ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Genetic polymorphisms, DNA methylation and antipsychotic induced weight gain in schizophrenia patients

Umarat Srisawat

A thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of Philosophy

July 2015

Acknowledgements

Firstly, I would like to express my heartfelt gratitude to both of my supervisors, Professor Gavin P Reynolds and Dr. Caroline F Dalton, for providing me with the opportunity to work with them and their brilliant supervision, encouragement and insightful advice throughout my time here. I have been extremely lucky to have both of you being my supervisors.

I would like to thank Dr.Helene Fachim who working with and being a nice friend sharing lovely memories; thanks for help and support. Many thanks to my colleagues and friends, Yasitha, Nat, Jen, Anna, Hanan, Yasin, Eva, Rachel, Kaled, Mariam, Mowafag, Tawfik, Pattrick, Richard, Clair, Marina, Sara, Malanie, Abbie, Nicola, Esadawi, Exta, Afnan, Amani, Callie, Nikhil, Chris, Rebecca, Robert, Amal, and Siriluk Veerasakul and other unmentioned friends both in BMRC and at University of Sheffield for their friendship, help and support.

I would like to thank Prof. Varanuj Chatsudthipong, Assoc.Prof. Kesorn Suwanprasert, Assist.Prof. Pintusorn Hansakul, Assist.Prof. Pritsana Piyabhan, Dr.Wanwarang Hiriote, Dr.Natwadee Poomipark, Assist.Prof. Surin Preawnim, Assist.Prof. Sophapun Ekarattanawong, Assist.Prof. Supaporn Vannasiri, Assist.Prof. Anyanee Burodom, Assist.Prof. Bhronprom Yoysungnoen, Assist.Prof. Jantarima Charoenphandhu, Dr. Naphatsanan Duansak, Nattapol Sukprasert, Watcharin Panunto and all the staff members at Physiology Division and other divisions at Department of Preclinical Science, Faculty of Medicine, Thammasat University for their support.

I would also like to thank all the members of staff at BMRC for all their help and guidance. Special thanks to Professor Nicola Woodroofe, Marguerite Lyons, and Sarah Wright for their help and support.

I am grateful to the Royal Thai Government for the funding for doing research, attending conferences and living in the UK. I am also very grateful to the Faculty of Medicine, Thammasat University for providing me an opportunity to study in the UK.

I would like to express my gratitude to my family, especially my mom and dad, my brother; a massive thanks to my husband Channarong, as well as his family members; million thanks for their endless love, continued support and encouragement.

This thesis is dedicated to all of my family, the Srisawats, the Sawangdees, and the Piromjits, in Thailand.

Publications

1. Srisawat U, Reynolds GP, Zhang ZJ, Zhang XