levels, as well as impaired glucose and lipid metabolism (Horikoshi et al., 2006; Spencer-Jones et al., 2006) in patients with type 2 diabetes. Though these studies did not investigate AMPK
gene variants in a psychiatric population, it appears these SNPs have an influential role not only in weight gain, but metabolic indices as well.

These exploratory genetic association studies must be interpreted with caution, but provide evidence in line with animal models that antipsychotic action may result in AMPK dysregulation. The elevated levels of AMPK phosphorylation due to antipsychotic administration may result in increased fatty acid synthesis resulting in the rapid and substantial weight gain observed across humans and rodent models, and this effect may be mediated by antipsychotic action at the H1R. Most importantly, in the context of this AIWG, the finding that clozapine had no effect on AMPK phosphorylation in H1R knockout mice suggests that histamine receptors may have a mediating role on the development of side effects (Kim et al., 2007).

1.5.6 The Adrenergic System in Antipsychotic-Induced Weight Gain

The adrenergic system may be implicated in the regulation of AIWG, given that a number of second-generation antipsychotic medications have affinity for the α1- and α2-adrenergic receptors. Some studies have indicated that the ratio of α2- to β3-adrenergic receptors expressed in the adipose tissue may regulate the onset of obesity through adipocyte hyperplasia (Lane et al., 2006; Saiz et al., 2008). The protein coding adrenoceptor alpha 1A (ADRA1A) gene was assessed in a sample of Chinese SCZ patients treated with SGAs. The tagSNP software was used to analyze the ADRA1A gene in this sample, and numerous SNPs were associated with AIWG (Liu et al., 2010). In addition, the α2A adrenoceptor (ADRA2A) ‘C’-allele of the 1291G/C
(rs1800544) polymorphism was significantly associated with higher weight in a Korean population (Park et al., 2006). Our group also reported that the rs1800544 SNP was associated with SCZ patients treated with SGAs (Sickert et al., 2009). Overall, SGAs exhibit a high affinity for adrenergic receptors; therefore, it is important to examine this gene system in the context of AIWG.

1.6 The Genetics of Obesity

1.6.1 General Obesity

General obesity is a complex phenotype that has been more comprehensively studied in the literature in comparison to AIWG. One standard definition by The World Health Organization (WHO) for overweight and obesity is abnormal or excessive fat accumulation that may impair health (Donald & Behan, 2012). A general understanding of factors that may contribute to the development of substantial weight gain in the general population may provide insight into how the AIWG phenotype develops.

Similar to AIWG, it has been suggested that the onset of obesity is a complex and dynamic state, resulting from both environmental and genetic factors. The heritability of obesity has been estimated to range between 64% and 84% (Stunkard et al., 1986), and has also been examined in twin studies. The rates of heritability have been reported to be similar between twins reared apart and twins reared together (Price & Gottesman, 1991). A large study examining 5000 Danish subjects reported a statistically significant relationship between the body mass index (BMI), a common measure of weight, of adopted children and their biological parents. No association was observed between adopted children and adoptive parents (Stunkard et al., 1986b). Interestingly, a
close correlation between the BMI of adopted and their biological siblings who were reared separately was also later reported (Sorensen et al., 1989). Thus, genetic factors have been known to influence obesity for some decades.

The prevalence of general obesity has been reported to be increasing worldwide, with rates ranging between 10% and 35% among adults (Hainer et al., 2008). Obesity has been defined as an excess of body fat, and this state can be measured using dual energy X-ray absorptiometry as well as isotopic dilution techniques (Goodpaster et al., 2002). However, given costliness, these techniques are not typically used in general obesity studies. Rather, measures such as body mass index (BMI), weight in kg/height in m², are commonly used to assess weight in obesity studies, and have been correlated to body fat content (Farooqi & O'Rahilly, 2006). Other measures including waist-to-hip ratio and leptin, which correlates with body fat mass (Comuzzie et al., 2001), have also been reported as measures of obesity and weight across studies. However, body mass index remains among the most commonly used assessment of weight in obesity studies. Clinically, obesity is measured as an excess accumulation of adipose tissue, which results in BMI that is > 30kgm⁻².

The ideal method approach in measuring a complex phenotype such as obesity is a complex matter. The measure of BMI follows a normal distribution, with no clear division between individuals who are ‘clinically obese’ (BMI>30) and the non-obese (O'Rahilly & Farooqi, 2006). Similarly, in studies that assess AIWG, individuals who exhibit greater than 7% weight gain during treatment are considered to be affected. This is essentially considered to be dividing individuals into cases versus controls. Unfortunately, defining the dependent variable in this capacity may not elucidate the underlying genetic mechanism, or reflect how the biology of
weight is regulated. Examining a dichotomous variable in complex phenotypes such as obesity and AIWG may result in a loss of information, as this method may significantly decrease statistical power. It has been suggested that statistical information may be lost if transforming continuous data to a discontinuous scale, and it may be beneficial to measure weight in a quantitative method (Comuzzie et al. 2001).

Other contributing factors toward the development of obesity include both an individual’s rate of energy expenditure and food intake. Measuring exact food and nutrient intake is difficult in an experimental setting. Nonetheless, twin studies have also shown that intake of nutrients, size and frequency of meals, as well as preference for particular foods is also likely to be governed by genetic factors (Wade et al., 1981).

Genetic association studies have provided information on the etiology of obesity, though no single variant has been attributed as casual (Loos et al., 2008; Speliotes et al., 2010). More recently, genome-wide association studies (GWAS) have revealed novel loci that may be implicated in obesity. For instance, the SH2B adapter protein 1 (SH2B1), and fat mass and obesity-associated (FTO) genes have been reported to be significantly associated with changes in body mass (Thorleifsson et al., 2008; Willer et al., 2009). Despite the strong association between a gene such as FTO and obesity, this gene does not appear to be associated with AIWG (Shing et al., 2014), suggesting that obesity and AIWG may be regulated by differing biological mechanisms.

A large-scale GWAS study examining obesity in 249,796 individuals reported that 32 loci were associated with this phenotype, while other genome-wide association studies have reported the melancortin-4 receptor (MC4R) rs17782313 SNP, located 188 kb downstream of the MC4R
gene, to be significantly associated with body mass index (Loos et al., 2008). A subsequent GWAS study conducted by Zhao et al. (2011) reported that polymorphisms across the fat mass and obesity (FTO), transmembrane protein 18 (TMEM18), neurexin 3 (NRXN3), melanocortin-4 receptor (MC4R), SEC16 homolog B (SEC16B), glucosamine-6-phosphate deaminase 2 (GNPDA2), TNNI3 interacting kinase (TNNI3K), glutaminyl-peptide cyclotransferase-like (QPCTL), and brain derived neurotrophic factor (BDNF) genes, were associated with common childhood obesity.

Though these genetic association findings were highly compelling, some limitations should be considered. The FTO and MC4R genes are considered as top obesity hits across GWAS studies (Frayling et al., 2007; Loos et al., 2008). However, the additive genetic effect of the FTO and MC4R genes was reported to be very low, in that the combination of these variants were reported to account for less than 2% of the variance in adult BMI (Willer et al., 2009; Bogardus et al., 2009). Thus, the question of the missing heritability for obesity was highlighted, and was attributed to factors such as the complex, multi-genic nature of obesity, as well as the possibility of copy number variation (CNV) contributing to obesity. The number of loci reported in the GWAS studies illustrates the daunting complexity of phenotypes such as obesity and AIWG. Nonetheless, looking closely at biological markers that influence weight regulation can provide valuable insight into the mechanisms and processes that govern obesity.
1.6.2 Environmental Factors and Obesity

Genetic factors may certainly predispose certain individuals to gain weight, however, environmental influences should also be considered when studying obesity. Accurate assessment of gene-environment interaction in obesity and weight phenotypes could provide further understanding of underlying biological mechanisms of weight gain. However, measuring environmental factors including diet can be difficult to ascertain, given that exposure to obesogenic environments will be different across individuals (O'Rahilly & Farooqi, 2006).

A well-described hypothesis of why modern-day individuals are susceptible to obesity is the idea of the “thrifty” genotype, which refers to our ancestors and their ability to efficiently store fats during periods of limited access to food resources. This situation may have been common for our ancestors, and obesity may have actually been a result of natural selection (Neel et al., 1962). Individuals who were more adept at storing fat would have been more likely to survive during long periods of famine (Qi & Cho, 2008). However, in a modern environment, the ‘thrifty’ genotype results in abundance of food resources, increased intake and then obesity, and perhaps the development of dangerous diseases such as type 2 diabetes. The problem lies in determining exactly which genes predispose an individual to obesity, given that recent studies have shown that 32 loci are associated with this phenotype (Speliotes et al., 2010), with each gene contributing a small effect to the overall variance. Nonetheless, there are some reports of using knowledge of the genetics of obesity to moderate lifestyle intervention.
1.6.3 Gene-Environment Studies in Obesity

A number of studies in the past decade have examined whether specific gene-environment interactions can influence weight gain. For instance, the Trp64 Arg variant of the beta-3 adrenergic receptor (ADRB3) gene was shown to interact with high energy intake, which led to a significant increase in obesity (Miyaki et al., 2003). Another approach includes examining the interaction between genetic variants and physical activity. Of interest, Andreasen et al. (2008) reported an interaction between physical activity and the FTO rs9939609 SNP in a sample consisting of Danish participants. Among the physically inactive subjects, significant differences in BMI were reported between the AA and TT genotypic groups.

In a study consisting of obese patients (BMI > 30), the patients were prescribed a lower-calorie diet, which was associated with a 25% reduction in spontaneous energy intake for 2.5 months, and a leptin SNP (−2549C >) was nominally associated with lower BMI loss (Mammes et al., 1998). In a follow-up study, the leptin receptor SNP Ser (T) 343 Ser(C) ‘C’-allele was associated with weight loss in women who were prescribed a low-calorie diet (Mammes et al., 2001). The melanocortin-3 receptor (MC3R) was also reported to have a nominal effect on the low-calorie diet and exercise on weight loss (Santoro et al., 2007).

Overall, the genetics of obesity encompass a vast number of studies and have been greatly explored in the general literature (Walley et al., 2006). While genetic factors alone do not appear to be the sole regulating components of obesity, they do play an important role in understanding the etiology. The approaches taken to understand genetic factors in obesity, and the subsequent findings, provide an effective springboard to begin to understand how AIWG may be regulated.
1.7 Leptin Genetics in Antipsychotic-Induced Weight Gain

1.7.1 Leptinergic System as a Critical Regulator of Weight Gain

Several studies over the past decades have reported dozens of peptides, hormones and other molecules that are all critical for the regulation of food and nutrient intake, energy homeostasis, satiety and appetite. These molecules all interact in complex ways to determine weight and metabolic measures. The hypothalamus has been identified as a key regulator of energy and weight regulation. The leptin hormone, a critical regulator of weight, has downstream effects upon receptors that are heavily expressed within the hypothalamus. Dysregulation of leptin has been reported to play a role in the pathogenesis of obesity. In the late 1990s, it was discovered that the ob gene encodes the hormone leptin, which is primarily expressed in adipose tissue. The ob/ob knockout mice have been reported to exhibit hyperphagia, decreased energy expenditure and decreased immune function, traits that are also exhibit by starved animals (Friedman & Halas et al., 1998). When leptin is administered to ob/ob knockout mice, these effects are reversed. Lean mice have been shown to decrease in adiposity when treated with leptin infusions. Thus, these initial findings determined a key physiological for leptin in informing an organism’s body during food deprivation (Friedman & Halas et al., 1998).

Leptin receptors are expressed in various hypothalamic nuclei: the arcuate nucleus, lateral hypothalamus, dorsomedial hypothalamus and paraventricular nucleus (reviewed in Cone et al., 2005). Leptin acts in a feedback loop in order to maintain stores of body fat. If an organism is starved and their body fat decreases, this leads to decreased levels of circulating leptin. As a
result, food intake will exceed energy expenditure. If an organism’s adiposity increases, leptin levels also increase. Leptin acts as part of a feedback loop to maintain constant stores of fat. During periods of starvation or decreased adiposity, leptin levels decrease, which in turn leads to a state of positive energy balance wherein food intake exceeds energy expenditure. Conversely, an increase in adiposity leads to an increase in the levels of leptin and a state of negative energy balance (Cone et al., 2005).

Within the hypothalamus, several different neuropeptides and neurotransmitters interact with leptin to regulate biological processes such as food intake and energy expenditure. The cocaine amphetamine regulated transcript, melanocortin-4 receptor, promelanin concentrating hormone, agouti related protein, neuropeptide-Y and ghrelin are examples of the most critical known molecules to regulate leptin signalling. Though the exact mechanism of how the leptin-melanocortin system is affected in humans when treated with antipsychotic medications is not well understood, olanzapine administration in rats has been shown to result in an increase in weight, along with levels of neuropeptide Y and reduced proopiomelanocortin expression (Weston-Green et al., 2012).

1.7.2 Leptin Genetics in Antipsychotic-Induced Weight Gain

In addition to dopamine and serotonin (described above), the leptin gene has also been extensively studied in AIWG. The gene-encoding leptin and leptin receptor have been of great interest, given that leptin has an established role in hunger and appetite signalling. To date, two SNPs, the functional promoter −2548A/G polymorphism of the leptin gene, as well as 223Gln/Arg polymorphisms in the leptin receptor, have been reported to be associated with AIWG. The most prominent and consisting finding with leptin and AIWG is the association with
the rs7799039 (−2548A/G) SNP (De Luca et al., 2007; Sicard et al., 2010). In 2008, Zhang et al. (2008) reported the −2548A/G G-allele was associated with AIWG in a male subset of patients. In addition, Gregoor et al. (2011) was able to demonstrate that the ‘G’-allele was associated with AIWG in a sample comprised of males of European ancestry. These findings are suggestive of a sex-specific effect for the (−2548A/G) SNP. An additional study by Calarge et al. (2009) reported the opposite allele, ‘A’-allele, as being associated with AIWG in an adolescent sample. It has been suggested the −2548A/G SNP may influence weight gain differently between age groups (e.g., adolescents vs. adults) (Reynolds et al., 2006; Lett et al., 2012). However, a recent study by Nurmi et al. (2013) reported that the G-allele of the rs7799039 SNP resulted in increased weight gain compared to A-allele homozygotes (P=1.4 × 10^{-4}) in a dominant model. Thus, the overall findings regarding the leptin promoter SNP rs7799039 (−2548A/G) indicate the GG genotypic group is the risk genotype for AIWG across samples.

The leptin receptor, primarily expressed in the hypothalamus (Leinninger et al., 2009), has also been examined within pharmacogenetic and AIWG studies. The LEPR 223Arg allele has been reported to be associated with lower risk for developing obesity in a patient sample consisting of adolescents treated with risperidone. The LEPR rs817983 (656N/K) SNP has been associated with weight gain resulting from risperidone treatment (Ruanō et al., 2007).

Leptin and leptin receptors remain compelling candidates to explore, and the involvement of this system is relevant to study in the development of AIWG. In addition to the leptin receptor, the hormone leptin has multiple targets that have not yet been extensively investigated in AIWG.
1.7.3 Cocaine and Amphetamine Regulated Transcript

The cocaine amphetamine regulated transcript (CART) is a neuropeptide highly expressed in the nucleus accumbens, hypothalamus and ventral tegmental area. The CART is reported to be a modulator of drug reward and drug re-enforcement (Jaworski & Jones, 2006; Jean et al., 2007). Though the role of CART in neural reward circuitry is its most extensively studied role, this neuropeptide is also an important mediator of energy homeostasis. It was initially suggested that CART had a role in food intake (Koylu et al., 1997), which was later supported by the finding that CART immunoreactivity was observed in brain areas that mediate feeding behaviour (Elmquist et al., 1999). More recently, it has been demonstrated that CART is co-expressed with another critical peptide involved in weight regulation, the neuropeptide Y, in the dorsomedial hypothalamus. Leptin has been shown to stimulate neurons within the dorsomedial hypothalamus, though its direct influence on CART remains a topic of further investigation (Lee et al., 2013). Interestingly, clozapine has been shown to reduce CART expression in the nucleus accumbens, and it has been suggested that the dysregulated food intake is induced by introduction of antipsychotic medications in rats (Beaudry et al., 2004).

1.7.4 Endocannabinoid System

The endocannabinoid system has recently been brought into the spotlight as a compelling contributing mechanism to weight and obesity. Stimulation of cannabinoid 1 receptors (CNR1) results in an increase of food intake and in body weight. The CNR1 receptors are stimulated by ghrelin and inhibited by leptin. The CNR1 antagonist, rimonabant, in combination with a healthy diet, has shown reductions in weight, waist circumference and cardiometabolic risk factors, and animal models also provide evidence for the role of the CNR1 in weight regulation (Jamshidi &
Taylor, 2001; Ravinet Trillou et al., 2003). Mice without CNR1 expression show reduced eating or hypophagia as well as resistance to obesity. In human studies, the CNR1 and acid amide hydrolase (FAAH) genes have been examined in terms of obesity and related phenotypes. In a study by Monteleone et al. (2010), the cDNA 385C/A (rs324420) SNP of the FAAH gene was reported to be associated with greater than 7% weight change in a sample of European ancestry patients diagnosed with psychosis. No association was found between the CNR1 359 G/A (rs1049353) SNP and weight gain. In 2010, Tiwari et al. conducted a study examining tag SNPs that provided improved coverage of the CNR1 gene. The CNR1 rs806378 T-allele was associated with second-generation AIWG in SCZ patients of European ancestry (P = 0.008). They then determined that the T-allele creates a binding site for the transcription factor arylhydrocarbon receptor translocator (ARNT), suggesting a functional effect. Finally, Nurmi et al. (2013) replicated the CNR1 SNP rs806378 T-allele association with body weight gain in a pediatric sample treated with risperidone for eight weeks. Overall, the role for the CNR1 gene in AIWG is among the most compelling findings in this field.

1.7.5 Neuropeptide Y

The neuropeptide Y (NPY) is an integral component of the feeding and appetitive neural circuitry governed by the hypothalamus, and has been described as a potent stimulator of food intake (Stanley et al., 1986). The NPY is expressed through the central nervous system, including the hypothalamic nuclei, and has been associated with altered feeding behaviour. Rats with overexpression of NPY resulted in increased body weight and lower metabolic efficiency (Zheng et al., 2013). In terms of AIWG, the neuropeptide Y receptor Y5 (NPY5R) rs6837793 SNP has been associated with weight gain in patients treated with risperidone (Ruaño et al., 2007). In the same
study, no association was reported between this SNP and in patients treated with olanzapine. The functional NPY promoter SNP rs16147 is associated with higher waist-hip ratio in women (Mutschler et al., 2013). In a recent study conducted by Tiwari et al. (2013), five polymorphisms (rs10551063, rs16147, rs5573, rs5574 & rs16475) in the NPY gene were investigated in SCZ patients treated with olanzapine or clozapine. Three NPY SNPs (rs16147, rs5573 & rs5574) were significantly associated with weight change, suggesting that NPY may have an influence on the onset of AIWG.

1.7.6 Ghrelin

The hormone ghrelin mediates perception of hunger by acting upon receptors expressed within the hypothalamus. Ghrelin acts upon ghrelin receptors, or growth hormone secretagogue receptor (GHS-R), located in the arcuate and paraventricular nuclei of the hypothalamus, as well as NPY receptors (Heiman & Witcher, 2006). In terms of antipsychotic action, it is currently unclear whether ghrelin has a major role in terms of regulating AIWG. Atypical antipsychotic medications have been associated with decreased morning fasting ghrelin levels in the first two weeks of treatment, and increased ghrelin levels for longer treatment durations (Sentissi et al., 2008; Jin et al., 2008). Overall, it has been suggested that ghrelin is up-regulated by second-generation antipsychotic medications, and that altered ghrelin signalling may contribute to the onset of AIWG (Yan et al., 2013).

Thus far, two published reports have shown that SNPs within the ghrelin gene were not associated with AIWG (Ujike et al., 2008; Ruano et al., 2007). However, low coverage of the
gene and sample sizes are limitations for these studies. Our group recently analyzed 18 polymorphisms across the ghrelin and ghrelin receptor genes, and also report no significant association with AIWG (Chowdhury & Shams et al., in prep).

1.7.7 Agouti Related Protein

The AGRP has a critical role within feeding behaviour, as the primary endogenous antagonist of melanocortin receptors, and its expression is down-regulated by leptin (Haskell-Luevano et al., 2001; Nijenhuis et al., 2001). Animal models provide evidence that the AGRP has a role in obesity, as AGRP over-expression in mice results in obesity, increased body length, hyperplasia, as well as hyperglycemia and hyperinsulinemia (Graham et al., 1997; Michaud et al., 1997) (Argyropoulos et al., 2002; Bonilla et al., 2006). In the context of AIWG, its exact role is unclear. Plasma levels of AGRP were reported to be no different between controls and SCZ patients treated with either ziprasidone or olanzapine, despite increases in both body mass and leptin levels (Ehrlich et al., 2012). We recently conducted a study investigating the role of AGRP in AIWG and reported no association in our local sample set (Chowdhury et al., 2014). Nonetheless, the AGRP is a critical regulator of weight and requires consideration in the context of AIWG.
1.7.8 Brain Derived Neurotrophic Factor

The brain derived neurotrophic factor (BDNF) has a widespread role in the central nervous system of regulating the development of neurons, as well as hippocampal neurogenesis and plasticity. Circulating levels of BDNF have been reported to be altered by the use of antipsychotics (Lipska et al., 2003). The BDNF 66 Val/Met polymorphism (rs6265) has an integral role in mental illness and cognitive function (Green et al., 2011). BDNF rs6265 has been associated with body mass index and eating behaviour in a GWAS study (Thorleifsson et al., 2009). Taken together, these findings provide a strong rationale study BDNF in the context of AIWG. The BDNF Val66Met (rs6265) polymorphism is the most well-studied SNP in the literature, and has been reported to be associated with antipsychotic-induced weight gain in males (p = 0.01) (Zhang et al., 2008). In this study, it was reported that the BDNF gene was a risk factor for weight gain in male SCZ patients on long-term antipsychotic treatment. A recent study conducted by Zai et al. (2012) found no association between the BDNF rs6265 and AIWG. However, a two-marker haplotype including the rs6265 and rs1519480 SNPs were reported to be significantly associated with weight change (Zai et al., 2012). The BDNF is a downstream effector of leptin and appears to have several regulatory roles aside from weight gain, elucidating the complexity of the AIWG phenotype.

1.7.9 Pro-Melanin Concentrating Hormone

The pro-melanin concentrating hormone (PMCH) is the pre-cursor of melanin concentrating hormone (MCH), and MCH levels are higher in leptin-deficient mice compared to controls (Qu et al., 1996). Knock-out PMCH mice are typically lean, hyperphagic and exhibit an increased metabolic rate (Shimada et al., 1998). The PMCH rs7973796 ‘G’-allele has been shown to be
associated with increased odds of obesity in the SCZ patient sample treated with olanzapine (Chagnon et al., 2007). The PMCH and MCH also have a regulatory role in skin pigment expression, but further understanding of its role in AIWG is warranted.

1.8 The Melanocortin System Genes

The melanocortin system is a neuronal pathway that is a critical regulator of energy homeostasis (reviewed in Cone et al., 2005). The unique circuitry projects and receives input from a vast number of peptides, regulators and hormones, and is largely expressed within the hypothalamus. The melanocortin system is among the forefront of investigated biological factors for obesity drug development, yet relatively understudied in the context of AIWG.

Peripheral hormone signals including leptin, insulin and ghrelin act upon a number of targets, including neuronal bodies expressed in the hypothalamus. Both POMC and CART cell bodies are expressed within the arcuate nucleus of the hypothalamus. Neurons expressing POMC send descending projections to the brainstem, innervating the nucleus of the solitary tract, and the medulla, nucleus ambiguous and spinal cord (reviewed in Cone et al., 2005). The POMC peptide produces the α-melanocyte stimulating hormone, a primary activator of melanocortin receptors. The POMC neurons are also targets of another critical regulator of weight, the serotonin 5-HT2C receptor (5HT2CR). Serotonergic compounds, including fenfluramine, activate POMC neurons, and administration of 5HT2CR agonists result in increased POMC expression levels in rodent models (Xu et al., 2011). Another critical upstream regulator for the melanocortin peptides are the NPY/AGRP-expressing neurons, and AGRP is an endogenous antagonist for both the
melanocortin-3 and melanocortin-4 receptors (see Figure 1). In mice with NPY/AGRP ablation, rapid decreases in food intake and body weight have been reported (Xu et al., 2005). The NPY/AGRP expressing neurons also produce γ-aminobutyric acid (GABA), which inhibit POMC neuron action, thus suppressing the anorexigenic effects of POMC. In a mouse model, mice lacking vesicular GABA transporter (VGAT) in NPY/AGRP-related protein neurons exhibit reduced body weight and GABA release (Vong et al., 2011).
The melanocortin system has been reported to have an integral role in terms of studies on obesity and metabolic abnormalities (Cone et al., 2005). The five melanocortin receptors (MC1R-MC5R) have differential physiological roles. The melanocortin-1 receptor (MC1R) is a regulator...
of pigmentation and anti-inflammation. The adrenocorticotropic hormone (ACTH) is the ligand for the melanocortin-2 receptor (MC2R), which regulates steroidogenesis, and the melanocortin-5 receptor (MC5R) plays a role in sebaceous gland section (further described in Yang et al., 2003; Cone et al., 2005). In terms of weight regulation, the melanocortin-3 receptor (MC3R) and the melanocortin-4 receptor (MC4R) have been the focus of several obesity studies over the past decade (reviewed in Santini et al., 2009) (see Table 1-2).

Adapted from Yang et al., (2003)

**Table 1-2. Melanocortin Receptor Expression and Function**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Primary Areas of Expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocortin-1 Receptor (MC1R)</td>
<td>melanocyte</td>
<td>anti-inflammation</td>
</tr>
<tr>
<td>Melanocortin-2 Receptor (MC2R)</td>
<td>adrenal Cortex</td>
<td>steroidogenesis, pigmentation</td>
</tr>
<tr>
<td>Melanocortin-3 Receptor (MC3R)</td>
<td>brain, intestinal tract, placenta</td>
<td>Weight homestasis, reproduction, cardiovascular function</td>
</tr>
<tr>
<td>Melanocortin-4 Receptor (MC4R)</td>
<td>hypothalamus, spinal cord</td>
<td>weight homestasis, sexual function</td>
</tr>
<tr>
<td>Melanocortin-5 Receptor (MC5R)</td>
<td>exocrine glands skeletal muscle, adipose tissue</td>
<td>exocrine gland secretion</td>
</tr>
</tbody>
</table>

Overall, the associations of both MC3R & MC4R and obesity provide a basis to study the melanocortin system in AIWG. If common variants of the MC3R and MC4R genes have a strong
regulating component within obesity, then common variants of these genes may also influence susceptibility and/or onset to AIWG.

Both MC3R and MC4R interact with leptin to regulate appetite, satiety and food intake signalling. Leptin is secreted from adipose tissue, and activates specific hypothalamic regions including pro-opiomelanocortin (POMC) expressing neurons. The pre-cursor polypeptide hormone (POMC) is cleaved into several peptides, including the α, β and γ melanocortin peptides, which stimulate receptors such as the MC4R and MC3R expressed in the paraventricular nucleus of the hypothalamus. Overall, the activation of the melanocortin receptors result in increased weight, as demonstrated in animal models and human genetic studies (Santini et al., 2009).

Mice with inactivated MC4R exhibit obesity and hyperphagia as well as altered metabolic indices including hyperglycemia. In humans, MC4R mutations have been shown to result in obesity, though the association varies between different ethnic populations. A frameshift mutation was associated with obesity in a study that examined 63 severely obese children (Yeo et al., 1998; Vaisse et al., 1998). MC4R mutations have been reported to be the most common single cause of monogenetic obesity and may be responsible for a range 0.5–4% across different ethnic populations (Miraglia et al., 2002). Recently, Lee et al. (2012) reported that MC4R mutations appeared to be more prevalent in patients of Northern European descent. A study by Lubrano-Berthelier et al., (2003) quantitatively analyzed the effect of a mutation on MC4R cell surface expression, and reported that 81.3% of pediatric obesity-associated heterozygous MC4R mutations led to intracellular retention of the receptor.

Two common MC4R variants have been reported to be protective against obesity (Val103Ile and Ile25Leu). The V103I MC4R polymorphism was negatively associated with obesity and lower
body mass index in a sample consisting of 7937 participants (Heid et al., 2004). More recently, the rs17782313 SNP was highlighted in a recent genome-wide association study investigating large obese populations (Loos et al., 2008). In the context of AIWG, it is currently unknown whether second-generation antipsychotic medication treatment has a direct impact upon the MC4R. However, second-generation antipsychotic medications have been shown to have an effect upon circulating leptin levels (Hägg et al., 2002; Atmaca et al., 2003). Patients treated with clozapine and olanzapine have been reported to have increases in weight, body mass index and leptin levels (Kraus et al., 1999). Given that leptin is stimulatory of MC4R, the exploration of its role in AIWG is warranted. Antipsychotic action could have substantial downstream effects on the two MC3R and MC4R receptors, resulting in the extreme weight gain observed in psychiatric patients treated with second-generation antipsychotic medications. Our group collaborated with a research group at Zucker Hillside Hospital, New York on a genome wide association study (GWAS). The results of the GWAS illustrated twenty SNPs at chromosome 18, exceeding a statistical threshold of P < 10^{-5} (see Figure 1-2).

*Reprinted with permission from the Archives of General Psychiatry (Malhotra et al., 2012).*

![Figure 1-2.](image.png)
The association between one SNP, rs489693, located near the melanocortin 4 receptor (MC4R) gene, and AIWG was found in the discovery sample, as well as three additional independent samples with rs489693 demonstrating consistent recessive effects. A subsequent meta-analysis resulted in a genome-wide significant effect \( P = 5.59 \times 10^{-12} \) (see Figure 1-3). Thus, the MC4R gene appears to have an important association with AIWG, and highly warrants investigation in psychiatric pharmacogenetic studies.

*Reprinted with permission from the Archives of General Psychiatry (Malhotra et al., 2012).*

**Figure 1-3.** Results from Genome Wide Association Study. A) Manhattan plot displaying chromosomal position for single-nucleotide polymorphisms on x-axis, and \(-\log_{10}P\) values on y-axis. Peak values are located on chromosome 18. B) Quantile-quantile plot displays statistical significance levels (\(-\log_{10}P\) values) of correlation and trend tests for change in BMI in the discovery cohort, plotted against expected values under the null hypothesis. With the exception of the most strongly associated SNPs on chromosome 18, there is no deviation from the diagonal (\(\lambda_{\text{genomic control}} = 1.00\)).
These data implicate MC4R in extreme SGA-induced weight gain and related metabolic disturbances. A priori identification of high-risk subjects could lead to alternative treatment strategies in this population.

The MC3R has a relatively less well-defined role in the context of obesity. Nonetheless, the MC3R has been reported to influence weight and feeding measures. Recent reports have shown that rare loss-of-function mutations of MC3R may partially indicate a role for this gene having a regulatory role in obesity (Mencarelli et al., 2008; Tao et al., 2004). Among the most frequently reported findings in human genetic studies are the reports of two common non-synonymous SNPs (Thr6Lys and Val81Ile) in high-linkage disequilibrium being associated with childhood adiposity (Feng et al., 2005). Interestingly, in vitro studies have shown that the two SNPs have been associated with reduced function and protein expression in comparison to the wildtype genotype (Feng et al., 2005). However, MC3R polymorphisms within the coding region and 5’ flanking sequences were reported to have no association with obesity in a sample assessing obese women (Li et al., 2000). A case-control study investigation of MC3R and MC4R mutations reported that the prevalence of rare MC4R variants was higher in obese participants compared to controls, while no difference was reported for MC3R mutations between the two groups (Calton et al., 2009). The MC3R rs3746619 SNP was examined in a study of SCZ patients treated with second-generation antipsychotic medications, and no association was reported (Moons et al., 2011). Altogether, the MC3R and MC4R appear to have non-redundant roles in regulating weight and appetite, given that mice with abolished MC3R and MC4R gain more weight than either MC3R -/- or MC4R -/--knock-out mice (Chen et al., 2000).

Given the prominent role of the melanocortin system in general obesity, namely the MC3R and MC4R, it is surprising that these factors have not yet been extensively studied as possible
underlying contributors to the onset of AIWG. This research delves into the exploration of identifying novel associations between melanocortin system gene variants and AIWG.

1.9 Methodologies and Statistical Considerations

1.9.1 Polymerase Chain Reaction and Genotyping

The primary method used for analyzing genetic associations presented in this work was conducted by utilizing genotyping methodology. Genotyping with polymerase chain reaction (PCR) allows for identification of a specific allele. DNA, which refers to any cell in an organism that contains a nucleus, from individuals is required for the genotyping process. For the purposes of our studies, DNA was extracted from blood cells, though it can be taken from saliva or cheeks as well. The polymerase chain reaction (PCR) is a common molecular biology technique used to amplify DNA in order to generate thousands to millions of copies of a pre-determined DNA sequence. (Holland et al., 1991). Further details are provided in Chapters 3, 4 and 5, as PCRs were utilized for all three experimental studies.

There are numerous ways to conduct a PCR within a laboratory setting, as well as various alterations. For the purposes of this thesis, genotyping was conducting use distinct methods: 1) TaqMan allele-specific assays, 2) Restriction Fragment Length Polymorphism and 3) Illumina GoldenGate genotyping assays. Full descriptions and protocols are available within each chapter, and general methodologies for the techniques are outlined below.
TaqMan allele-specific assay

The TaqMan assay used for our studies utilizes a general PCR guideline with some small alterations with the procedure/mechanism (Bartlett et al., 2003). The TaqMan assays provide optimized genotyping of SNPs. The assay uses a 5’ nuclease assay for detecting and amplifying specific SNPs in purified DNA sequences. The forward and reverse primers amplify the sequence of interest using two TaqMan probes. The TaqMan MGB probes provide a fluorescent signal for the amplification of each allele. One probe labelled with the VIC dye detects Allele 1, and the FAM probe detects allele 2 (Holland et al., 1991).

Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) is a relatively older technology, which we utilized in Chapter 4. At times, it is not feasible to design or obtain primers via TaqMan technology, due to non-binding of primers to a given fragment of a DNA sequence. This was the case for our analysis of the POMC rs3754860 polymorphism. Thus, we used RFLP methodology in order to genotype this SNP and determine an association with our phenotype. RFLP distinguishes the difference in homologous DNA sequences, which can be detected by the presence of fragments of different lengths after digestion of the DNA samples. The digestion is conducted with the use of specific restriction endonucleases. Once the digestion is complete, gel electrophoresis is conducted and ethidium bromide staining is used to visualize unique DNA patterns at a pre-specified genotype. (http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechRFLP.shtml).
**Electrophoretic Mobility Shift Assay**

The electrophoretic mobility shift assay is a methodology that can be used to determine the existence of a potential interaction between DNA and protein. A control lane (DNA without a probe) is used in one lane, and the visualization of the band on the gel will exhibit unbound DNA. In the second lane, if a DNA fragment is able to bind to a protein, the DNA will bind to the protein complex and a band will appear in the gel. (Fried et al., 1989; Smith et al., 2009).

**1.9.2 Analyses, Sample Size and Power Calculations**

**Genetic Analyses**

The primary statistical analyses used to assess differences between genotypic groups were performed using SPSS 15.0. Categorical variables were tested using a \( \chi^2 \) test, and continuous variables were determined using both analysis of variance (ANOVA), and analysis of co-variance (ANCOVA). Demographic and clinical characteristics of the samples assessed in our studies were determined using ANOVA. Though BMI has been reported to be a consistent measure across obesity and AIWG literature (O’Rahilly & Farooqi, 2006), patient height data was unavailable across all of our local samples. Thus, we used the comparison of mean weight change (\%) across genotypic categories as our primary genetic association test. This method incorporates baseline weight of patients into our calculations.
Haplotype Analyses

Linkage disequilibrium (LD) refers to the correlation across alleles located near each other, which may be transmitted across generations, or descend from an ancestral chromosome (Reich et al., 2001). This grouping of alleles at adjacent locations which may combine together, may also be referred to as a haplotype. The level of LD between two or more SNPs is expressed as either D’ or $r^2$, with values closer to 1.0 representing relatively higher correlations (Li et al., 2003).

In our studies, both a 2-marker and 3-marker sliding window haplotype analyses across genes of interest (MC4R, MC3R) were analyzed. We used the sliding window approach so that we could systematically explore additional haplotypes in areas of lower correlation or linkage disequilibrium.

Gene-gene Interaction Analysis

The AIWG phenotype is multifactorial, with a number of genetic and environmental components that may influence the onset of AIWG in a given individual. However, both gene-gene and gene-environment interactions require analyses by specific statistical methods, aside from the ANCOVA described above. When multiple genes are assessed for an interaction effect, a number of cells may have very few or no data points, and this is referred to as the ‘curse of dimensionality’ (Bellman et al., 1961). If there are too few data points, then large standard errors may result, which can lead to an increase in the number of Type I errors (Concato et al., 1996; Peduzzi et al., 1996; Hosmer and Lemeshow, 2000). The multifactor dimensionality reduction
(MDR) uses a data-reduction approach that can address the limitations of using parametric tests in gene-gene interaction analyses (Hahn et al., 2003). More recently, the Model-Based Multifactor Dimensionality Reduction (mbmdr) was proposed as a statistical tool, given that this program can analyze quantitative traits and has increased power compared to the MDR program (Cattaert et al., 2011). Thus, we utilized mbmdr for our gene-gene interaction analyses.

**Power Calculations, Sample Size and Correction for Multiple Testing**

Our current samples include 266 patients assessed for drug-induced weight gain. This sample size will continue to increase over the next few years as more subjects are recruited. The power of a genetic association depends on multiple variables. Factors that contribute toward statistical power in genetic association studies include the degree of linkage disequilibrium in the population, minor allele frequency, effect size, sample size, standard deviation or variance of the population mean. Greater than 80% power is generally accepted in order to determine genetic effects.

In genetic association studies, it is typical to divide empirical p-values by the number of tests performed (Bonferroni correction) (Abdi et al., 2007). The Bonferroni method has been suggested to be too conservative and applies to a global null hypothesis, as opposed to the specific individual hypotheses. An additional consideration is that type I errors cannot decrease without an increase in type II errors (the probability of accepting the null hypothesis when the alternative is true), and type II errors are no less false than type I (Perneger et al., 1998). In the case of our MC3R study (Chapter 5), the variants are highly correlated or linked, thus the Bonferroni method was not an appropriate test to implement. Some tests that include the
calculation for the LD between SNPs include SNP Spectral Decomposition (SNPSpD) (Nyholt 2004) and False Discovery Rate (FDR) (Benjamini et al., 2001). The FDR test is more suitable and appropriate for correcting for a large number of tests (e.g., assessing over 10,000 genes across individuals in a study) (Benjamini et al., 2001). For the purposes of our studies consisting of 266 patients, the SNPSpD was the appropriate test to apply for correction for multiple testing in our study assessing the melanocortin-3 receptor (Chapter 5), where SNPs were highly linked.
Chapter 2 Hypotheses and Experimental Approach
2.0 Overview of Experiments, Hypotheses and Objectives

2.1.1 General Overview

The general aims of our research include the exploration of the effects of melanocortinergic system gene variants in antipsychotic induced weight gain (AIWG), as well as any additive effects of these genes. The genetic effects of leptin, serotonin and dopamine gene variants have been extensively investigated in the literature, and several variants from these systems have been examined on our samples. The present work will focus on downstream targets of leptin and constituents of the leptin-melanocortin system that have been determined to have a role in weight regulation in healthy populations. Our data are presented in three separate papers, two of which have been published in peer-reviewed journals; the third is in preparation for publication.

2.2 Study One: Genetic Association Study between Antipsychotic-Induced Weight Gain and the Melanocortin-4 Receptor Gene.

2.2.1 Background

The first paper (Chapter 3) focuses on the melanocortin-4 receptor (MC4R). The purpose of this study was to explore the potential effect of MC4R variation in the AIWG phenotype. Several genome-wide association studies detected a signal on chromosome 18, and the MC4R rs17782313 has been associated with obesity phenotypes in healthy populations (Loos et al., 2008). However, the MC4R gene had not yet been investigated in psychiatric pharmacogenetic
studies. We hypothesized that MC4R variation influences AIWG, and that a gene association will be found between MC4R SNPs and this phenotype.

An additional objective in this study included the investigation of a potential functional role of the MC4R rs8087522 SNP, located in the promoter region of this gene. We hypothesized that this SNP alters protein expression.

2.2.2 Hypothesis and Objectives of Study

**Hypothesis:** the MC4R gene variants are associated with AIWG. Melanocortin-4 receptor (MC4R) mutations have been reported to be a cause of monogenic obesity, where one single mutation is associated with disease, and common MC4R variants have been identified to be associated with obesity (Loos et al, 2008).

2.3 Study Two: Investigation of Melancortin-System Gene Variants in Antipsychotic-Induced Weight Gain

2.3.1 Background

The second paper (Chapter 4) examines the role between pro-opiomelanocortin, cocaine amphetamine regulated transcript and agouti related protein in AIWG. The POMC, CART and AGRP are integral to the leptin-melancortin system, and have been associated with general obesity and weight gain (reviewed in Santini et al., 2009). The CART is an especially intriguing target in the context of AIWG. It has been suggested that the CART peptide has a potential role
in dysregulated reward and food intake as a result of antipsychotic treatment (Beaudry et al., 2004).

2.3.2 Hypothesis and Objectives of Study

Hypothesis: one or more variants across the POMC, CART and AGRP genes will be associated with AIWG in our sample. The pro-opiomelanocortin, cocaine amphetamine regulated transcript, and agouti related proteins have significant roles in general weight regulation. The CART peptide has been reported to be influenced by clozapine administration in a rodent model (Beaudry et al., 2004). However, the association between any of these three genes and AIWG has remained relatively unexplored.

2.4 Study Three: An Exploratory Investigation of Melanocortin-3 Receptor Gene Variants in Antipsychotic-Induced Weight Gain

2.4.1 Background

The third paper (Chapter 5) examines the potential relationship between the melanocortin-3 receptor and AIWG. In addition, this paper explores the potential gene-gene interaction between MC4R and MC3R with AIWG. This is based upon the finding that in animal models, MC3R and MC4R have non-redundant roles in weight gain. Mice that lack both MC3R and MC4R receptors gain more weight in comparison to MC3R -/- or MC4R -/- mice (Chen et al., 2000). Thus, we will conduct pair-wise interactions between MC3R and MC4R SNPs. The melanocortin-3 receptor has two functional variants (rs3746619 & rs3827103), which have been associated with
obesity (Feng et al., 2005) and reduced in vitro protein expression. Given the biological roles of MC3R and MC4R described above, gene-gene epistatic effects will occur between melacortinergic genes and AIWG.

2.4.2 Hypothesis/Objectives of Study

We hypothesize that MC3R variants are associated with antipsychotic induced weight gain. Furthermore, we hypothesize that an interaction between MC3R and MC4R SNPs in AIWG will be detected. Ultimately, a gene-gene interaction analysis within the wider leptin-melanocortin system may provide improved modelling of AIWG risk.
Chapter 3

Genetic association study between antipsychotic-induced weight gain and the melanocortin-4 receptor gene

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A link to the published paper can be found at
http://www-ncbi-nlm-nih-gov.myaccess.library.utoronto.ca/pubmed/22310352
3.1 Abstract

Antipsychotic induced weight gain (AIWG) may result in the metabolic syndrome in schizophrenia (SCZ) patients. Downstream variants of the melanocortin-4 receptor (MC4R) gene have been associated with obesity in various populations. Thus, we examined single-nucleotide polymorphisms (SNPs) in the MC4R region for association with AIWG in SCZ patients. Four SNPs (rs2229616, rs17782313, rs11872992, and rs8087522) were genotyped in 224 patients who underwent treatment for SCZ and were evaluated for AIWG for up to 14 weeks. We compared weight change (%) across genotypic groups using analysis of covariance for three SNPs ($r^2=0.8$). European-ancestry patients who were rs8087522 A-allele carriers (AG+AA) on olanzapine gained significantly more weight than non-carriers ($P = 0.027, n = 69$). These observations were marginal after correction for multiple testing. We performed in vitro electrophoretic mobility shift assay that suggested that the presence of the A-allele may create a transcription factor-binding site. Further investigation is warranted for both these exploratory findings.
3.2 Introduction

Schizophrenia (SCZ) is a debilitating psychiatric disease that affects 1% of the general population. The widely prescribed atypical antipsychotic medications are effective in treating core positive symptoms of SCZ. Despite the success of atypical antipsychotic drugs in symptom reduction, their clinical use remains burdensome. This is due to their association with substantial weight gain and the metabolic syndrome. The metabolic syndrome is composed of several factors, including insulinemia, abdominal obesity and hypertension (Meyer et al., 2005). Both severe weight gain and the metabolic syndrome can have potentially fatal consequences, including development of diabetes mellitus and coronary heart disease (Müller et al., 2006).

Studies have confirmed that the rates of the metabolic syndrome in SCZ samples are between 40 and 50% (McEvoy et al., 2005; Meyer et al., 2005; Callaghan, 2005). The underlying mechanisms of onset of weight gain and the metabolic syndrome in SCZ patients have yet to be elucidated. Approximately 30–40% of patients experience substantial weight gain and the metabolic syndrome after initial treatment with second-generation antipsychotic drugs. Discovery of genetic factors associated with metabolic side effects can lead to improved treatment regimes and novel targets for drug development.

Genetic mechanisms are among the strongest known factors in regulating second-generation antipsychotic (SGA)-induced weight gain. A high inter-individual variability exists between patients who are treated with SGAs in terms of the side effect profile. This is demonstrated by the finding that up to 30% of patients develop weight gain and the metabolic syndrome, whereas some patients do not (Müller et al., 2006). One of the strongest reports supporting genetic regulation of this side effect is from the recent twin and sibling study that reported heritability of
SGA-induced weight gain is between $h^2 = 0.6–0.8$ (Gebhardt et al., 2010). Thus, pharmacogenetic-based algorithms have a high potential to identify individuals who are at risk for development of antipsychotic-induced weight gain (AIWG). These, in turn, can greatly aid in the decision-making process for antipsychotic treatment.

In general, obesity has become an epidemic in North America (Wang et al., 2006). The pathophysiology of appetite and weight regulation is an area that is being extensively investigated in order to develop counteracting strategies and treatment. The leptinergic system has been reported to be heavily involved in physiological food intake and weight regulation. In an intact homeostatic feedback system, leptin activates pro-opiomelanocortin neurons in the arcuate nucleus of the hypothalamus. The pro-opiomelanocortin peptide is the precursor for neuropeptides, including $\alpha$-melanocyte-stimulating hormone ($\alpha$-MSH) (Santini et al., 2009). The $\alpha$-MSH activates the melanocortin receptor-4 (MC4R), and the signals from MC4R-expressing neurons are hypothesized to modulate various pathways through downstream effectors that result in energy expenditure (Arch et al., 2005). Animal model studies utilizing gene targeting found that mice lacking the MC4R receptors showed a mature-onset obesity syndrome, associated with metabolic abnormalities such as hyper-insulinemia and hyperglycemia (Huszar et al., 1997; He et al., 2010; Srisai et al., 2011). Furthermore, mutations of MC4R can cause monogenic obesity in humans.

Recently, a more common genetic variant located 188 kb downstream from the MC4R gene (rs17782313) has been associated with increased fat mass, weight and waist circumference (Loos et al., 2008; Speliotes et al., 2010). The MC4R gene was recently identified as a gene for obesity susceptibility in a genome-wide association study (GWAS) of 249 796 individuals (Speliotes et al., 2010). The minor C-allele of this MC4R variant has previously been associated with higher
intake of total energy and dietary fat, resulting in greater long-term weight change in a healthy population (Loos et al., 2008). Another well-studied polymorphism, the MC4R 103I (rs2229616) marker, has been associated with decreased waist circumference along with other measures of the metabolic syndrome (decreased glycosylated hemoglobin) (Brönner et al., 2006; Heid et al., 2005).

Interestingly, the same rs17782313 marker was not significantly associated with AIWG in a sample of pediatric and adolescent patients who were naive to antipsychotic medication using a GWAS approach (Malhotra et al., 2012). However, our group, in collaboration with Malhotra et al., (2012) discovered and replicated an association between another marker at MC4R rs489693 and AIWG. The discovery cohort in this study consisted of antipsychotic-naive adolescents who were administered one of several antipsychotics (olanzapine, risperidone, quetiapine or aripiprazole) and weight gain was measured for up to 12 weeks. The rs489693 marker was also associated with weight gain in a sample comprising SCZ patients who had been given clozapine with no prior second-generation antipsychotic treatment. The association of rs489693 was again replicated in a third sample that consisted of SCZ patients who were prescribed antipsychotic treatments in a naturalistic setting. Taking into consideration the strong positive associations of MC4R polymorphisms with obesity and AIWG, we evaluated whether additional MC4R gene polymorphisms are associated with AIWG. In this study, we investigated the possible association between rs17782313 and rs2229616, which have been consistently implicated with obesity in the literature (Loos et al., 2008; Speliotes et al., 2010; Brönner et al., 2006; Heid et al., 2008). In addition, we also investigated rs8087522 and rs11872992, which are both located in the promoter region of the MC4R gene.
3.3 Materials and Methods

3.3.1 Subjects

A total of 224 patients with chronic SCZ or schizoaffective disorder were included in this study, and sample characteristics have been described previously (Tiwari et al., 2010). Approval from the institutional ethics committee and informed consent were obtained for all patients. Patients were primarily recruited from three different sites. Sample-A: Charite University Medicine (Berlin, Germany) (DJ Muller, n= 93). Patients were diagnosed with either SCZ or schizoaffective disorder according to the DSM-IV and ICD-10 criteria. They were given antipsychotic medication and assessed for up to 6 weeks (Table 1). Sample-B: Case Western Reserve University in Cleveland (Ohio, USA) (HY Meltzer, n=76). DSM III-R-diagnosed SCZ patients were longitudinally studied and were either treatment-refractory or intolerant to treatment with typical antipsychotics. Prior to treatment with clozapine, patients underwent a washout period of 2–4 weeks during which no medications were given unless deemed clinically essential by the physician. The dosage of clozapine was not fixed and serum levels were monitored to ascertain compliance. Patients were treated with clozapine for a minimum of 6 weeks and weight change (%) was calculated from baseline weight (for details see Masellis et al., 1998 and Basile et al., 2002). Sample-C: Hillside Hospital in Glen Oaks (New York, USA) (JA Lieberman, n=55). DSM-IV-diagnosed patients with chronic SCZ or schizoaffective disorder that had shown suboptimal response to previous atypical anti-psychotic drug treatment were included in this study. Despite previous treatment, these patients had continued to express positive symptoms and poor cognitive functioning over a period of 2 years. In a 14-week double-blind study, patients were randomly assigned to receive clozapine (500 mg day\(^{-1}\)), olanzapine
(20 mg day\(^{-1}\)) risperidone (8 mg day\(^{-1}\)) or haloperidol (20 mg day\(^{-1}\)) (for details see Volavka et al., 2002 and Müller et al., 2005). Patient ethnicity was ascertained using self-reported ancestry information over the last three generations.

### 3.3.2 Genetic Analysis

Venous blood (10–20 ml) was obtained from study participants and DNA was extracted at the Centre for Addiction and Mental Health using the high-salt method (Lahiri & Nurnberger et al., 1991). All single-nucleotide polymorphisms SNPs; rs17782313, rs8087522, rs11872992 and rs2229616; Figure 1) were genotyped using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA). PCR was performed in a final volume of 10ml, using 20 ng genomic DNA and 1 x TaqMan Universal PCR master mix. The following conditions were applied for amplification: 95 °C for 10 min, followed by 50 cycles of 92 °C for 15 s and 60 °C) for 1 min. Alleles were called using the Allelic Discrimination module of the Sequence Detection software supplied with the ABI model 7500 machine. Genotyping was performed blind to the subject’s weight gain status. Ten percent of the sample was randomly re-genotyped for quality control and 100% concordant genotype calling was observed.

### 3.3.3 Data analysis

Statistical analyses were performed for individual SNP using SPSS 15.0. Categorical variables were tested by \(\chi^2\) test. Continuous variables were tested by analysis of variance or covariance. Weight change (%) from baseline was compared across the genotypic categories by analysis of covariance using duration of treatment as a covariate. Distribution of age and sex across genotypic groups was assessed. If either age or sex, or both, showed a significant effect, they
were included as covariates in the analysis of covariance. Haplotype blocks were examined and weight change (%) with UNPHASED 3.1 (Dudbridge et al., 2003). Only haplotypes with frequencies greater than 5% were included in the analyses. Linkage disequilibrium and Hardy–Weinberg equilibrium were determined using Haploview 4.1 (Barret et al., 2006). Power calculations were performed using Quanto 1.2.4. Assuming a minor allele frequency of 0.20 in a sample size of n=224, in an additive model we had more than 80% power to detect a mean weight difference of 1.5% between carriers and non-carriers of the risk genotype (Gauderman et al., 2007).

3.3.4 Electrophoretic Mobility-Shift Assay

We performed an electrophoretic mobility-shift assay (EMSA) as described by Tiwari et al (2010). We initially analyzed in silico for presence of binding sites using the Genomatix MatInspector program. Biotin-labeled and unlabeled double-stranded oligonucleotides for each allele of rs8087522 (A and G), and unlabeled oligonucleotides with the specific consensus-binding site for the transcription factor for Paired Related Homeobox 2 (PRX2) and MSH homeobox 2 (MSX2) were synthesized. Nuclear extract was prepared from SH-SY5Y neuroblastoma cells (ATCC:CRL2266, Manassas, VA, USA) using the NE-PER Nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL, USA). The complementary pairs of nucleotides were suspended in STE Buffer (10 mM Tris (pH 8.0), 50 mM NaCl, 1 mM EDTA) and annealed using a thermocycler (Step-1: 95 °C for 5 min; Step-2: 95 °C (-1°Ccycle, 70 times); Step-3: HOLD at 4 °C 1. The re-annealed double-stranded oligonucleotides (20 fmol) were incubated with 2ml of the nuclear extract, 1 x binding buffer, 2.5% glycerol, 5 mM MgCl2, 50 ng ml⁻¹ poly-(dIdC)and 0.05% NP-40 for 20 min at room temperature. For the competition experiments,
unlabeled oligonucleotides at 200-fold molar excess (4 pmol) were pre-incubated with the nuclear extract before adding the biotin-labeled oligo-nucleotides. DNA–protein complexes were fractioned on 5% polyacrylamide gel in 0.5 x TBE for 35 min. Complexes were electrotransferred to a nylon membrane at 380 mA for 45 min, and cross-linked at 120 mJ cm$^{-2}$ for 45 s. The biotinylated oligonucleotides were detected by chemiluminescence using the chemiluminescent nucleic acid detection module of the Light Shift Chemiluminescent EMSA kit (Pierce). The processed nylon membrane was exposed to an X-ray film for 45 s.

3.4 Results

3.4.1 Genetic analysis

The demographic characteristics of the study sample are presented in Table 1. Significant differences in clinical variables were observed among clinical sites; however, similar rates of change in weight were observed (Table 3-1). The three SNPs, rs17782313, rs8087522 and rs11872992, analyzed in this study were in Hardy–Weinberg equilibrium (P >0.05; Table 3-2).
**Table 3-1.** Demographic characteristic from each of the clinical sites.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sample A (n=93)</th>
<th>Sample B (n=76)</th>
<th>Sample C (n=55)</th>
<th>Total sample (n=224)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>55 (59.1%)</td>
<td>49 (64.4%)</td>
<td>46 (83.6%)</td>
<td>150 (66.9%)</td>
<td>0.008</td>
</tr>
<tr>
<td>Female</td>
<td>38 (40.9%)</td>
<td>27 (35.5%)</td>
<td>9 (16.4%)</td>
<td>74 (33.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>35.01±12.0</td>
<td>32.84±8.0</td>
<td>40.5±9.3</td>
<td>35.63±10.50</td>
<td>p&lt;0.001b</td>
</tr>
<tr>
<td><strong>Initial body weight (kg)</strong></td>
<td>80.7 ± 15.6</td>
<td>74.3 ± 13.6</td>
<td>84.82 ± 17.6</td>
<td>79.5 ± 15.9</td>
<td>p&lt;0.002b</td>
</tr>
<tr>
<td><strong>Weight change (kg)</strong></td>
<td>3.32 ± 3.9</td>
<td>3.56 ± 4.5</td>
<td>4.53 ± 6.5</td>
<td>3.69 ± 4.8</td>
<td>0.570b</td>
</tr>
<tr>
<td><strong>Weight change (%)</strong></td>
<td>4.06 ± 4.7</td>
<td>5.09 ± 6.4</td>
<td>5.85 ± 8.4</td>
<td>4.85 ± 6.4</td>
<td>0.555b</td>
</tr>
<tr>
<td><strong>Study duration (weeks)</strong></td>
<td>5.09±1.6</td>
<td>6.00±0</td>
<td>11.83±3.7</td>
<td>7.03±3.4</td>
<td>p&lt;0.001b</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European-Americans</td>
<td>91 (97.8%)</td>
<td>55 (72.4%)</td>
<td>11 (20.0%)</td>
<td>157</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>African-Americans</td>
<td>2 (2.2%)</td>
<td>21 (27.6%)</td>
<td>33 (60.0%)</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>11 (4.91%)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clozapine</td>
<td>12</td>
<td>76</td>
<td>11</td>
<td>99</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>5</td>
<td>0</td>
<td>11</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Olanzapine</td>
<td>15</td>
<td>0</td>
<td>21</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Risperidone</td>
<td>28</td>
<td>0</td>
<td>12</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

a The samples were included from three different clinical sites

b Kruskal-Wallis Test
Table 3-2. List of SNPs in MC4R tested for association with antipsychotic Induced Weight Gain at Genotypic Level

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Total sample</th>
<th>European Americans Clozapine/Olanzapine</th>
<th>European Americans Clozapine Only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All Drugs</td>
<td>Weight Change (%), n=224</td>
<td>Weight Change (%), n=96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P-value</td>
<td>P-value</td>
</tr>
<tr>
<td>rs11872992</td>
<td>GG</td>
<td>5.03±6.6(176)</td>
<td>0.460</td>
<td>4.37±5.7(68)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>3.49±4.7(34)</td>
<td>3.776±4.9(16)</td>
<td>3.09±4.1(12)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>7.80±10.2(3)</td>
<td>7.80±10.2(3)</td>
<td>2.01±2.8(2)</td>
</tr>
<tr>
<td>rs8087522</td>
<td>GG</td>
<td>3.99±5.8(91)</td>
<td>0.231</td>
<td>3.22±4.8(42)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>4.73±6.3(89)</td>
<td>5.27±5.8(33)</td>
<td>5.55±6.0(27)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>6.73±6.4(27)</td>
<td>4.92±5.2(10)</td>
<td>4.92±5.2(10)</td>
</tr>
<tr>
<td>rs17782313</td>
<td>TT</td>
<td>4.97±6.4(118)</td>
<td>0.306</td>
<td>4.50±5.9(51)</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>4.75±6.5(73)</td>
<td>5.15±5.6(27)</td>
<td>5.24±5.5(24)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>4.62±6.0(10)</td>
<td>3.38±4.3(7)</td>
<td>3.41±5.2(5)</td>
</tr>
</tbody>
</table>

\(^a\)Mean ± SD (number of individuals).

\(^b\)SNPs with low numbers in a genotypic group were merged with the heterozygous genotypic group.

\(^c\)For GG vs AA+AG.

\(^c\)All the p-values were calculated using ANCOVA. rs8087522, European Americans on Clozapine: GG vs AA+AG, 2.43±4.3 vs 5.38±5.7, (p=0.027); Bonferroni corrected p= 0.081.
The rs2229616 SNP was monomorphic in our sample and thus was excluded from our statistical analyses. No variation of the SNP was detected, and thus there was only one genotypic group to assess. Duration of treatment varied across clinical sites and thus was entered as a covariate in all association analysis. In the total sample, we observed no significant association between genotypes and weight change (Table 2). However, our sample consisted of patients of different ethnicities (mostly of European and African ancestry) and receiving drugs with different propensities to cause weight gain. Clozapine and olanzapine have been reported to be associated with the highest risk of clinically significant weight gain and show overlapping side-effect profiles (Nasrallah et al., 2008). Thus, we performed a sub-analysis stratifying patients by their self-reported ethnicities and whether they received clozapine or olanzapine. Patients classified in the African American group were too low in number to allow analysis with sufficient power. In the patients of European ancestry who received either clozapine or olanzapine, no significant genotypic association was observed for rs11872992 and rs17782313, whereas a non-significant trend was found for the rs8087522 marker, where the GA or AA genotypes gained more weight than GG homozygotes (P=0.11) (Table 3-2). Both clozapine and olanzapine have a significantly higher propensity to induce weight gain compared with other antipsychotic drugs (Nasrallah et al., 2008).

We stratified the European-ancestry sample by individual drug type to uncover associations with weight gain in relation to these two individual drugs. In patients of European ancestry who were treated with clozapine (n=69), we found that carriers of the MC4R rs8087522 ‘A’-allele (AA + AG) gained significantly more weight (5.38% ± 5.7) than the GG homozygotes (2.43% ± 4.3; P= 0.027) (Table 3). However, these results were not significant after taking into account the multiple SNPs that we have analyzed (3 SNPs, Bonferroni-corrected, P=0.081) (see figure 3-2).
Allelic analyses also suggested that the ‘A’-allele carriers gained more weight (5.60% ± 5.4) than carriers of the ‘G’-allele (3.3% ± 5.4; \( P = 0.04 \)) (Table 3-2). There were no significant allelic or genotypic associations observed with weight gain in patients of African American ancestry, owing to the very small sample size. In the allelic analyses in the European sub-sample, the minor C-allele of the rs17782313 marker, a trend for association (\( P = 0.09 \)) with weight gain was observed in the combined sample of clozapine- and olanzapine-treated patients. A non-significant trend for allelic association was also observed with rs11872992, whereas no allelic association was observed for the rs8087522 marker (Table 3) despite its modest association in the genotypic analyses. Linkage disequilibrium was calculated using Haplovie 4.1. \( D' \)-values
were high for all pairwise comparisons among the SNPs and $r^2$ was low (Figure 1). Two marker haplotype analyses were conducted and no associations were found for the three SNPs and weight gain ($P > 0.05$) (Figure 3-1).

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3.4.2. Analysis of Electrophoretic-mobility shift assay

The SNP rs8087522, associated with clozapine-induced weight gain, is present in the promoter region of MC4R and is in the vicinity of a genomic region that has been reported to code for transcription factor-binding sites, according to the University of California Santa Cruz (UCSC) genome browser. EMSA is an *in vitro* method to assess DNA–protein interactions that reflect transcription factor-binding sites, which may be relevant to gene expression. An *in silico*
prediction of transcription factor binding using a 31-bp oligonucleotide surrounding the SNP rs8087522 was performed using MatInspector (Genomatix, 2010). Presence of the A-allele was predicted to bind to 20 transcription factors. The transcription factors PRX2-binding protein-1 (PRX2, matrix similarity of 1.00) and MSX2 protein (matrix similarity of 0.995) had the highest similarity with the region encompassing the A-allele (Table 3-2). Therefore, these two transcription factors were chosen for further analysis. An EMSA was performed and putative binding of a nuclear protein to the A-allele was observed (Figure 3-2).

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Table 3-3. Oligonucleotides for EMSA

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotides</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs8087522-A</td>
<td>5’-GCCAGTAGTGTTCAATTTAATACCTGAAA-3</td>
<td>This Study</td>
</tr>
<tr>
<td></td>
<td>Rev-5’-TTTCAGGTATTTTAATTGAACCACCTGACG-3’</td>
<td></td>
</tr>
<tr>
<td>rs8087522-G</td>
<td>5’-GCCAGTAGTGTTCAATTTAATACCTGAAA-3</td>
<td>This Study</td>
</tr>
<tr>
<td></td>
<td>Rev-5’-TTTCAGGTATTTTAATTGAACCACCTGACG-3’</td>
<td></td>
</tr>
<tr>
<td>PRX2 (S8)</td>
<td>5’-TAACCTAATTAAC-3’</td>
<td>(Norris &amp; Kern, 2001)</td>
</tr>
<tr>
<td></td>
<td>R5’-GTAAATTAGTTA-3’</td>
<td></td>
</tr>
<tr>
<td>MSX-2</td>
<td>F5’-GGCGAATTAGGAATTAGG-3’</td>
<td>(Towler et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>R5’-CCTAATTCCTAATTCGCC-3’</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-2. EMSA for SNP rs8087522. Identification of nuclear proteins binding to the A-allele (lanes 1–5) and G-allele (lanes 6–8). In the competition experiment, using 200-fold excess of unlabeled A-allele containing an oligonucleotide, binding is significantly reduced, suggesting that binding of this nuclear protein is specific to the A-allele. We tested two known transcription factors (PRX and MSX2) for binding to the A-allele. Oligonucleotides containing specific binding sites for these transcription factors (lanes 4 and 5) were unable to compete out the A-allele. EMSA, electrophoretic mobility-shift assay; MSX, MSH homeobox; PRX, Paired Related Homeobox.

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Oligonucleotides containing specific binding sites for transcription factors PRX2 and MSX2 were unable to compete out the labeled oligonucleotide with the A-allele, indicating that these may not be the transcription factors binding at this site (Figure 3-2). Binding of nuclear protein to the G-allele was likely to be result of non-specific binding as the unlabeled G-allele containing the oligonucleotide could not disrupt this binding.
3.5 Discussion

In this study, based on the strong biological rationale for MC4R in weight regulation, we investigated MC4R polymorphisms for their potential roles in AIWG. We investigated rs17782313 and rs2229616, which have been associated with obesity previously (Loos et al., 2008; Masellis et al., 1998), as well as two SNPs in the MC4R promoter region (rs8087522 and rs11872992) that could have potential roles in gene expression.

The rs2229616 marker has been reported to be mildly protective against weight gain in the general population (Brönner et al., 2006; Heid et al., 2008). However, it was monomorphic in our sample and was therefore excluded from statistical analyses. We did not observe any significant associations with the rs17782313 and rs11872992 polymorphisms and AIWG in the total sample set. There was no significant association between rs17782313 or rs11872992 and weight gain in a subset of European-ancestry patients who received clozapine or olanzapine treatment. Notably, the rs17782313 minor C-allele had a non-significant trend for association with weight gain in our allelic analyses in our European-ancestry patients (P=0.09). This same allele has been previously associated with increased body mass index in a GWAS of 16 876 individuals of European descent (Renström et al., 2009). However, the rs17782313 marker was not significantly associated with weight gain or metabolic parameters in the recent GWAS assessing a pediatric drug-naïve population exposed to antipsychotic medication (Malhotra et al., 2012). Given that our hypothesis-driven study design and sample were different from those of the pediatric GWAS investigation by Malhotra et al (2012) direct comparisons are not possible.

Overall, more work is needed to elucidate the role of this SNP in AIWG.
By contrast, we observed a modest association with the rs8087522 variant and AIWG in the subset of European patients who were exposed to clozapine. Our genetic results indicated that patients of European American ancestry who were carriers of the rs8087522 AA genotype were more likely to gain weight when given clozapine for treatment. In the allelic analysis, a modest trend of association with weight gain was found for carriers of the A-allele. This polymorphism is in the promoter region of MC4R (Figure 3-1), suggesting a potential regulatory role for transcription factor binding. In order to discern the functional relevance of this marker, we performed in silico predictions and observed that several transcription factors bind to the A-allele. Using EMSA we provide suggestive evidence that the rs8087522 A-allele, but not the G-allele, is bound by a yet unknown nuclear protein, and thus may influence MC4R gene expression.

To our knowledge, this is the first study investigating the role of the rs8087522 marker in AIWG. The limitations of the study include a relatively small sample size, compared with other studies, but it is important to note that we have sufficient power. One caveat with our sample is that patients in some sub-samples were not drug-naïve when they entered our study. Thus, they may have already gained weight owing to prior antipsychotic exposure. Despite this, clozapine, the antipsychotic with the highest risk for weight gain, was administered for the first time to a substantial number of patients (every patient in Sample-B), and our analyses showed that these patients did gain a considerable amount of weight. In this study, we report a nominally significant association between clozapine-induced weight gain and the rs8087522 variant in our European American-ancestry sample. Furthermore, antipsychotic-induced side-effect profiles vary across ethnicities, and we did not have sufficient power to detect an effect for AIWG in our
African American-ancestry sample. Further investigation of MC4R gene variants in African Americans is warranted.

Our collaborative group reported that the rs489693 marker is associated with AIWG in three separate samples (Malhotra et al., 2012). The region where this marker is located has been highlighted in several GWASs regarding obesity (Loos et al., 2008; Speliotes et al., 2010). The rs489693 is located 190 kb downstream from the MC4R gene and has no known functional relevance. There is no gene located in the region, but the variant has been associated with weight gain in the general population. It may be controlled by one or more remote regulatory sites located at considerable distance from rs489693 (for example, chromosomal folding) (Espinoza et al., 2011). The rs489693 marker may also be linked to another ‘true’ marker that is the actual biological regulator of changes in weight gain. We also examined possible combinations of markers across the MC4R that were significant either in the study by Malhotra et al. (2012) or within our Toronto-based AIWG samples. There were no significant findings for these marker combinations.

To summarize, we did not replicate the reported association of rs17782313 with obesity (Loos et al., 2008; Speliotes et al., 2010; Renström et al., 2009) in AIWG. Similarly, we found no association with the rs11872992 SNP, which is located within the promoter region of MC4R, and AIWG. A strong biological rationale remains for the study of MC4R and metabolic parameters, including weight gain. The MC4R has a significant regulatory role in food intake and energy homeostasis. Furthermore, the MC4R genetic findings may be used in an attempt to define new targets for discovery of novel medications that do not have severe weight-gain side effects. A recent study reported that an MC4R agonist has potent anti-obesity efficacy in a rodent model (He et al., 201). Furthermore, recent studies have shown that in some cases the MC4R protein, if
altered by mutation, is unable to reach the plasma membrane as the receptor may misfold in the endoplasmic reticulum, and these defective MC4R proteins are therefore targeted for degradation (Granell et al., 2010). The authors suggest that drugs, which improve MC4R folding or decrease the endoplasmic reticulum-associated degradation of the receptor, may treat some forms of hereditary obesity. Understanding the common variants of the MC4R gene may also have implications in drug development for general obesity as well as AIWG.

In conclusion, we report a nominally significant association between rs8087522 and AIWG, and we found that this SNP affects potential transcription factor-binding sites, which may result in functional effects. A recent case-control study reported no association between the rs8087522 and high weight (Beckers et al., 2011). Our overall findings still suggest that the rs8087522 marker in the MC4R gene may be of interest for further investigation in AIWG and weight regulation in general.
Chapter 4

Investigation of melanocortin-system gene variants in antipsychotic induced weight gain


A link to the published paper can be found at

4.1 Abstract

The common use of second-generation antipsychotic medications may result in substantial weight gain and metabolic syndrome in a subset of schizophrenia patients. Distinct populations of neurons expressed in the hypothalamus have regulatory roles in weight control and energy homeostasis involving the cocaine amphetamine and regulated-transcript (CART), the polypeptide pro-opiomelanocortin (POMC) and the agouti related protein (AGRP). Thus, we investigated the potential role of CART, POMC and AGRP genetic variants in antipsychotic induced weight gain. Five CART single nucleotide polymorphisms (SNPs) (rs10515115, rs3763153, rs3857384, rs11575893, rs16871471), three POMC SNPs (rs6713532, rs1047521, rs3754860), and one AGRP SNP were genotyped in 218 patients treated with different antipsychotics for chronic schizophrenia and evaluated for AIWG. We compared weight change (%) across genotypic groups using analysis of covariance. In our overall sample, the CART and AGRP variants were not significantly associated with AIWG. The POMC SNP rs3754860 was significantly associated with AIWG (p_corrected = 0.036). In our sub-sample accounting for ethnicity (European descent) and drug-type (olanzapine and clozapine), the POMC rs3754860 and rs1042571 SNPs reached a trend for association (p = 0.097 and p = 0.089, respectively) with AIWG. In this exploratory study, we found that POMC gene variants were nominally associated with antipsychotic induced weight gain.
4.2 Introduction

Antipsychotic drugs are an essential component in the drug treatment of schizophrenia. However, the variability of treatment outcome and side effect manifestation remains a critical problem in terms of effective treatment regime and compliance (reviewed in Lett et al. 2012). One devastating and potentially fatal effect of antipsychotic drug use is substantial weight gain and metabolic disturbances. The use of atypical antipsychotics such as clozapine and olanzapine are associated with dramatic secondary effects in the basal metabolism ultimately increasing risk for obesity, diabetes and heart disease (Ray et al. 2009). Despite the serious impact on overall physical and psychological health, mechanisms and predictors for antipsychotic induced weight gain (AIWG) remain unclear. Pharmacogenetic approaches may provide distinct advantages in the search for the factors associated with AIWG and its predictors. Weight gain may be considered a robust phenotype for pharmacogenetic studies because of evidence on the heritability of weight regulation shown by twin, adoption and family studies (Gebhardt et al. 2010, Ternouth et al. 2011, Wehmeier et al. 2005). Recent studies suggest that genes expressed in the hypothalamus regulating energy homeostasis may have a role in AIWG (Brandl et al., 2012, Tiwari et al., 2012; Malhotra et al., 2012).

In the arcuate nucleus of the hypothalamus, distinct populations of neurons influence feeding behaviour (Cone et al., 2005). Adipocytes release leptin, which, in turn, stimulate neurons expressing cocaine amphetamine and regulated transcript (CART) and polypeptide pro-opiomelanocortin (POMC) inhibit feeding behaviour (anorexigenic effects). Additionally, leptin acts upon the agouti related protein (AGRP), reducing the AGRP expression, which is an
endogenous antagonist for melanocortin receptors. Thus, an intact leptin-melanocortin system results in an anorexigenic effect (Santini et al. 2009).

4.2.1 Proopiomelanocortin

Sub-chronic olanzapine treatment has been shown to result in down regulation of pro-opiomelanocortin expression in the rat hypothalamus (Ferno et al. 2011). However, Davoodi et al., (2009), reported that female rats treated with olanzapine gained body weight and exhibited increased food intake, but showed no difference in POMC mRNA expression compared to controls (Davoodi et al. 2009). Genetic studies assessing healthy human populations for weight-related measures have provided evidence that certain POMC gene variants may predispose some individuals to become obese (Baker et al., 2005; Ternouth et al., 2011).

4.2.2 Cocaine Amphetamine Regulated Transcript

CART is a hypothalamic neuropeptide, and hypothalamic expression levels of both POMC and CART are decreased during food deprivation in rodents (Brady et al. 1990, Savontaus et al. 2002, Ziotopoulou et al. 2000). CART is also directly stimulated by leptin (Cheung and Mao 2012). Acute and chronic treatment with clozapine has been shown to reduce the expression of CART mRNA in the shell of the nucleus accumbens (Beaudry et al. 2004). Two studies reported different CART variants (Phe34Leu and Delta A1457) associated with obesity in Italian children and adolescents (del Giudice et al. 2001, Rigoli et al. 2010) but other CART variants were reported not to be associated with obesity in Danish Caucasians (Echwald et al. 1999), Pima Indians (Walder et al. 2000) or severely obese Caucasian children (Challis et al. 2000).
4.2.3 Agouti Related Protein

AGRP regulates feeding behaviour by activating melanocortin receptors, and its expression is down-regulated by leptin (Lett et al. 2012, Santini et al. 2009). Mice that are either leptin deficient (ob/ob), or leptin receptor deficient (db/db) exhibit increased levels of AGRP mRNA in the hypothalamus (Ollmann et al. 1997). The AGRP over-expression in mice results in obesity, increased body length, hyperplasia, as well as hyperglycemia and hyperinsulinemia (Graham et al. 1997; Michaud et al. 1997). Notably, administration of olanzapine in rats resulted in an up regulation of AGRP in the arcuate nucleus (Ferno et al. 2011). Polymorphisms of the AGRP gene have been implicated in genetic association studies across populations. The GG genotypic group for the c.199G-->A) polymorphism were significantly associated with fatness and abdominal adiposity in a healthy human population (Argyropoulos et al. 2002). Additionally, the functional AGRP promoter SNP -38C/T has been reported to be related in body measures in African American populations (Argyropoulos et al. 2002, Bonilla et al. 2006), indicating that ethnicity can play a major role in this context.

Given the roles of POMC, CART and AGRP in weight regulation, we evaluated whether these variants are associated with AIWG in schizophrenia subjects treated mostly with clozapine. We assessed SNPs that were associated with weight measures in the literature as well as utilized tagSNPs covering the common variations across POMC, CART and AGRP genes.
4.3 Materials and Methods

4.3.1 Subjects

A total of 218 patients with chronic schizophrenia (SCZ) or schizoaffective disorder according to DSM-III or DSM-IV criteria were included in this study. Approval from the institutional ethics committee and informed consent were obtained for all patients. The patient ethnicity was ascertained using self-reported ancestry information over the last three generations. The sample characteristics have been described in more detailed previously (Tiwari et al. 2010). **Sample-A:** Charité University Medicine (Berlin, Germany) (DJ Müller, n=88). Patients were treated with antipsychotic medication and assessed for up to 6 weeks in this naturalistic study. The SCZ patients were treated with medications deemed most suitable, including fluphenazine, aripiprazole, quetiapine, ziprasidone, amisulpride, clozapine, olanzapine, haloperidol, and risperidone [for details see Tiwari et al., 2010]. **Sample-B:** Case Western Reserve University in Cleveland (Ohio, USA) (HY Meltzer, n=73). Schizophrenia patients were treated with clozapine. They then underwent a washout period of 2–4 weeks during which no medications were given unless deemed clinically essential by the physician. Serum levels were monitored to ascertain compliance [for details see Masellis et al, 1998 and Basile et al., 2002]. **Sample-C:** Hillside Hospital in Glen Oaks (New York, USA) (JA Lieberman, n=48). Patients diagnosed with schizophrenia had shown suboptimal response to previous atypical antipsychotic drug treatment. These patients were included in this 14-week double-blind study and were randomly assigned to receive clozapine, olanzapine, risperidone or haloperidol [for details Volavka et al., 2002 and Müller et al., 2005]. Briefly, patients were primarily recruited from three separate recruitment
sites, and patients were mostly treated with antipsychotic drugs that carry high risk for weight gain (see Table 1) varying across the sample sites for up to 14 weeks.

**Table 4-1.** Demographic characteristics from each of the clinical site

<table>
<thead>
<tr>
<th>CHARACTERISTICS</th>
<th>SAMPLE A (N=88)</th>
<th>SAMPLE B (N=73)</th>
<th>SAMPLE C (N=57)</th>
<th>TOTAL SAMPLE (N=218)</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>50</td>
<td>47</td>
<td>48</td>
<td>145</td>
<td>0.003</td>
</tr>
<tr>
<td>Female</td>
<td>38</td>
<td>26</td>
<td>9</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>35.78 ± 12.2</td>
<td>33.38 ± 8.6</td>
<td>40.47 ± 9.1</td>
<td>36.21 ± 10.6</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Initial body weight (kg)</strong></td>
<td>79.5 ± 15.9</td>
<td>74.8 ± 13.7</td>
<td>84.9 ± 17.4</td>
<td>79.26 ± 16.0</td>
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<td><strong>Weight change (kg)</strong></td>
<td>3.26 ± 3.9</td>
<td>3.81 ± 4.4</td>
<td>4.43 ± 6.4</td>
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<td><strong>Weight change (%)</strong></td>
<td>4.00 ± 4.8</td>
<td>5.42 ± 6.3</td>
<td>5.73 ± 8.4</td>
<td>4.93 ± 6.4</td>
<td>0.21</td>
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<tr>
<td><strong>Study duration (weeks)</strong></td>
<td>5.09 ± 1.6</td>
<td>6.00 ± 0.00</td>
<td>11.57 ± 3.9</td>
<td>7.07 ± 3.5</td>
<td>P &gt; 0.0001</td>
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<td><strong>Ethnicity</strong></td>
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<tr>
<td>European Americans</td>
<td>87</td>
<td>52</td>
<td>13</td>
<td>152</td>
<td>P &gt;0.0001</td>
</tr>
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<td>African Americans</td>
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<td>21</td>
<td>33</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Clozapine</td>
<td>12</td>
<td>73</td>
<td>12</td>
<td>97</td>
<td>P &gt;0.0001</td>
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<td>Haloperidol</td>
<td>5</td>
<td>0</td>
<td>11</td>
<td>16</td>
<td></td>
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<tr>
<td>Olanzapine</td>
<td>15</td>
<td>0</td>
<td>22</td>
<td>37</td>
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<tr>
<td>Risperidone</td>
<td>27</td>
<td>0</td>
<td>12</td>
<td>39</td>
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<tr>
<td>Others</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

a The samples were included from three different clinical sites
b Includes antipsychotic drugs: fluphenazine, aripiprazole, quetiapine, ziprasidone and amisulpride
4.3.2 Genotyping

Blood samples were collected from the clinical sites and sent to the Centre for Addiction and Mental Health (CAMH) in Toronto, Canada. Genomic DNA was extracted from white blood cells using the high-salt method (Lahiri and Nurnberger, 1991). The POMC (rs3754860, rs6713532, rs1047521) (see Figure 4-1a) and CART (rs10515115, rs3763153, rs3857384, rs11575893, rs16871471) (see Figure 4-2b) variants were genotyped using TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA). The AGRP SNP rs1338993 (see Figure 4-3c) was genotyped Illumina GoldenGate Assay (Illumina, San Diego, California, USA).

The POMC rs3754860 was genotyped using PCR restriction enzyme digestion and agarose gel electrophoresis. This SNP is present 1798bp 5’ to exon 1 (a C/T substitution), was genotyped using primers F: 5’-GGC AAC ATA GTG AAA CCC TGT C 3’ and R: 5’-TCC AAA TGG ACC CAA CTT 3’, annealing temperature 72 °C and 1.5 mmol MgCl2 and 2.5 mmol 10X Buffer w/ KCl. The PCR product (298 bp) was digested with the restriction enzyme RsaI (New England Biolabs). The PCR products with the C-allele were digested into two fragments of 190 and 108 bp, whereas the T-allele did not carry the Rsal restriction site and was not digested (298bp fragment). The digested products were resolved in a 2.5% ethidium bromide stained agarose gel and reference DNA samples of known genotypes were included in each run.
Figure 4.1. Schematic gene diagram of POMC, CART, and AGRP genes. The black filled box represents the coding region and the gray box represents the non-coding region. Standard color scheme has been implemented (Haploview 4.1). The two dark-shaded diamonds represent D=1 and LOD<2; the white diamond, D'<1 and LOD<2. The values in the box represent r².  

a) Proopiomelanocortin (POMC) (chr2p23) gene structure and linkage disequilibrium in European-ancestry patients. Location of polymorphisms with respect to the exons. The black filled box represents the coding region and the gray box represents the non-coding region. Standard color scheme has been implemented (Haploview 4.1). The two dark-shaded diamonds represent D=1 and LOD<2; the white diamond, D'<1 and LOD<2. The values in the box represent r².  

b) CART (5q13) gene structure and linkage disequilibrium in European ancestry patients.  

c) AGRP (16q22) gene structure.
Laboratory staff was blind to the clinical data. Ten percent of the total sample was randomly re-genotyped to confirm genotyping accuracy. Haploview 4.1 (Barrett et al. 2005) was used to select tagSNPs. All polymorphisms that had a minimum allele frequency more (MAF) than 5% and Hardy-Weinberg equilibrium (HWE) p > 0.001 in the CEPH population (Utah residents with ancestry from northern and western Europe) of the HapMap database (The International HapMap Consortium, 2007) were included for tag SNP selection.

4.4 Statistical Analysis

The statistical analyses were conducted using SPSS 15.0. Continuous variables were tested using analysis of variance (ANOVA) or co-variance (ANCOVA), and categorical variables were tested using $\chi^2$ tests. Our primary dependent measure was percentage (%) weight change from baseline, compared across genotypic groups using ANCOVA with duration of treatment as a covariate.

Haplotypes were constructed and weight change (%) was compared using UNPHASED 3.1 (Dudbridge 2003), and only haplotypes with frequencies greater than 5% were included in the analyses. Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium were determined using Haploview 4.1 (Barrett et al. 2005). Power calculations were carried out using Quanto 1.2.4. (Gauderman et al., 2006). Assuming an additive model, a minor allele frequency of 0.15 and a sample size of n=218, we had more than 80% power to detect a mean difference of 2.5%
between carriers and non-carriers of the risk genotype. The demographic characteristics of the study sample are presented in Table 1.

4.5 Results

Significant differences in clinical variables were observed among clinical sites; nonetheless, comparable rates of change in weight were observed (Table 1). All SNPs analyzed in this study were in Hardy–Weinberg equilibrium ($p > 0.05$; Table 3). Linkage disequilibrium was calculated using Haploview 4.1. (Barrett et al., 2005).

The duration of drug treatment significantly differed between clinical sites and was therefore entered a covariate in the association analyses. In the overall sample, the POMC rs3754860 SNP was associated with weight gain in patients of all races treated with all drugs ($p = 0.004$, Bonferroni corrected: $p = 0.036$). Subjects that were G-allele carriers, GG homozygotes (5.35% ± 6.8) and GA (5.04% ± 4.8) showed increased weight gain compared to the AA homozygotes (-2.00 ± 7.7) (Table 2). No significant observations were observed for CART and AGRP SNPs (Table 2).

Table 4-2. List of SNPs in POMC, CART & AGRP tested for association with AIWG at the genotypic level
<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype</th>
<th>Total Sample – All Patients on All Medications (%)*, n=218</th>
<th>p-valueb</th>
<th>Weight Change European ancestry-clozapine or olanzapine (%)*, n=87</th>
<th>p-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>POMC</td>
<td>rs3754860 (Rsal)</td>
<td>CC</td>
<td>5.35 ± 6.8 (118)</td>
<td>0.004</td>
<td>3.92 ± 5.9 (41)</td>
<td>0.139c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>5.04 ± 4.8 (66)</td>
<td></td>
<td>5.65 ± 5.0 (31)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>-2.00 ± 7.7 (9)</td>
<td></td>
<td>0.04 ± 1.28 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs6713532</td>
<td>TT</td>
<td>4.01 ± 5.9 (87)</td>
<td>0.224</td>
<td>4.08 ± 5.4 (46)</td>
<td>0.637</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TC</td>
<td>4.77 ± 6.0 (83)</td>
<td></td>
<td>4.88 ± 6.0 (35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>6.56 ± 8.2 (32)</td>
<td></td>
<td>2.61 ± 2.3 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs1042571 (C8246T)</td>
<td>GG</td>
<td>4.80 ± 6.4 (151)</td>
<td>0.413</td>
<td>4.50 ± 5.0 (58)</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>5.21 ± 6.2 (44)</td>
<td></td>
<td>5.10 ± 6.3 (21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>2.29 ± 7.2 (9)</td>
<td></td>
<td>-0.35 ± 6.3 (6)</td>
<td></td>
</tr>
<tr>
<td>CART</td>
<td>rs10515115 (-2815T&gt;A)</td>
<td>TT</td>
<td>3.88 ± 4.9 (42)</td>
<td>0.526</td>
<td>3.27 ± 4.9 (19)</td>
<td>0.659</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TA</td>
<td>5.08 ± 7.1 (98)</td>
<td></td>
<td>4.66 ± 5.8 (40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>4.99 ± 6.01 (68)</td>
<td></td>
<td>4.46 ± 5.4 (27)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs3763153 (-1474T&gt;C)</td>
<td>AA</td>
<td>4.92 ± 6.3 (100)</td>
<td>0.264</td>
<td>3.01 ± 4.8 (32)</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG</td>
<td>5.10 ± 6.1 (92)</td>
<td></td>
<td>5.42 ± 6.0 (43)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>1.92 ± 9.0 (11)</td>
<td></td>
<td>4.24 ± 5.6 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs3857384 (-1157C&gt;T)</td>
<td>AA</td>
<td>5.14 ± 7.3 (8)</td>
<td>0.294</td>
<td>-</td>
<td>0.718</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG</td>
<td>6.02 ± 6.8 (68)</td>
<td></td>
<td>4.04 ± 5.5 (22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>4.16 ± 6.0 (132)</td>
<td></td>
<td>4.45 ± 5.5 (64)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs11575893 (IVS1+172C&gt;T)</td>
<td>TT</td>
<td>5.41 ± 4.8 (6)</td>
<td>0.828</td>
<td>5.87 ± 9.2 (2)</td>
<td>0.787</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TC</td>
<td>5.05 ± 7.5 (43)</td>
<td></td>
<td>4.94 ± 3.8 (15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>4.68 ± 6.1 (164)</td>
<td></td>
<td>4.17 ± 5.7 (69)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs16871471</td>
<td>TT</td>
<td>5.32 ± 7.0 (9)</td>
<td>0.928</td>
<td>4.50 ± 4.2 (25)</td>
<td>0.718</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TA</td>
<td>5.03 ± 6.4 (59)</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>4.82 ± 6.5 (131)</td>
<td></td>
<td>4.21 ± 6.0 (58)</td>
<td></td>
</tr>
<tr>
<td>AGRP</td>
<td>rs1338993</td>
<td>AA</td>
<td>4.13 ± 5.9 (146)</td>
<td>0.152</td>
<td>4.46 ± 5.8 (70)</td>
<td>0.716</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>5.98 ± 6.7 (49)</td>
<td></td>
<td>5.06 ± 5.1 (13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>7.52 ± 8.1 (22)</td>
<td></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± s.d. (number of individuals)

b SNPs with low numbers in a genotypic group were merged with heterozygous genotypic group

c For AA versus GA+GG: All P-values were calculated by ANCOVA. rs3754860, European Americans on olanzapine or clozapine: AA versus GA+GG, 0.03 ± 1.1 versus 4.54 ± 5.6 (P = 0.097).

Our overall study sample consisted of patients of two primary and distinct ethnicities (European and African ancestry). The patients were all also treated with a variety of different antipsychotics. Due to these differences in the overall sample consisting of 218 subjects, further sub-analyses were carried out.
Significant differences in the amount of AIWG between Africans and Europeans were observed in our sample (Table 1). In addition, the patients of African American ancestry (n = 55) exhibited different minor allele frequencies (data not shown) than the Europeans, and were therefore excluded from any further analyses to avoid risk for spurious findings.

**Refined Sub-Sample Analysis**

Both clozapine and olanzapine have been established as antipsychotics being associated with the higher propensity for weight gain and have similar pharmacological profiles (Nasrallah 2008). Thus we conducted a refined sub-analysis that incorporated patients by their self-reported ethnicities (European-ancestry vs. African-American ancestry) and whether these patients received either clozapine or olanzapine.

In our refined sample of European-ancestry patients treated with either olanzapine or clozapine (n=87), the POMC rs3754860 SNP reached a trend for association with weight gain for AA homozygotes (0.03% ± 1.1) vs. G-allele carriers, GA+GG (4.54% ± 5.6) (p = 0.097); (Table 2). The POMC SNP rs1042571 reached a trend for association with change in weight gain percentage in patients of European ancestry, treated with either olanzapine or clozapine (p = 0.089). The GG (4.50% ± 5.0) and GA (5.10% ± 6.3) genotypic groups gained more weight than AA homozygotes (-0.35% ± 6.3). No allelic associations were observed in our sample of patients of European ancestry, treated with either clozapine or olanzapine (Table 4-3).
### Table 4-3. Allelic differences in weight change (%) in patients of European ancestry

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>HapMAP CEU</th>
<th>European-ancestry-all drugs</th>
<th>European ancestry-clozapine or olanzapine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Allele Frequency</td>
<td>p-Value</td>
</tr>
<tr>
<td>POMC</td>
<td>rs3754860</td>
<td>C</td>
<td>0.63</td>
<td>0.71 (193)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>0.33</td>
<td>0.29 (75)</td>
</tr>
<tr>
<td></td>
<td>rs6713532</td>
<td>T</td>
<td>0.75</td>
<td>0.72 (210)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>0.23</td>
<td>0.28 (82)</td>
</tr>
<tr>
<td></td>
<td>rs1042571</td>
<td>G</td>
<td>0.76</td>
<td>0.82 (245)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>0.24</td>
<td>0.17 (51)</td>
</tr>
<tr>
<td>CART</td>
<td>rs10515115</td>
<td>A</td>
<td>0.59</td>
<td>0.50 (158)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>0.41</td>
<td>0.50 (140)</td>
</tr>
<tr>
<td></td>
<td>rs3763153</td>
<td>A</td>
<td>0.66</td>
<td>0.68 (198)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>0.34</td>
<td>0.32 (92)</td>
</tr>
<tr>
<td></td>
<td>rs3857384</td>
<td>G</td>
<td>0.93</td>
<td>0.86 (258)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>0.07</td>
<td>0.14 (42)</td>
</tr>
<tr>
<td></td>
<td>rs11575893</td>
<td>C</td>
<td>0.89</td>
<td>0.89 (268)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>0.11</td>
<td>0.11 (32)</td>
</tr>
<tr>
<td></td>
<td>rs16871471</td>
<td>A</td>
<td>0.76</td>
<td>0.92 (231)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>0.24</td>
<td>0.18 (49)</td>
</tr>
<tr>
<td>AGRP</td>
<td>rs1338993</td>
<td>A</td>
<td>N/A*</td>
<td>0.93 (211)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>N/A*</td>
<td>0.07 (7)</td>
</tr>
</tbody>
</table>

*Data not available in hapmap

### 4.6 Discussion

In this study, we investigated the role of three candidate genes, pro-opiomelanocortin (POMC), cocaine amphetamine regulated transcript (CART), and agouti related protein (AGRP) in AIWG.
We included SNPs that were previously associated with weight measures across various ethnic populations, and utilized tagSNP to capture meaningful coverage of these genes.

We found a significant association between the POMC SNP rs3754860 and percentage change in weight in our overall sample ($p_{corrected} = 0.036$). This signal became non-significant in our refined sample of European-Ancestry patients treated with clozapine and olanzapine. A previous report on this gene variant showed no association between this SNP in BMI and diabetes mellitus in patients of Caucasian origin with chronic heart failure (Bienertova-Vasku et al. 2009). A study conducted by (Baker et al. 2005) also reported no association between the POMC rs3754860 SNP and waist-to-hip ratio in a family study comprised of over 1000 individuals.

The POMC rs1042571 SNP reached a trend for association with AIWG in patients of European ancestry treated with clozapine or olanzapine. No allelic association was detected in this sub-sample. The POMC rs1042571 has also been associated with measures including BMI, waist, visceral adipose tissue, and subcutaneous adipose tissue in obese patients of Hispanic descent (Sutton et al. 2005) and more recently, was associated with an obesity phenotype: the ratio between fat and protein intake in Dutch males (Ternouth et al. 2011).

Despite the biological evidence supporting a role for both the CART and AGRP genes in weight regulation (Cheung & Mao et al., 2012), we did not observe any significant associations with polymorphisms of these genes in the total sample set. To the best of our knowledge, these genes and their impact on AIWG have not yet been thoroughly investigated in pharmacogenetic studies. Plasma values of total cholesterol, LDL-cholesterol, and HDL-cholesterol were
associated with Delta A1457 CART variant in Chinese diabetic subjects (Fu et al. 2002) but not in Caucasians (Challis et al. 2000); (Vasseur et al. 2007).

Some limitations of this study should be considered. These include a relatively small sample size, heterogeneity of the sample to differing ethnicities, and different medication treatments between sub-samples. We did not observe any significant association in sub-groups of patients of European ancestry who received clozapine or olanzapine. Our findings with the SNP rs3754860 suggest that the heterogeneity and population stratification in the overall sample may be contributing to our initial positive association in the total sample.

In summary, we demonstrated a potential influence of a POMC genetic variation in AIWG. However, we could not detect a positive association between AGRP and CART SNPs in AIWG. Nonetheless, our finding, in combination with recent reports of the melanocortin system (Chowdhury et al., 2012; Tiwari et al., 2012), underline the importance of the role of this system in antipsychotic induced weight gain and deserves further investigation in larger sample sets.
Chapter 5

An Exploratory Investigation of Melanocortin-3 Receptor Gene Variants in Antipsychotic-Induced Weight Gain

This manuscript is in preparation

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5.1 Abstract

Second generation antipsychotics treat core symptoms of schizophrenia (SCZ) and other disorders. However, the use of this class of medications may result in the development of severe side effects such as substantial weight gain and metabolic syndrome. Early diagnosis of metabolic disturbances through genetic testing could reduce the morbidity and mortality of patients. Based on the melanocortin-3 receptor (MC3R) gene’s role in the regulation of weight and food intake, we investigated the potential role of MC3R single nucleotide polymorphisms
(SNPs) in antipsychotic induced weight gain (AIWG). Ten MC3R SNPs (rs6127698, rs6024731, rs1543570, rs6024730, rs6014649, rs1926064, rs6014646, rs11697509, rs3827103) were selected for coverage of common variation in the gene using tagSNP software. The SNPs were genotyped with the Illumina GoldenGate Genotyping Assays in 266 chronic SCZ patients who underwent treatment with various antipsychotics and were evaluated for weight gain for up to 14 weeks. We compared weight change (%) across genotype groups using analysis of covariance. In this study, we observed that the MC3R gene was associated with AIWG. Specifically, genotypic associations were found between the correlated MC3R polymorphisms in our total sample set. Genotypic associations were also found between MC3R SNPs (rs6014649, rs11697509, rs3746619, rs3827103, rs1543570) and weight gain (p<0.05) in a refined sub-sample consisting of European ancestry patients treated with either olanzapine or clozapine (n=104). This finding requires validation in larger sample sets. However, the potential role of MC3R in AIWG may further provide understanding toward the genetic etiology of AIWG, which could in turn lead to greatly novel drug target development for obesity or psychiatric medications.
5.2 Introduction

Between 40-80% of schizophrenia (SCZ) patients treated with second-generation antipsychotic medications experience substantial increase in body weight, or antipsychotic induced weight gain (AIWG) (Umbricht et al, 2001; Green et al., 2000). Second-generation antipsychotic medications – namely clozapine and olanzapine - are associated with the most dramatic weight increases in patients (Allison et al., 1999; Lett et al., 2012). Despite the effectiveness of second-generation antipsychotics, AIWG and associated metabolic side effects greatly impair patient quality of life and may lead to conditions such as type 2 diabetes and cardiovascular diseases (Rummel-Kluge et al., 2010; Correll et al., 2011). Various neural systems regulating appetite, satiety and lipid metabolism have been implicated in the etiology of AIWG, but a clear mechanism by which this side effect develops remains largely unknown (Roerig et al., 2011). Further understanding of AIWG and predicting this side effect could lead to improved treatment in psychiatric patients.

The onset of AIWG is highly variable between patients, and a genetic component has been cited as among one of the leading factors of susceptibility to develop this side effect (Gebhardt et al., 2010). The antipsychotic action at the serotonergic (Reynolds et al., 2002; Reynolds et al., 2006), and histaminergic (Kroeze et al., 2003) systems has been reported to be associated with AIWG, though no causal mechanism has been verified. Adding to the complexity of AIWG is that the pharmacological action of antipsychotic drugs is complex, as various antipsychotics have differing receptor-binding profiles, which may have downstream effects on weight and metabolism (Nasrallah et al., 2008).
The Melanocortin System

The melanocortin receptors are encoded by a gene family (MC1R-MC5R) and have preferential binding to separate ligands, resulting in different functions. The biological roles of the melanocortin receptors have been well described in their capacity to regulate melanogenesis (MC1R), (Sulem et al., 2007) mediate cortisol levels via interaction with the adrenocorticotropic hormone (MC2R) (Schiöth et al., 2005), and have been implicated in obesity (MC3R-MC5R). The MC3R gene has been reported to have mixed associations with high body mass index and weight measures in the literature, while MC4R mutations have been reported to cause monogenic obesity (Huszar et al., 1997; Farooqi et al., 2003). The MC5R gene has been implicated in lipid metabolism (Sánchez et al., 2009; Rodrigues et al., 2013) but its function in weight regulation is not known.

In the context of AIWG, a recent genome wide association study yielded a highly significant association between the MC4R rs489693 in AIWG, and this association was subsequently replicated three distinct samples (Malhotra et al., 2012) Additionally, an MC4R SNP (rs8087522) located in the promoter region of the gene, was associated with clozapine induced weight gain (Chowdhury et al., 2013). A study conducted by Moons et al., (2011) examined the MC3R Thr6Lys (rs3746619) in olanzapine induced weight gain and reported no significant association. Nonetheless, the MC3R gene is an important candidate to examine in terms of a contributory role to the development of AIWG. The double-variant combination of MC3R Thr6Lys (rs3746619) and Val81Ile (rs3827103), located in the 5’UTR, have been reported to be associated with higher body weight measures such as body mass index (BMI), and body fat mass in obese pediatric patients. (Feng et al., 2005). The two mis-sense MC3R variants have been associated with decreased cyclic AMP generation in vitro (Feng et al., 2005).
One shortcoming of previous *MC3R* gene association studies is the limited coverage of the *MC3R* gene. In this study, we captured 100% of the common variation of the *MC3R* gene in order to comprehensively investigate the role of this gene in AIWG.

### 5.3 Methods

#### 5.3.1 Samples

Our samples have been described in detail in other studies (Masellis et al., 1998; Volavka et al., 2002; Tiwari et al., 2010). Briefly, patients with schizophrenia or schizoaffective disorder according to DSM-III-R or DSM-IV criteria were recruited at three different sites: Patients in Sample A: (N=88; DJM; Charité University Medicine, Berlin, Germany) received various antipsychotics in a naturalistic study design (Müller et al., 2012) Sample B: (N=74; HYM; Case Western Reserve University, Cleveland, Ohio, USA) consisted of patients who were treated with clozapine (first exposure to a second generation antipsychotic) for six weeks (Kane et al., 1988; Masellis et al., 1998). In Sample C: (N=54; JAL; Hillside Hospital in Glen Oaks, New York, USA), patients were treated with clozapine, risperidone, olanzapine or haloperidol in a double-blind study design (Volavka et al., 2002). For Sample D: (N=48, DJM2, Centre for Addiction and Mental Health, Toronto, ON), patients were prescribed antipsychotic medication and followed up for a minimum of 6 weeks (Goncalves et al., 2013). All ancestry information across the four samples was self-reported. All subjects gave written informed consent prior to study entry in accordance with institutional ethics guidelines and the Declaration of Helsinki.
Due to the heterogeneity among the samples in terms of ethnicity and medication, we selected patients of European ancestry treated with high-risk medication for AIWG (clozapine, olanzapine) to create a more homogeneous subset (N=104). The baseline weight and duration of treatment significantly influenced the amount of weight gain during the study (see Table 5-1), and therefore we used these as covariates in our analyses. Details on all samples are provided in Table 5-1.

### 5.3.2 Genotyping

DNA extraction was performed with a standard high-salt method (Lahiri & Nurnberger et al., 1991). The MC3R SNP genotyping was conducted using customized GoldenGate Genotyping Assays (Illumina Inc, San Diego, CA, USA). The MC3R SNPs were selected from information available in the literature (Feng et al., 2005; Moons et al., 2011; Santos et al., 2011), and tag SNPs from the European ancestry population: CEU, in the HapMap database ($r^2 \geq 0.8$, minor allele frequency $\geq 0.05$; Haploview 4.2, Barrett et al., 2005). Ten MC3R SNPs within and near the MC3R gene (rs6014646, rs6024730, rs6024731, rs6014649, rs11697509, rs6127698, rs3746619, rs3827103, rs1926064, rs1543570) were genotyped in the DNA samples. Samples with less than 95% successful genotyping were excluded from our study. To ensure genotyping accuracy, 10% of samples were randomly re-genotyped, and the concordance rate was 100%.

### 5.3.3. Statistical Analysis

Categorical variables were analyzed with Pearson’s $\chi^2$, and continuous variables with analysis of covariance (ANCOVA) using percentage (%) weight change as outcome and baseline weight
(kg) and treatment duration (weeks) as covariates. The *Statistical Package for the Social Sciences* (SPSS), version 15.0, was used for our genetic association tests. Hardy-Weinberg-equilibrium, linkage disequilibrium and haplotype associations were calculated using Haplovew Version 4.2. The change in weight change percentage (%) was compared across haplotypes using in UNPHASED version 3.1.4. Correction for multiple testing was carried out by estimating the number of independent tests using single nucleotide polymorphism spectral decomposition (SNPSpD) (Nyholt, 2004; Li & Ji, 2005).

### 5.4 Results

Demographic and clinical characteristics of the samples are shown in Table 5-1. Weight gain was significantly correlated with treatment duration (p< 0.0001), and with baseline weight (p=0.005). We observed significant differences across different variables, including baseline weight, age, ethnicity, sex and study medication across the samples. Weight change (%) from baseline weight did not differ significantly among samples (Table 5-2). Joint statistical analysis of weight change was conducted for the combined samples, and confounding factors were factored into the analyses. In our total sample (N = 266), eight correlated SNPS near the *MC3R* gene were associated with AIWG (Figure 5-1).

<p>| Table 5-1. Demographic Characteristics |</p>
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sample A (n=88) a</th>
<th>Sample B (n=73) a</th>
<th>Sample C (n=57) a</th>
<th>Sample D (n=48) a</th>
<th>Total Sample (n=266)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>Male</td>
<td>50 (56.8%)</td>
<td>47 (64.3%)</td>
<td>48 (84.2%)</td>
<td>32 (66.7%)</td>
<td>177 (66.5%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>38 (43.1%)</td>
<td>26 (35.6%)</td>
<td>9 (15.8%)</td>
<td>17 (35.4%)</td>
<td>90 (33.8%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.78 ± 12.2</td>
<td>33.38 ± 8.6</td>
<td>40.47 ± 9.1</td>
<td>37.9 ± 12.4</td>
<td>36.52 ± 11.0</td>
<td>0.002</td>
</tr>
<tr>
<td>Initial body weight (kg)</td>
<td>79.5 ± 15.9</td>
<td>74.8 ± 13.7</td>
<td>84.9 ± 17.4</td>
<td>81.8 ± 19.4</td>
<td>79.7 ± 16.7</td>
<td>0.005</td>
</tr>
<tr>
<td>Weight change (kg)</td>
<td>3.26 ± 3.9</td>
<td>3.81 ± 4.4</td>
<td>4.43 ± 6.4</td>
<td>3.89 ± 7.1</td>
<td>3.77 ± 5.3</td>
<td>0.261</td>
</tr>
<tr>
<td>Weight change (%)</td>
<td>4.00 ± 4.8</td>
<td>5.42 ± 6.3</td>
<td>5.73 ± 8.4</td>
<td>3.89 ± 7.1</td>
<td>4.74 ± 6.5</td>
<td>0.649</td>
</tr>
<tr>
<td>Study Duration (weeks)</td>
<td>5.09 ± 1.6</td>
<td>6.00 ± 0.0</td>
<td>11.57 ± 3.9</td>
<td>11.20 ± 5.2</td>
<td>7.79 ± 4.1</td>
<td>p &lt;0.001</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>European Americans</td>
<td>87 (98.9%)</td>
<td>52 (71.2%)</td>
<td>13 (22.8%)</td>
<td>26 (54.1%)</td>
<td>178 (66.9%)</td>
<td></td>
</tr>
<tr>
<td>African Americans</td>
<td>1 (0.01%)</td>
<td>21 (28.8%)</td>
<td>33 (57.9%)</td>
<td>2 (0.04%)</td>
<td>57 (21.4%)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>0</td>
<td>11 (19.2%)</td>
<td>1 (0.02%)</td>
<td>29 (0.11%)</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p &lt;0.001</td>
</tr>
<tr>
<td>Clozapine</td>
<td>12 (13.6%)</td>
<td>73 (100%)</td>
<td>12 (21.1%)</td>
<td>27 (56.3%)</td>
<td>124 (46.6%)</td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td>5 (0.056%)</td>
<td>0</td>
<td>11 (19.3%)</td>
<td>0</td>
<td>16 (0.06%)</td>
<td></td>
</tr>
<tr>
<td>Olanzapine</td>
<td>15 (17.4%)</td>
<td>0</td>
<td>22 (38.6%)</td>
<td>8 (16.7%)</td>
<td>45 (16.9%)</td>
<td></td>
</tr>
<tr>
<td>Risperidone</td>
<td>27 (30.6%)</td>
<td>0</td>
<td>12 (21.1%)</td>
<td>5 (10.4%)</td>
<td>44 (16.5%)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>28 (31.8%)</td>
<td>0</td>
<td>0</td>
<td>9 (18.8%)</td>
<td>37 (13.9%)</td>
<td></td>
</tr>
</tbody>
</table>

*Samples were included from four different clinical sites.

*b Includes antipsychotic drugs: fluphenazine, aripiprazole, quetiapine, ziprasidone and amisulpride.
b) The linkage disequilibrium structure of the MC3R SNPs is displayed, with the numbers representing \( r^2 \) values, and the intensity of red relating to the \( r^2 \)-squared values between SNP pairs.

**Figure 5-1.** a) Schematic diagram of the MC3R gene with the nine single nucleotide polymorphisms we examined for weight percentage change in psychiatric patients treated with either clozapine or olanzapine.

b) The linkage disequilibrium structure of the MC3R SNPs is displayed, with the numbers representing \( r^2 \) values, and the intensity of red relating to the \( r^2 \)-squared values between SNP pairs.
The allele frequency of rs1926064 SNP was low (p = 0.034) in our samples and was not analyzed further. Of the remaining nine MC3R SNPs, eight (rs6014646, rs6024730, rs6024731, rs6014649, rs11697509, rs3746619, rs3827103 rs1543570) were associated with AIWG in our overall sample (see Table 5-2). To account for differing ethnic backgrounds in the patient population, we ran analyses on a sub-sample consisting of patients of European ancestry treated with olanzapine or clozapine (see Table 5-3).

Table 5-2. Genotypic associations between MC3R and Antipsychotic Induced Weight in Overall Sample

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>N = 260</th>
<th>Weight-gain% a</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6014646</td>
<td>AA</td>
<td>92</td>
<td>3.51 ± 6.8</td>
<td>0.007</td>
</tr>
<tr>
<td>rs6014646</td>
<td>AT</td>
<td>123</td>
<td>4.50 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>rs6014646</td>
<td>TT</td>
<td>57</td>
<td>7.57 ± 8.2</td>
<td></td>
</tr>
<tr>
<td>rs6024730</td>
<td>GG</td>
<td>141</td>
<td>3.76 ± 6.1</td>
<td>0.001</td>
</tr>
<tr>
<td>rs6024730</td>
<td>AG</td>
<td>103</td>
<td>5.09 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>rs6024730</td>
<td>AA</td>
<td>17</td>
<td>10.18 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>rs6024731</td>
<td>AA</td>
<td>114</td>
<td>3.77 ± 6.4</td>
<td>0.001</td>
</tr>
<tr>
<td>rs6024731</td>
<td>AG</td>
<td>115</td>
<td>4.87 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>rs6024731</td>
<td>GG</td>
<td>26</td>
<td>9.10 ± 9.1</td>
<td></td>
</tr>
<tr>
<td>rs6014649</td>
<td>GG</td>
<td>189</td>
<td>3.64 ± 5.8</td>
<td>8.66E-06</td>
</tr>
<tr>
<td>rs6014649</td>
<td>AG</td>
<td>64</td>
<td>6.77 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>rs6014649</td>
<td>AA</td>
<td>9</td>
<td>12.86 ± 9.4</td>
<td></td>
</tr>
<tr>
<td>rs11697509</td>
<td>CC</td>
<td>167</td>
<td>3.58 ± 5.9</td>
<td>3.79E-05</td>
</tr>
<tr>
<td>rs11697509</td>
<td>CG</td>
<td>83</td>
<td>5.63 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>rs11697509</td>
<td>GG</td>
<td>12</td>
<td>12.64 ± 8.7</td>
<td></td>
</tr>
<tr>
<td>rs6127698</td>
<td>TT</td>
<td>66</td>
<td>3.78 ± 6.9</td>
<td>0.175</td>
</tr>
<tr>
<td>rs6127698</td>
<td>TG</td>
<td>116</td>
<td>4.51 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>rs6127698</td>
<td>GG</td>
<td>81</td>
<td>5.75 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>rs3746619</td>
<td>CC</td>
<td>173</td>
<td>3.66 ± 5.9</td>
<td>6.75E-05</td>
</tr>
<tr>
<td>rs3746619</td>
<td>AC</td>
<td>77</td>
<td>6.08 ± 6.6</td>
<td></td>
</tr>
<tr>
<td>rs3746619</td>
<td>AA</td>
<td>11</td>
<td>12.03 ± 8.8</td>
<td></td>
</tr>
<tr>
<td>rs3827103</td>
<td>GG</td>
<td>178</td>
<td>3.77 ± 5.9</td>
<td>1.37E-04</td>
</tr>
<tr>
<td>rs3827103</td>
<td>AG</td>
<td>73</td>
<td>5.93 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>rs3827103</td>
<td>AA</td>
<td>11</td>
<td>12.03 ± 8.8</td>
<td></td>
</tr>
<tr>
<td>rs1543570</td>
<td>CC</td>
<td>153</td>
<td>3.53 ± 6.0</td>
<td>3.00E-03</td>
</tr>
<tr>
<td>rs1543570</td>
<td>AC</td>
<td>82</td>
<td>5.85 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>rs1543570</td>
<td>AA</td>
<td>27</td>
<td>7.97 ± 8.2</td>
<td></td>
</tr>
</tbody>
</table>

a. Mean ± S.D. (number of individuals)
The *MC3R* SNPs rs6014646, rs6024730, rs6024731, rs6014649, rs11697509, rs3746619, rs3827103, & rs1543570 were associated with AIWG in our sub-sample consisting of patients of European ancestry. The rs6014649, rs3746619, rs11697509, rs3827103, and rs1543570 SNPs consisted of genotypic groups with n = 3 or lower (see Table 5-3). Thus, the group sizes for the minor allele homozygotes consisted of low numbers, and the homozygote groups were merged with heterozygote groups to examine dominant models. Of interest, the two highly linked SNPs in the literature, rs3746619 & rs3827103, were also associated with AIWG in our samples, among additional SNPs (see Table 5-3).

The total number of independent tests according to the SNPSpD method was four. After correction for multiple testing (α = 0.0125), five *MC3R* SNPs remained significant (rs6014649, rs3746619, rs11697509, rs3827103, rs1543570). In our allelic analyses in the European sub-sample treated with clozapine and olanzapine, eight SNPs (rs6014646, rs6024730, rs6024731, rs6014649, rs11697509, rs6127698, rs3746619, rs3827103, rs1543570) had significant allelic associations with AIWG. The allelic associations are listed within Table 5-4. The rs6127698 SNP had no significant allelic association with AIWG.
Table 5-3. Genotypic associations between $MC3R$ and Antipsychotic Induced Weight in European Patients treated with antipsychotic medications

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>N = 104</th>
<th>Weight-gain%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6014646</td>
<td>AA</td>
<td>45</td>
<td>2.65±6.0</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>46</td>
<td>4.40±4.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>12</td>
<td>7.7±7.5</td>
<td></td>
</tr>
<tr>
<td>rs6024730</td>
<td>GG</td>
<td>61</td>
<td>3.06±5.6</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>37</td>
<td>4.97±5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>5</td>
<td>8.28±6.5</td>
<td></td>
</tr>
<tr>
<td>rs6024731</td>
<td>AA</td>
<td>55</td>
<td>2.84±5.8</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>39</td>
<td>5.00±4.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>8</td>
<td>7.62±8.3</td>
<td></td>
</tr>
<tr>
<td>rs6014649</td>
<td>GG</td>
<td>84</td>
<td>3.14±5.4</td>
<td>0.003b</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>18</td>
<td>7.21±5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>2</td>
<td>12.03±6.1</td>
<td></td>
</tr>
<tr>
<td>rs11697509</td>
<td>CC</td>
<td>78</td>
<td>3.06±5.4</td>
<td>0.000464b</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>22</td>
<td>5.91±5.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>2</td>
<td>14.5±6.0</td>
<td></td>
</tr>
<tr>
<td>rs6127698</td>
<td>TT</td>
<td>28</td>
<td>3.07±5.1</td>
<td>0.429</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>49</td>
<td>4.13±5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>21</td>
<td>5.18±6.6</td>
<td></td>
</tr>
<tr>
<td>rs3746619</td>
<td>CC</td>
<td>81</td>
<td>3.07±5.5</td>
<td>0.006b</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>21</td>
<td>6.47±5.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>2</td>
<td>12.03±6.1</td>
<td></td>
</tr>
<tr>
<td>rs3827103</td>
<td>GG</td>
<td>81</td>
<td>3.18±5.5</td>
<td>0.007b</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>21</td>
<td>6.47±6.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>2</td>
<td>12.03±6.1</td>
<td></td>
</tr>
<tr>
<td>rs1543570</td>
<td>CC</td>
<td>73</td>
<td>3.11±5.6</td>
<td>0.007b</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>28</td>
<td>5.50±5.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>3</td>
<td>12.15±4.3</td>
<td></td>
</tr>
</tbody>
</table>

a. Mean ± S.D. (number of individuals).

b. SNPs with low numbers in a genotypic group were merged with the heterozygous genotypic group. All p-values were calculated using ANCOVA. All p-values corrected with Nyholt testing. rs6014649: AA+AG (7.68% ± 5.7) vs. GG (3.01% ± 5.5), $p = 0.004$; rs3746619: AA+AC (6.96% ± 5.7) vs. CC (3.04% ± 5.5), $p = 0.016$; rs11697509: GG+CG (6.94% ± 6.0) vs. CC (2.94% ± 5.4), $p = 0.008$; rs3827103, AA+AG (6.96% ± 5.7) vs. GG (3.04% ± 5.5), $p = 0.016$; rs1543570, the 6.14% ± 5.4) vs. GG (2.96% ± 5.7), $p = 0.044$
**Table 5-4.** Allelic differences in weight change (%) in patients of European ancestry

<table>
<thead>
<tr>
<th>MC3R Polymorphism</th>
<th>Allele</th>
<th>European Ancestry All Medications</th>
<th>P-value</th>
<th>European Ancestry Olanzapine/clozapine</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6014646</td>
<td>A</td>
<td>0.667</td>
<td>0.0293</td>
<td>0.668</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.333</td>
<td></td>
<td>0.332</td>
<td></td>
</tr>
<tr>
<td>rs6024730</td>
<td>G</td>
<td>0.776</td>
<td>0.0649</td>
<td>0.778</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.224</td>
<td></td>
<td>0.222</td>
<td></td>
</tr>
<tr>
<td>rs6024731</td>
<td>G</td>
<td>0.725</td>
<td>0.0469</td>
<td>0.737</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.275</td>
<td></td>
<td>0.263</td>
<td></td>
</tr>
<tr>
<td>rs6014649</td>
<td>G</td>
<td>0.905</td>
<td>0.00373</td>
<td>0.898</td>
<td>0.00049</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.095</td>
<td></td>
<td>0.102</td>
<td></td>
</tr>
<tr>
<td>rs11697509</td>
<td>C</td>
<td>0.87</td>
<td>0.00341</td>
<td>0.866</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.130</td>
<td></td>
<td>0.134</td>
<td></td>
</tr>
<tr>
<td>rs6127698</td>
<td>T</td>
<td>0.548</td>
<td>0.201</td>
<td>0.566</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.452</td>
<td></td>
<td>0.434</td>
<td></td>
</tr>
<tr>
<td>rs3746619</td>
<td>C</td>
<td>0.895</td>
<td>0.01</td>
<td>0.883</td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.105</td>
<td></td>
<td>0.117</td>
<td></td>
</tr>
<tr>
<td>rs3827103</td>
<td>G</td>
<td>0.895</td>
<td>0.01</td>
<td>0.883</td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.105</td>
<td></td>
<td>0.117</td>
<td></td>
</tr>
<tr>
<td>rs1543570</td>
<td>C</td>
<td>0.861</td>
<td>0.032</td>
<td>0.84</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.139</td>
<td></td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

European panel; CEU: Utah (USA) residents with northern and western European ancestry by the Centre d'Etude du PolymorphismeHumain (CEPH) (http://hapmap.ncbi.nlm.nih.gov/).

**Power Analysis**

The power analysis for this study was conducted using Quanto 1.2.4. Our study had more than 80% power to detect a difference of 3.6% between risk-genotype carriers and non-carriers in our sample of patients in an additive model, assuming a minor allele frequency of 0.05. The variance explained for this phenotype was 2.91%.
Haplotype Analysis

Haplotype analyses were conducted using UNPHASED 3.1.4. Our haplotype analyses were conducted by using the marker distribution in our haplotype block (see Figure 5-1). We determined the estimated additive values (EAV) for the haplotypes. The EAV indicates the means of quantitative variable (weight change %) for a haplotype relative to other haplotypes within a haplotype window. All of the statistical analyses in this study were based on alpha < 0.05 as nominally significant. The first haplotype assessed in AIWG was the rs6014646-rs6024730-rs6024731-rs6014649 T-A-A-A was associated with AIWG (p = 0.004893, EAV T-A-A-A = 0.1328, C.I.: 0.036 - 0.230). The second tested haplotype was rs3746619-rs3827103-rs1543570 A-A-A, and was significantly associated with AIWG (p = 0.01777, EAV A-A-A = 0.1059 , C.I.: 0.0158 - 0.196). Given the exploratory nature of our analysis, we did not correct the haplotypes for multiple testing.

Gene-Gene Interaction Analysis

Gene-gene interaction analyses were performed between *MC3R* and *MC4R* SNPs utilizing stepwise linear regression from the mbmdr software. For the purposes of comprehensiveness, all *MC3R* SNPs from this study (rs6014646, rs6024730, rs6024731, rs6014649, rs11697509, rs6127698, rs3746619, rs3827103, rs1926064, rs1543570) were assessed with top *MC4R* SNPs associated with AIWG in the study conducted by Malhotra et al., (2012), (rs12967878,
rs12970134, rs2045439, rs489693, rs646749, rs6567160), and a recent study conducted by our own group (rs8087522, rs11872992, rs17782313) (Chowdhury et al., 2012).

The significance was determined using a permutation test with 10,000 simulated replicates (Calle et al., 2010). The gene-gene interaction analyses resulted in no significant interaction between any \textit{MC3R} and \textit{MC4R} SNPs.

\section{5.5 Discussion}

We investigated the association between common MC3R polymorphisms and antipsychotic induced weight gain (AIWG). The melanocortin receptors system has biological functions in energy homeostasis, pigmentation, and glucorticoid mediation. Several common MC4R gene variants as well as mutations have been reported to be associated with weight gain and development of obesity in non-psychiatric populations. However, MC3R gene variation had not yet been broadly examined in AIWG.

Our current study found that eight of the nine tested MC3R SNPs are associated with AIWG. The rs6024730, rs6024731, rs6014649, rs11697509, rs6127698, rs3746619, rs3827103 SNPs were associated with AIWG in the overall sample. However, our total sample (N=266) is comprised of various ethnicities. Therefore, we examined a sub-sample comprising of patients of European-ancestry, treated with either clozapine or olanzapine (N=104). Five SNPs remained associated with AIWG under a dominant model.

Two functional \textit{MC3R} SNPs, 6Thr>Val (rs3746619) and 81Val>Ile (rs3827103), have been reported to be associated with obesity across different populations (Feng et al., 2005) and are
associated with a reduction in cAMP signaling \textit{in vitro} (Feng et al., 2005). However, a recent study by Cieslak et al. (2013), reported that MC3R 81Ile allele was not associated with obesity in a Polish population. Four non-synonymous coding variants (82S, N128S, L249F, and R257S) were identified in a sample of Obese Belgian sample (Zegers et al., 2013). These mutations have been reported to be associated with obesity in previous studies as well (Zegers et al., 2011; Calton et al., 2009; Mencarelli et al., 2011). The MC3R 6Lys–81Ile haplotype has also been associated with substrate oxidation in response to moderate exercise in obese children (Obregón et al., 2011). In a study conducted by Moons et al. (2011), the \textit{MC3R} 6Thr>Val SNP was not associated with olanzapine induced weight gain in SCZ patients. In our study, we found that the two non-synonymous polymorphisms, 6Thr>Val (rs3746619) and 81Val>Ile (rs3827103), were associated with AIWG in our sample. The sample described in Moons et al. (2011) consisted of male schizophrenia patients. Our sample was comprised of both male and female patients, and the sample was assessed for weight gain for up to six weeks. Thus, there are notable differences between the patients described in Moons et al. (2011), and the population described in the current study.

A recent study conducted by Santos et al. (2011) reported that the \textit{MC3R} rs6014646 ‘T’-allele was nominally associated with weight loss in a clinical trial consisting of an obese adult population. The association between the \textit{MC3R} rs6014646 SNP and AIWG did not survive correction for multiple testing. In addition, the \textit{MC3R} rs6014646-rs6024731, rs6024731-rs6014649, rs6014649-rs11697509, rs11697509-rs6127698, rs6127698-rs1543570 haplotypes achieved statistically or near-statistically significant results in relation to weight loss (p > 0.05).
However, given the high correlation between the $MC3R$ SNPs in this study (Figure 5-1), the haplotype results must be interpreted with caution and require replication in independent samples for validity.

The melanocortin-4 receptor (MC4R) has a highly established role in general obesity (reviewed in Friedman et al., 2000; Blakemore 2014). Recently, two separate studies highlighting the genetic association between $MC4R$ variants and AIWG (Chowdhury et al., 2012; Malhotra et al., 2012) have been reported. We reported that the $MC4R$ rs8087522 promoter SNP may have a nominal association with olanzapine induced weight gain in European ancestry patients, and that this SNP may have a functional role in regulating transcription factor binding.

In addition, a genome wide association study found several SNPs located near $MC4R$ to be associated with AIWG (Malhotra et al., 2012). However, our gene-gene interaction analysis showed no significant interaction between the $MC4R$ SNPs recently reported in the literature and the $MC3R$ SNPs in this study. The results from this analysis suggest that $MC3R$ and $MC4R$ may act independently in their roles in regulating AIWG. Though the results found in our study are compelling, some limitations should be considered. Our sub-sample of patients of European ancestry was low in sample size, and the numbers within genotypic groups became low in our sub-analyses. Nonetheless, there may still be a small effect of $MC3R$ on the onset of AIWG. We had sufficient power to detect small effects of the $MC3R$ on AIWG. These findings require replication and further study in independent sample sets. Another consideration is that the SNPs were highly correlated in our sample set (see Figure 5-1). Thus, one $MC3R$ SNP may be the critical regulator of AIWG, while the remaining SNPs may serve as a proxy. Investigation of $MC3R$ SNPs in an independent sample set may further elucidate the specific polymorphisms which play a role in AIWG.
Overall, our results are indicative of an influential role for the *MC3R* gene in AIWG. Future studies could assess *MC3R* SNPs in larger sample sets in order to validate these findings, and to further determine whether the MC3R gene interacts with other leptin-melanocortin system genes which may result in increased risk to gain weight.
Chapter 6 General Discussion
6.0 General Discussion, Conclusions and Future directions

6.1 Summary of Findings

The three main studies (Chapters 3-5) presented in this thesis focused upon demonstrating a role for associations between melanocortin system genes and antipsychotic induced weight gain (AIWG). The main experiments in this study demonstrated the following results: (a) the MC4R rs8087522 SNP was nominally associated with AIWG, and that the rs8087522 ‘A’-allele may have a functional effect, (b) each of the POMC, AGRP and CART genes were not significantly associated with AIWG in our samples, and the roles of these genes may require further exploration in larger sample sizes, (c) the correlated MC3R SNPs rs6014649, rs3746619, rs11697509, rs3827103, rs1543570 SNPs are significantly associated with AIWG.

In the first study, we investigated whether the MC4R gene is associated with AIWG in SCZ samples. MC4R is located in on 18q22, and the gene encodes a protein that functions as g-protein coupled receptor. The protein interacts with peptides including the adrenocorticotropic and melanocyte stimulating hormones. The MC4R gene is comprised of one exon, with no intron, and mutations in this gene have been a reported cause of autosomal dominant obesity (Yeo et al., 1998; Vaisse et al., 1998). To the best of our knowledge, our group was the first to examine the MC4R gene and its possible association with AIWG in a psychiatric sample. The MC4R SNP rs17782313 was initially identified in a genome wide association study examining risk variants for obesity in an adult and pediatric population (Loos et al., 2008). Interestingly, the MC4R rs17782313 and MC4R rs11872992 SNPs were reported to be associated with high BMI in this study (Zegers et al., 2011). This rs17782313 SNP is located approximately 188 kb away
from the MC4R gene. Since the publication of this study, dozens of articles have been published indicating that the MC4R rs17782313 is associated with several weight measures including high BMI, as well as dysregulated eating behaviours (Zobel et al., 2009; Stutzmann et al., 2009; Renström et al., 2009; Cauchi et al., 2009; Grant et al., 2009; Timpson et al., 2009; Li et al., 2010; Kring et al., 2010; Hardy et al., 2010; Cheung et al., 2010; Liu et al., 2010; Petry et al., 2010; Valladares et al., 2010; Orkunoglu-Suer et al., 2011; Croteau-Chonka et al., 2011; Tao et al., 2012; Thomsen et al., 2012; Lombard et al., 2012; Xi et al., 2012; Yang et al., 2013; Ortega-Azorín et al., 2012; Dwivedi et al., 2013; Zlatehlavek et al., 2013; Mejía-Benítez et al., 2012; Xi et al., 2013; Sull et al., 2013; Marcadenti et al., 2013; Dušátková et al., 2013; Jääskeläinen et al., 2013; Hortsmann et al., 2013; Ho-Urriola et al., 2014; Mutombo et al., 2013; Srivastava et al., 2014; Yilmaz et al., 2014). Overall, the MC4R rs17782313 ‘C’-allele is associated with risk for obesity (Loos et al., 2008). Despite some reports that the MC4R rs17782313 is not significantly associated with obesity (Vasan et al. 2012; Valette et al., 2012; Arrizabalaga et al., 2014), the majority of studies have reported that rs17782313 SNP has an association with weight measures. A recent meta-analysis examined 61 studies for rs17782313 SNP in obesity, and reported a significant risk for obesity (obesity risk (OR=1.18, 95% CI=1.15-1.21, p<0.001) (Xi et al., 2012).

In our first study, we investigated whether the MC4R SNPs rs8087522, rs17782313 & rs11872992 were associated with AIWG. We found that the MC4R rs8087522 SNP ‘A’-allele was associated with clozapine induced weight gain, and that the ‘A’-allele may regulate or create a transcription factor binding site. We interpreted these findings with caution, considering that the sample size of patients treated with clozapine was relatively low (n = 69). The MC4R
rs8087522 SNP has been previously reported not to be associated with obesity in a case control study comprised of obese and healthy individuals (Beckers et al., 2011). In a recent study (Czerwensky et al., 2013), MC4R rs17782313 ‘C’-allele carriers were reported to be at risk for AIWG in a sample of patients treated with atypical antipsychotics (clozapine, olanzapine, risperidone, paliperidone, quetiapine, or amisulpride), and were assessed in a naturalistic study design. Limitations of this study include that patients were assessed for antipsychotic treatment for only four weeks.

The MC4R rs489693 SNP has been examined within obesity, though this SNP has not been reported as a primary hit in the context of obesity GWAS studies. Our research group conducted a collaborative research project with a laboratory affiliated with Zucker Hillside Hospital (Long Island, NY) and the GWAS revealed that the rs489693 was significantly associated with AIWG in a pediatric sample treated with second generation antipsychotic medications, along with more than ten other SNPs located near the MC4R gene (Malhotra et al., 2012). Furthermore, the association between rs489693 was associated with AIWG in three distinct samples, a rarity in psychiatric genetics. Replication of the association provides strong evidence that the rs489693 SNP has a role in AIWG. However, further study of the exact influence of the MC4R rs489693 upon weight regulation and AIWG are required. Previously, the MC4R rs489693 SNP was associated with obesity in a large sample size of over 30 000 participants collected from various consortiums (Heard-Costa et al., 2009). However, the specific investigation of this SNP remains relatively unexplored. More recently, the MC4R rs489693 SNP 'A'-allele was significantly associated with AIWG in a sample of patients treated with olanzapine, clozapine, risperidone, paliperidone, quetiapine or amisulpride (Czerwensky et al., 2013). However, the 'C'-allele was
associated with high BMI in a pediatric sample treated with risperidone (Nurmi et al., 2013). Nonetheless, the rs489693 ‘A’-allele association across distinct samples remains a strong finding in the pharmacogenetics of AIWG (Malhotra et al., 2012; Czerwensky et al, 2013).

The potential functional role of SNPs such as rs17782313 and rs489693 are currently unknown. The two SNPs are located downstream of MC4R gene, approximately ~200,000 base pairs away from the gene. Examinations of the Encyclopedia of DNA Elements (ENCODE) functional information using the UCSC genome browser and HaploReg did not indicate a potential functional effect. Given the evidence described above, it appears that certain MC4R SNPs are implicated within the realm of general obesity (rs17782313), and that the rs489693 specifically appears to be more strongly associated with AIWG. A recent study by Yilmaz et al., (2014), reported that the rs17782313 was more associated with depressed mood and over-eating behaviours, while no association was detected between either rs489693 or rs8087522 and over-eating measures. Nonetheless, further study of potential functional effect would help to determine how exactly MC4R SNPs may act in order to alter the onset of AIWG.

In the second manuscript, Chapter 3, we investigated whether hypothalamic peptide genes, including POMC, CART and AGRP were associated with AIWG. These critical key endogenous ligands that bind to MC4R were examined in Chapter 3. As discussed above, leptin has been reported to account for a small portion of the variance of AIWG. However, the role of leptin’s biological targets located in the hypothalamus has not yet been extensively studied in terms of AIWG. Leptin stimulates POMC and CART expressing neurons within the arcuate nucleus of the hypothalamus, eventually resulting in decreased food intake and body weight (Santini et al.,
The POMC gene encodes for different neuropeptides, including the adrenocorticotropic hormone, and α-melanocortin stimulating hormone. The α-melanocortin stimulating hormone exerts its anorexigenic effects largely through melanocortin-3 and melanocortin-4 receptor. To the best of our knowledge, after review of the literature the genetics of the proopiomelanocortin system and AIWG had not yet been thoroughly examined. Adult male Sprague Dawley rats treated with olanzapine exhibit increased weight gain, and decreased proopiomelanocortin expression in the arcuate nucleus (Weston-Green et al., 2011). This finding supports the biological rationale to study POMC gene variants in AIWG and the hypothesis that variation within the POMC gene would be associated with AIWG. The CART is a neuropeptide which is highly expressed within the hypothalamus and the nucleus accumbens. In 2004, Beaudry et al., demonstrated that CART mRNA levels are altered in the nucleus accumbens of rats treated with clozapine. Recently, leptin has been shown to have a stimulatory effect on CART production in mice that were not exposed to acute chronic stress (Xu et al., 2014). Within human genetics obesity studies, the findings with CART remained mixed. The CART variants Phe34Leu and Delta A1457 were associated with obesity in Italian children and adolescents (del Giudice et al. 2001; Rigoli et al. 2010). Though relatively little is known in regard to the role of CART in AIWG, the CART has been cited as a critical regulator of weight, energy homeostasis and is regulated by leptin (Hunter et al., 2004). Thus, the investigation of CART was relevant in the context of AIWG.

The agouti-related protein (AGRP) is an endogenous antagonist of the MC4R. Olanzapine administration in rats has been reported to result in an up-regulation of AGRP in the arcuate nucleus in rats (Ferno et al., 2011). However, AGRP plasma levels do not differ between patients
treated with olanzapine or ziprasidone (Ehrlich et al., 2012). In terms of AGRP and AIWG, our study was unable to demonstrate an association between AGRP SNPs and this phenotype. We utilized tagSNPs for full coverage of the AGRP gene, however, no associations were detected. Thus, it appears that AGRP may not have a critical role in mediating AIWG. However, studies in larger sample sets are required to rule out minor contribution of AGRP to AIWG.

Overall, Chapter 3 demonstrated that genes that have a high profile in terms of weight regulation in general obesity do not necessarily translate to being implicated in AIWG. The POMC, CART and AGRP were not associated with AIWG in our samples. However, our findings do not definitively negate the potential roles of these genes in AIWG, especially if the effect sizes are small. Investigation of these SNPs in additional sample sets is warranted. The role of the NPY is further discussed in published work by our group (Tiwari et al., 2013), and also appears to have significant role in the development of AIWG. The NPY rs16147 ‘C’-allele was associated with weight change, as well as two additional NPY polymorphisms (rs5573 and rs5574). A significant gene-gene interaction between the NPY rs16147 and cannabinoid receptor 1 rs806378 was also detected, illustrating complex epistatic interactions that may be contributory toward AIWG.

In Chapter 4, we examined the role of the melanocortin-3 receptor in AIWG. The melanocortin-3 receptor has been reported to have a role in weight regulation according to animal studies – MC3R knockout mice exhibit increased weight despite hypophagia. Furthermore, mice which are lacking both MC3R and MC4R genes have been reported to be more obese than either MC3R -/- or MC4R -/- mice (Chen et al., 2000). Thus, it appears that MC3R has role distinct from
MC4R in weight regulation, and that these two receptors have their own, non-redundant functions. The MC3R has not been extensively studied in AIWG, either in human or animal studies, and it is unknown whether antipsychotic treatment alters MC3R expression, or MC3R plasma levels. Two non-synonymous SNPS (Thr6Lys & Val81Ile) have been associated with higher weight in pediatric samples, and reduced MC3R expression in an in vitro study (Feng et al., 2005). The MC3R variants associated with weight gain were more prevalent among obese children of African American ancestry compared to children of European ancestry (Feng et al., 2005). Thus, the exact impact of MC3R in obesity is not well understood; however, it remains a compelling candidate gene for AIWG.

The MC3R rs3746619 was not significantly associated with AIWG in a sample of schizophrenia patients treated with second generation antipsychotic medications (Moons et al., 2011). However, that study had a limited sample size (n = 261), and patients were treated with medications in a naturalistic manner. Furthermore, only the rs3746619 SNP was assessed in this sample, while previous obesity studies had shown that the combination of this SNP and an additional SNP in high LD were associated with higher weight in children (Thr6Lys and Val81Ile). Our study investigated SNPs that covered the common genetic variation in the MC3R gene, in order to comprehensively understand the impact of MC3R variations in AIWG. Our sample size is also limited, however, our power calculation determined that at a given reasonable minor allele frequency, we could detect a genetic effect within our sample. We were able to determine that five MC3R variants were associated with AIWG in our samples, under a dominant model, and corrected for multiple testing. However, these findings have not yet been replicated in an independent sample with patients assessed for AIWG, and should therefore be
considered as preliminary. Future studies would include larger sample sets to validate whether the MC3R has an influence on the development of AIWG.

Given that the MC3R and MC4R have been postulated to have non-redundant roles in terms of weight gain and energy homeostasis in animal models (Chen et al., 2000). Thus, it was imperative to assess potential interactions between the two genes. We used the R-based MB-MDR package to assess pairwise MC3R x MC4R interactions with percentage change in weight as the phenotype. No significant interactions were detected between MC3R SNPs in our study, or the MC4R SNPs (Chowdhury et al., 2013) or the findings from our collaborative study (Malhotra et al., 2012). Overall, our findings with these analyses indicate that the MC3R and MC4R do not act together to contribute to the development of AIWG. To the best of our knowledge, this study was the first to attempt to examine the potential additive effects of MC3R and MC4R in AIWG. However, the potential of epistatic effects between biological factors that have an association with AIWG warrant further investigation in larger sample sets.

The evidence provided in this thesis provides support for the melanocortin system as an important regulator of AIWG, and genes encoding peptides within the system may provide insight as to which patients may go on to develop AIWG. However, further molecular genetic studies, and recently available databases such as the ENCODE may help in investigating the exact mechanism of AIWG, and more precisely which genetic variants are actually contributing to the development of AIWG phenotype.
6.2 Obesity and Antipsychotic Induced Weight Gain

Obesity has become a serious worldwide health concern, and a risk factor for many severe health problems including type 2 diabetes and heart disease. The interaction between environmental and genetic factors likely contributes to the onset of obesity. Adding to the complexity of understanding the biological processes contributing to obesity is the many genetic risk factors that have been identified. Genome wide association studies have highlighted both the fat mass and obesity-associated (FTO) gene and and the melanocortin-4 receptor as being highly associated with obesity (Loos et al., 2008; Speliotes et al., 2010). The MC4R is a critical regulator of weight and is expressed within hypothalamic nuclei, a key component of the brain in regulating appetite and satiety. Ligands such as α-melanocyte stimulating hormone and agouti related protein interact with the melanocortin receptor in order to regulate hunger signaling. MC4R rare mutations are associated with severe obesity (Vaisse et al, 1998; Hebebrand et al., 2010). The MC4R non-synonymous SNPs (rs2229616 / Val103Ile and rs52820871 / Ile251Leu) have been reported to be associated with obesity (Geller et al., 2004; Heid et al., 2005; Stutzmann et al., 2009; Young et al., 2007; Wang et al., 2010; Meyre et al., 2009; Xiang et al., 2006). The MC4R 103Ile variant has been reported to increase MC4R function, resulting in lower body weight. Thus, the MC4R 103Ile variant has been reported to have a protective effect (Xiang et al., 2006). In addition, the MC4R SNP rs17782313 has been reported to be associated with obesity in GWAS (Loos et al., 2008; Speliotes et al., 2010). However, in our study, the MC4R rs17782313 SNP was not associated with AIWG (Chowdhury et al., 2012). In our collaborative study (Malhotra et al., 2012), the MC4R rs17782313 was among the top SNPS associated with AIWG, though this signal was not subsequently replicated among independent samples. To the best of our knowledge, the top MC4R SNP associated with AIWG (rs489693:}
Malhotra et al., 2012; Czwerensky et al., 2014) has not been reported to be associated with general obesity. We also investigated common variations of three independent genes (POMC, CART & AGRP) and found no association with AIWG (Chowdhury et al., 2014). Variants of these genes have not been associated with general obesity in genome wide association studies, though the roles of POMC, CART and AGRP have been well established in studies investigating the physiology of weight gain in animal models (Biebermann et al., 2012). Interestingly, the melanocortin-3 receptor SNPs investigated in our study (rs3746619 and rs3827103) were both significantly associated with AIWG as well as general obesity (Feng et al., 2005). Nonetheless, the MC3R variants have not been implicated with general obesity in large scale GWAS investigations. Thus, taken together, these findings indicate that distinct genetic mechanisms may govern general obesity vs. AIWG. In addition, a distinct epistatic effect of melanocortinergic system SNPs may be important in the development of AIWG.

6.3 Multi-Gene Modeling

In Chapter 5, we utilized the model based multifactor dimensionality reduction (mbmdr) software to determine whether an interaction between the MC3R and MC4R genes contributed to AIWG in an additive manner. In our study, we found no evidence of an interaction effect across the variants between the two genes. Nonetheless, the effect of the combination of SNPs across genes may provide crucial insight toward the genetic regulation of AIWG. Currently, there are no reliable biomarkers or tests that can predict the onset of AIWG.
Our group recently used the most promising findings from across our multiple previous and
ongoing studies to attempt to develop a predictive model which could help determine which
patients were at risk for gain weight. The SNPs used in the model include variations from the
gamma-aminobutyric acid receptor subunit alpha-2 (GABRA2), translocator protein-18 (TSPO),
NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1), orexin receptor (HCRTR2),
glicentin-related polypeptide -1 encoding gene (GCG), melanocortin-4 receptor (MC4R),
cannabinoid receptor 1, (CNR1) and neuropeptide Y (NPY) genes.

The rationale of investigating the CNR1 and the NPY in AIWG is more extensively described in
Chapter 1 of this thesis (see sections 1.7.4 and 1.7.5, respectively). Tiwari et al., (2010)
investigated the potential role of CNR1 in AIWG. Twenty SNPs across the CNR1 gene were
examined in psychiatric patients treated with antipsychotic medications. The CNR1 rs806378
polymorphism was found to be associated with weight gain in patients of European ancestry
treated with either clozapine or olanzapine. Carriers of the rs806378 T-allele (CT + TT
genotype) gained more weight (5.96%), than the CC homozygote group (2.76%, p=0.008)
(Tiwari et al., 2010). In another paper, five NPY polymorphisms were analyzed in our
antipsychotic treated sample set, and the NPY rs16147 was significantly associated with weight
change (p=0.012). Patients who received either clozapine or olanzapine and carrying the C-allele
gained significantly more weight with CC+CT gaining 5.82%±5.6 compared to individuals with
TT-genotype who gained on average 2.25%±4.8 (p=0.001) (Tiwari et al., 2013).

Our group has also investigated the glucagon-like peptide gene (GLP-1), a critical endogenous
 glucose regulator. It has been postulated that GLP-1 inhibits AMPK activity, resulting in reduced
food intake (Burmeister et al., 2013) in mice. Recently, a GLP-1 analog, liraglutide, has been demonstrated to treat weight gain in Type 2 Diabetes patients (Flint et al., 2013). Our group investigated four GLP-1 (GCG) gene polymorphisms in AWIG, and found that the rs13429709 ‘C’-allele was associated with this phenotype (p = 0.002) (Brandl et al., 2014).

Recently, our group has also examined the orexin receptors, orexin receptor 1 (OX1R or HCRTR1) and orexin 2 receptor (OX2R or HCRTR2). Both receptors are expressed in the hypothalamus, and have established roles in feeding behaviour (Sakurai et al., 1998). In our sample, the OX2R rs3134701 and rs4142972 SNPs were nominally associated with AIWG (Tiwari et al., submitted).

Finally, our group has also been highly involved in examining the role of mitochondrial genes in AIWG. The mitochondria organelle is a primary energy source for neurons, and has several distinct functions. It has been reported that clozapine and olanzapine may impact mitochondrial function. For instance, clozapine has been proposed to inhibit activity of complex I, and may be involved in oxidation of mitochondrial specific proteins (Baig et al., 2010). Our group found that two mitochondrial system genes, including the NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75KDa (NDUFS1), and the translocator protein-18 kDa (TSPO), were associated with AIWG. The NDUFS1 gene is among the most conserved mitochondrial genes, and is a part of the complex I system. Complex I protein expression has been reported to be down regulated in postmortem brains of SCZ patients (Maurer et al., 2001; Prabakaran et al., 2004). The TSPO gene is involved in biosynthesis of steroids, and may also have a role in general weight regulation (Giannaccini et al., 2011). The TSPO ligand, PK1195, has been reported to lower lipid
accumulation in the liver, and free and LDL cholesterol, as well as blood glucose levels in zebrafish (Gut et al., 2013). The TSPO gene has been associated with weight gain in our samples assessed for AIWG (Pouget et al, submitted).

We incorporated genotypic information from individual’s SNPs to determine risk for AIWG development. The mode of inheritance for each SNP was determined using post hoc pair-wise comparison of mean AIWG among the genotypic groups. The total risk score for each individual was the sum of the individual scores for each genotype of the SNPs included in the model. Based on the post hoc comparisons, genotypes were re-coded as ‘0’ = no risk or as ‘1’ = at-risk for AIWG. The total risk score for each individual was determined by adding genotypes across the nine loci leading to a score ranging from 0 (no-risk) to 9 (highest risk). Finally, the score was then entered as a factor in an ANCOVA analysis in order to determine the cumulative effect of these variants on AIWG, with baseline weight and duration of treatment as covariates in the model. In our model evaluation of the nine SNPs, individuals who carried from zero up to three risk variants gained the least amount of weight (0.81% ± 3.7) compared to individuals with four or more risk alleles. This nine SNP model can explain 68.1% of the variance in AIWG (United States Patent 61946003, filed 2014). If this genetic model can predict AIWG in other samples, it could be a clinically useful tool for physicians to utilize in the future to determine which medications are most beneficial to individual patients. The nine SNP model comprised in our laboratory requires replication in an independent sample set in order for validation of its predictive utility (Full details of model described in our US patent 61946003, 2014).
6.4 Postulated Mechanisms of Antipsychotic Induced Weight Gain

Combination models such as the one described above provide evidence that the interaction across several genes may be a way to determine which individuals are predisposed to gaining weight when treated with antipsychotic medication. Despite these models, the exact mechanism of combined genetic effects in AIWG is still not well understood. The mechanisms by which a subset of patients exhibit substantial weight gain after second generation antipsychotic treatment remains to be determined in terms of the detailed biological mechanisms.

Full understanding of AIWG is extremely complicated, since this phenotype is multi-faceted and governed by several factors. The complexity is well illustrated by the association of various SNPs reported in the literature that are described as being contributory to the variance of AIWG. Some considerations for discerning a primary mechanism include the potential overlap between several gene systems, and that signal transduction pathways stemming from primary antipsychotic medication receptor targets results in virtually thousands of possible candidates in AIWG risk. Recently, with the availability of databases such as the ENCODE, the role of enhancers, amplifiers, regulators, and transcription factors are becoming extremely important to examine in molecular genetic studies. The finding that the RNA molecule microRNA-137 has an influence in schizophrenia risk (Ripke et al., 2011; Lett et al., 2014; Ripke et al., 2014), demonstrates that factors targets other than the classic dopamine and serotonin system gene variants are important to investigate in order to further understand genetic mechanisms. It is important to consider that even if a receptor has not been identified in the receptor binding profile for antipsychotic medications, that this does not exclude the receptor from having a potential regulatory effect of AIWG (Panariello et al., 2011). One example of this situation is
the mechanism of the HR1 mediated AMPK phosphorylation in mice treated with clozapine (Kroeze et al., 2003). It has also been shown in animal models that AMPK activity is mediated by the melanocortin pathway. Administration of a melanocortin agonist (MT-II) was shown to overcome high-fat diet-induced leptin resistance in mice, and alter AMPK activity (Masuzaki et al., 2009). More recently, an in vivo study examined whether α-MSH affects AMPK via direct intracellular signaling cascades. The α-MSH was shown to result in dephosphorylation of AMPK, and this is mediated by enzymes including extracellular-signal-regulated kinases (ERK - s1/2) (Damm et al., 2012). The authors proposed that further study of mechanisms could lead to development of drugs for the treatment of general obesity. Whether antipsychotic medications alter AMPK phosphorylation via melanocortin mediation remains to be determined. In terms of genetic studies, a study conducted by our group (Souza et al., 2011), detected no association between AMPK subunit polymorphisms and melanocortin SNPS in AIWG. Nonetheless, altered AMPK activity due to antipsychotic administration could be one of many potential mechanisms that could be a reason for the onset of AIWG.

Finally, an additional topic of importance in the scope of this research is the understudied aspect of the metabolic syndrome which may develop shortly after antipsychotic treatment. The prevalence of type 2 diabetes in schizophrenia patients has been reported to be three times higher than the general population, even prior to treatment (Nasrallah et al., 2008). Thus, it appears that some schizophrenia patients may have a genetic predisposition toward diabetes, adding to the complexity of examining altered metabolic indices associated with antipsychotic treatment (Tschoner et al., 2007). The development of type 2 diabetes may occur as a result of the weight gain. However, within 6 weeks of SGA treatment, hyperglycemia has been reported to occur in a
subset of patients (Tschoner et al., 2007). The effects of antipsychotics on glucose dysregulation per se have been proposed to be due to the inhibitory effects of muscarinic and serotonergic antagonism upon insulin secretion (Hahn et al., 2011). Therefore, the onset of type 2 diabetes may be independent of the substantial weight gain effect, and be a regulated by a distinct mechanism.

6.5 Limitations and Considerations

6.5.1 Sample Characteristics

Though our studies indicate a role of the melanocortinergic system in AIWG, some limitations should be considered for the interpretation of our results. Firstly, our sample was comprised of patients with distinct genetic ancestries. Differing genetic ancestries are a consideration within our studies, as allele frequencies can vary greatly across different populations. As a result of the varying allele frequencies between ethnic populations, the risk of disease and susceptibilities to developing disease may vary greatly (http://depts.washington.edu/cgph/pdf/PopStrat.pdf). In order to account for the differing ethnicities in our studies, we separated our total sample based on self-reported ancestry. Participants were asked to disclose their ancestry, and this was how ethnicity was determined for our studies. In the future, genetically determined ancestry will be more technically accurate. For instance, genetic markers can be assessed across individuals in order to determine a population’s genetic structure. The ancestry informative marker (AIM) panel examines a set of 62 markers polymorphisms across the genome that have been selected to highlight differences in allele frequencies, among populations from different geographical
regions (Bauchet et al., 2007). This method is considered more advantageous as it determines ancestry based on individuals’ genetic information as opposed to self-report.

Another consideration for our analyses is that within our samples, approximately 40% of patients in our sample have been previously treated with other antipsychotic medications. Notably, the findings across the three studies were conducted in the same clinical samples. In the context of AIWG studies, previous antipsychotic treatment can be limitation. If a patient was previously treated with antipsychotic medication prior to baseline measurement of weight for a study, it is difficult to ascertain what portion of their overall weight gain is due to the antipsychotic under investigation. However, in our analyses, a sub-set of patients were treated with a second generation antipsychotic medication for the first time (Sample B) (described in Masellis et al., 1998; Tiwari et al., 2010).

6.5.2 Sample Size and Power

Our current total sample of psychiatric patients assessed for AIWG (n= 266) has more than 80% power to detect a genotypic relative risk of as low as 3.6%, if we genotype polymorphisms with minor allele frequencies of 20% and set the critical p-value at $\alpha = 0.05$. Our sample size is well characterized for weight gain assessments, and is one of the largest in the world. We had adequate statistical power to provide meaningful results. Moreover, our experiments across our papers began with specific hypotheses and used evidence from the pharmacology, appetite biology and literature. However, our investigation of the melanocortin system should be considered exploratory and novel, and should be investigated in independent sample sets.
Given that our total sample was divided based upon ethnicity for our sub-analyses, a number of our statistical analyses were conducted in pools of relatively low sample sizes (n = 104). Thus, for our sub-sample of patients of European ancestry treated with olanzapine or clozapine, our sample size was reduced from 266 patients to approximately 104 patients. The issue with a limited sample size is that genotypic group sizes become very small, and the comparisons between groups become difficult to interpret. Furthermore, an additional issue in genetic association studies is that low minor allele frequencies may result in very low group sizes. In fact, in our analyses of the MC3R and MC4R genes, in order to assess various types of genotypic models (e.g. dominant vs. recessive), we were required to merge genotypic groups with the heterozygote group to have large enough sample sizes to have meaningful comparisons. The low sample size for the European ancestry group treated with risk medications was further problematic when we examined gene-gene interaction between MC3R x MC4R in Chapter 5. We detected no gene-gene interaction between MC43R x MC4R, and this may be due to lack of power from small group sizes.

Often, samples are not large enough to detect the small effect sizes expected in complex traits, and the inability to replicate the same allelic associations across separate samples points to common limitations among many psychiatric genetic studies. For instance, whether our samples were too low in size to detect an effect for the POMC, CART, or AGRP genes, or they have no influence on the development of AIWG, is difficult to determine. It is important to acknowledge that our collaborative group (Malhotra et al., 2012), recently demonstrated that effects can be detected across relatively low sample sizes. The MC4R rs489693 SNP associated with AIWG was identified in a patient group consisting of 139 subjects and was replicated in three
independent studies. These were also relatively small, with (73, 40, and 92 subjects). In an overall meta-analysis, the effect size for the weight gain was 3 and the p value was $2 \times 10^{-12}$ (Malhotra et al., 2012). Thus, the importance of MC4R in weight gain in AIWG is further highlighted, as well as the ability to detect large effect sizes in small samples. Future studies with larger sample sizes may be able to further understand the role of such genes in the cause of AIWG.

6.6 Correction for Multiple Testing

An additional consideration for our findings regarding the MC4R and MC3R genes is a caveat that is often seen in psychiatric genetic studies – the possibility that a statistically significant result may be a false positive due to multiple testing. This is important to consider in the context our studies, given that the SNPs across the melanocortinergic genes, in addition to many other SNPs, were investigated in the same local samples. Despite the hypothesis driven and exploratory nature of our studies, the issue of correction for multiple testing must be considered. For our studies investigating melanocortin system genes in AIWG, the statistical threshold was set at $p = 0.05$, the general accepted value. The p-value of 0.05 is a probability which indicates that five tests out of 100 conducted would show a positive finding, even if the null hypothesis were true. Simply put, the higher the number of performed tests, the higher the probability of discovering false positives. The process of correcting for multiple testing is a fine balance, as correction that is too stringent may not allow for observing true positive associations within a dataset (Perneger et al., 1998). For the purposes of our studies, either Bonferroni (Abdi, 2007) or SNPSpD (Nyholt, 2004), were utilized to correct for multiple testing. Bonferroni correction is
appropriate for genetic association studies when the SNPs are fully independent, that is, not in linkage disequilibrium, or are not correlated with each other. The Bonferroni correction divides the significance threshold \((p = 0.05)\) by the number of conducted tests. Thus, we utilized Bonferroni correction for the investigation of the MC4R gene (Chapter 2), and for the POMC, AGRP, and CART (Chapter 3) genes. In terms of studying the MC3R gene (Chapter 4), there was relatively high correlation across the 10 SNPs, and the rs3746619 and rs3827103 were in complete linkage disequilibrium. Thus, utilizing the SNPSpD (Nyholt, 2004) method was appropriate in this case.

Overall, the melanocortin system genes examined within this thesis were relatively unexplored in terms of previous studies investigating AIWG. Prior to our studies, the leptin and leptin receptor genes were explored by various research groups in order to determine whether certain variations of these genes explained risk for development of AIWG (reviewed in Brandl et al., 2012). However, a limited number of studies have investigated the melanocortin receptors in the context of AIWG. One common inconsistency observed in AIWG studies include a conundrum in which opposite alleles are associated with conferred risk for a phenotype.

### 6.7 Replication in Independent Sample Sets

An additional obstacle commonly encountered in psychiatric pharmacogenetic studies is that associations between SNPs and a given phenotype are typically difficult to replicate, or the same allele is not associated with a given phenotype when researchers attempt to replicate the initial finding (Lin et al., 2007). An in-depth analytical study by Lin et al., (2007) suggested that the
observed effect of a genetic variant may differ between studies due to correlation differences between the marker SNP and the actual causal variant. The examination of a single locus and a disease resulting in inconsistent findings may be due to overlooking of other genetic loci effects as well as environmental factors that could work in tandem with the candidate gene or locus. So-called ‘flip-flop’ associations may be due to a causative variant that is in variable LD with a non-casual variant – where the non-casual variant is reported to be positively associated with a phenotype. However, when an independent research group attempts to replicate the association, a negative association or the previously associated allele may come to have a protective role as opposed to risk. Additional factors to be considered when observing flip-flop phenomenon in a dataset include the fact that LD can vary greatly across different ancestral populations. Finally, sampling variation may also result in opposite allele findings between studies. Statistical models have indicated that an $r^2$ value as low as 0.3 between markers should still be examined carefully to explain opposite-allele effects (Lin et al., 2007). Overall, replication samples consisting of a carefully characterized and specific phenotype, in the case of our research focus – AIWG, are often difficult to obtain. We were able to collaborate with a research group who examined a sample of pediatric patients treated with risperidone - Research Units on Pediatric Psychopharmacology (RUPP) patients - for weight gain measures. Variations within the LEP and CNR1 genes were associated with AIWG in the RUPP sample (Nurmi et al., 2013). However, the ‘C’-allele of the MC4R rs489693 was nominally associated with AIWG, which displays an opposite allele association. Overall, the MC4R rs489693 ‘A’-allele as the risk allele has been replicated across various samples assessed for AIWG (Malhotra et al., 2012; Czerwensky et al., 2014). Closer examination of LD patterns between both our local sample and the RUPP sample were not deemed to be the cause of the A-allele being positive in our sample, and the C-allele in
theirs for the MC4R rs489693. This same phenomenon or the “flip-flop phenomenon” was observed with our MC3R findings and the subsequent attempt to replicate within the RUPP sample. The opposite-allele effects within the MC3R must be examined more carefully in future sample sets.

6.8 Future Directions

6.8.1 Measures of Weight Change

In the studies presented across Chapters 3, 4 & 5, our phenotype was measured as percentage weight change. More specifically, AIWG was measured as the change in weight between baseline and after a minimum of six weeks of being treated with antipsychotic medication. We were able to use this measure of weight change in our studies, given that this information was consistently available across the clinical data in our samples. In the general literature describing AIWG, the US Food and Drug Administration (FDA) reports a 7% threshold for assessing weight gain. Patients who gain more than 7% weight after the initiation of antipsychotic treatment are considered to have gained a significant amount of weight. We chose to examine a quantitative phenotype (percentage change in weight), as this approach is more a more informative analysis compared to examining a categorical phenotype.

Body mass index (BMI) is the most common method to measure weight in general obesity studies (reviewed in Section 1.6). Assessing BMI is a relatively inexpensive and reliable method for screening weight changes, and has been shown to be correlated with direct measures of body fat (Mei et al., 2002; Garrow et al., 1985). In addition to measuring body fat, waist circumference (abdominal weight) has also been implicated as an important measure in obesity studies. Abdominal weight has been reported to be an indicator for metabolic diseases (Rosito et al.,
Alternative methods of measuring weight include measuring body fatness via skinfold thickness measurements (with calipers), underwater weighing, bioelectrical impedance, dual-energy x-ray absorptiometry (DXA), and isotope dilution (Prentice et al., 2001). Some considerations for utilizing alternative measures to BMI are the costliness, as well as the potential difficulty to replicate across studies at different sample sites. Overall, BMI has a strong correlation to body fatness, and is considered to be a reliable indicator of weight. Specifically measuring adiposity could also be a more precise way to measure AIWG in future pharmacogenetic studies.

6.8.2 Candidate Gene Study vs. Genome Wide Association Study

Limitations of the MC3R and MC4R findings may partially be attributed to the candidate gene study approach. Our studies primarily utilized a candidate gene approach, which has some limitations. Studying one gene at a time may lead to ambiguous results and non-replication across samples. Genome wide association studies (GWAS) have become an additional way to investigate genetic associations within pharmacogenetic studies. The GWAS is a more recent methodology in comparison to the candidate gene approach. The GWAS essentially consists of genotyping of hundreds of thousands of SNPs on array chips, and then using that large number of tests as a correction for association with a phenotype (Pearson et al., 2008; Manolio et al, 2010). A GWAS typically examines numerous polymorphisms and determines associations with a given trait. One of the cited advantages for using GWAS is that this methodology utilizes a hypothesis free approach. The GWAS thus also allows for identification of novel gene associations with a phenotype (Pearson et al., 2008; Manolio et al, 2010).
In terms of AIWG, the findings from GWAS studies are mostly limited and open to interpretation in terms of the results. Previously, Adkins et al., (2011), conducted a genome wide association study on 738 schizophrenia patients assessed in the Clinical Antipsychotic Trial of Intervention Effectiveness (CATIE) study. In this study, 492 900 SNPs were analyzed across twelve weight regulation and related metabolic variables, including a blood lipid panel, glucose, hemoglobin A1c, blood pressure and heart rate. Mixed models were used to predict glucose and triglycerides with fasting time, and output the residuals as fasting time-adjusted measures.

Several genes including the meis homeobox 2 (MEIS2), protein kinase cAMP-dependent, regulatory, type II, beta. (PRKAR2B), G protein-coupled receptor 98 (GPR98), formin homology 2 domain containing 3 (FHOD3), ring finger protein 144A (RNF144A), astrotactin 2 (ASTN2), SRY-related HMG-box (SOX5) and activating transcription factor 7 interacting protein 2 (ATF7IP2) were associated with antipsychotic induced metabolic side-effects. There are several limitations of this study, primarily due to the fact that CATIE was not designed to assess genetics of weight gain measures. The primary caveat as outlined by the authors included that patients were not antipsychotic naïve, and that the assessed sub-sample with available DNA and GWAS data had lower symptom severity. The authors attempted to adjust for these potential confounders using mixed modeling. However, it is difficult to extrapolate from the findings in this study, given that the initial study design was not ideal for assessing weight gain. Also, the blood levels of the medication were not checked and thus adherence was not confirmed. Another example of utilizing the GWAS method was presented in our collaborative study conducted with the Malhotra et al., (2012) research group, and discussed above. The patients assessed in the discovery sample were antipsychotic naïve and the association was detected across separate
samples, illustrating that GWAS can be effective in the study of complex phenotypes in small and well characterized populations.

However, there are number of limitations of the GWAS approach that should be considered. Typically, a large sample size is needed to conduct a GWAS experiment, and the number of tests require a heavy correction penalty for multiple testing (Manolio et al, 2010). The GWAS approach is valuable for detecting novel variants. However, the functional effects of these novel hits are not characterized in these studies. The unknown function or method of regulation of genetic variants is currently a prominent barrier in genetic association studies. Recently, expression quantitative trait loci (eQTLs), which act as regulatory elements of genetic expression, have been highlighted in the literature. A combination of varying methodologies, including GWAS, genome sequencing, the study of eQTLs (Lappalainen et al., 2013), and use of novel databases such as the Encyclopedia of DNA Elements (ENCODE) project are beginning to provide some answers.

6.8.3 Remote Regulatory Site Databases

The US National Human Genome Research Institute initiated a novel and large undertaking – and launched the ENCODE project – a large public research consortium. The primary objective of the ENCODE project was to discover functional, regulatory elements of the human genome. One of the key, critical findings of the ENCODE project was that molecular biological functions could be attributed to over 80% of the genome. The primary mechanism or function of these regulatory regions was the mediation of expression level of coding DNA. This finding was
revolutionary in the molecular genetic field, due to the novel understanding that coding genes located within the human genome may be regulated by multiple regulatory sites, and that these sites may be located very far from the gene itself. Thus, the ENCODE findings provided further steps towards understanding the functional roles of SNPs which are located within intergenic regions. Recently, the ENCODE database has been incorporated into newer platforms and provide additional information regarding gene regulation (e.g. HaploReg v2: 

The ENCODE project and its methodology and aims are relevant to the research in this thesis. For instance, the MC4R rs489693 was significantly associated with AIWG. Yet, this SNP is located a considerable distance, 188,000 base pairs, away from the MC4R gene, and its regulatory function is unknown. Projects such as ENCODE may help to determine whether the MC4R rs489693 is part of an enhancer site, or perhaps the rs489693 is in LD with a SNP which is the true mediator of a biological function, for instance, a SNP that might truly regulate MC4R expression. Additionally, we were unable to uncover whether the MC4R rs8087522 ‘A’-allele regulated transcription factor binding, despite our investigation using electrophoretic mobility shift assay. In a recent study conducted by Evans et al., (2014), MC4R SNPs reported to be associated with obesity were checked against expression quantitative trait loci (eQTLs), to determine whether MC4R SNPs are associated with MC4R expression levels. The authors of this study proposed that the transcriptional insulator, CCCTC-binding factor (CTCF), can bind to the intergenic MC4R segment (that also contains rs489693). The authors suggested that intra-chromosomal interactions regulated by CTCF could potentially bring gene variants in close
proximity to the MC4R promoter (Evans et al., 2014). Nonetheless, further exploration is needed to uncover potential functional regulators of the MC4R gene.

6.8.4 Whole Genome Sequencing

In order to uncover additional variants, whole genome sequencing may be a complimentary methodology. High throughput sequencing allows for thorough examination of the genome, and can aid in identifying rare variants. This methodology allows for an organism’s entire genome to be read at one time. A variety of different platforms provide sequencing platforms, and this approach provides a vast amount of genetic information. The decreasing cost of sequencing methodology has made this a plausible technique to collect genetic information (El-Metwally et al., 2013). The 1000 Genomes Project was initiated in 2008, whole genome sequencing was performed on a set of humans for various ancestral backgrounds (Durbin et al., 2010). This method remains promising in terms of studying the MC4R and MC3R genes. However, it is important to consider that collecting genetic information on whole genomes from individuals results in the compilation of an enormous dataset. Theoretically, whole genome sequencing could provide information six billion nucleotides, or three billion base pairs, in one individual’s DNA to researchers. The bioinformatic and analysis processes for determining associations between genetic information provided from sequencing requires enormous resources, and may not be feasible for all laboratories to implement at this point in time. Whole genome sequencing findings are advantageous for discovery of novel variants. However, with the increasingly common use of databases such as 1000 Genomes (www.1000Genomes.org), a repository of very rare variants has become available for researchers to investigate. However, the clinical utility of very rare variants in psychiatric pharmacogenetics is questionable. For instance, if a mutation or
rare variant is determined to be causative of AIWG in one individual out of a thousand, this finding would be difficult to translate to other patients.

6.8.5 Epigenetics

Novel methods that have been utilized in other fields of psychiatric genetics include studying epigenetic mechanisms. However, utilizing epigenetic approaches have not yet been widely investigated in AIWG studies. Epigenetic studies in pharmacogenetics warrant further investigation due and may provide novel information for AIWG mechanisms. Epigenetic changes primarily occur in 1 of 2 ways, 1) DNA methylation, or 2) histone modification (reviewed in Jaenisch, & Bird, 2003, reviewed in Meaney et al., 2010). DNA methylation primarily refers to a methyl group being added to a DNA nucleotide, which may a result in changes in gene expression. Histone modification refers to alteration of chromatin structure via acetylation of the histone protein, which may affect gene transcription (reviewed in Meaney et al., 2010). The CYP 1A2 enzyme is the major metabolizer of clozapine and olanzapine, the two antipsychotic medications with the highest propensity for weight gain in patients (Ghotbi et al., 2009). The CYP 1A2 has been reported to have the expected inverse correlation between DNA methylation and CPA 1A2 mRNA (Ghotbi et al., 2009). A long term, high fat diet has been reported to influence the methylation of the MC4R exon in mice (Widiker et al., 2010). These findings, among many others, are suggestive that epigenetic analysis could be informative in the field of pharmaco-epigenetics in the future. However, most epigenetic studies have been explored in cancer chemotherapy research, and are only beginning to be conducted in the field of psychiatry (Ptak & Petronis, 2010).
6.8.6 Environmental Factors

A common limitation across pharmacogenetic/weight gain studies include the exclusion of potential environmental factors which may influence weight. For instance, measures of diet, smoking and food intake are rarely assessed in many studies examining the effects of AIWG. Thus, the relative contribution of such environmental factors to the development of AIWG is unknown (Panariello et al., 2010).

**Environmental Treatment Factors**

A randomized clinical trial conducted by Wu et al., (2008), reported that lifestyle intervention and additional medication off-set the weight gain effects of antipsychotic medication treatment. Treatment with metformin, an anti-diabetic drug, and lifestyle intervention have resulted in better outcome for patients. The lifestyle intervention was administered by providing information from a psychoeducational program which highlighted information about proper nutrition, exercise and behavioural techniques. In this study, lifestyle intervention alone, metformin treatment alone, and the combination of lifestyle intervention and metformin treatment all resulted in decreased measures of BMI, insulin levels, waist circumference, and insulin resistance index (IRI) compared to the placebo group. Furthermore, the combination of lifestyle intervention and metformin were more efficacious in weight reduction in comparison to either lifestyle intervention alone or metformin treatment alone. The findings from this study clearly demonstrate that environmental factors can affect weight gain, and that factor such as exercise, diet and additional treatments should be carefully considered in future studies.
6.9 Concluding Remarks

The investigation of antipsychotic induced side-effects, including weight gain, is a relatively new sub-field within psychiatric genetics. Until recently, the most likely candidate genes that were reported to be associated with antipsychotic induced weight are SNPs across the dopamine, serotonin and leptin genes. Overall, the reported associations between various SNPs and AIWG were relatively inconsistent across the literature. Furthermore, with each SNP explaining a small amount of variance in AIWG (~1-2%), the next step forward became utilizing statistical modeling to determine whether a combination of SNPS contributed in an additive manner to the onset of AIWG. It has been proposed that a combination of accounting for age, baseline BMI, 5HT2C and leptin promoter gene variants accounted for 30% of the variance of short term AIWG (Reynolds et al., 2006). However, this model has not been successfully tested in an independent sample set in order to validate its predictive potential. AIWG is a complex trait, and likely has several different contributing genes contributing a small effect. Our patent includes eight genes (nine SNPs) in an additive model (US patent 61946003, 2014). Nonetheless, it is likely that some genes have a higher influence on the development of this phenotype over others. Overall, the findings presented in this thesis are novel and unique, and may be a basis for clinical genetic testing in the future. The MC4R locus has generated much interest. We collaborated with colleagues at Zucker Hillside Hospital, New York to determine an association between the MC4R gene and AIWG. Our collaborators conducted a genome wide association and found that MC4R rs489693 was significantly associated with weight gain in a pediatric sample treated with second generation antipsychotic medications. Our group was able to replicate this finding in our schizophrenia samples. Replication of the MC3R in independent sample sets is warranted, in order to further understand whether this gene has a role in AIWG. The MC3R opposite allele
was reported to be associated with antipsychotic weight gain the RUPP sample set, illustrating the difficulty of replication with complex phenotypes. The advancements of ENCODE and related databases, whole genome sequencing, and epigenetic studies may further help in understanding the mechanism as to why AIWG and its associated metabolic abnormalities occur within a large subset of patients.
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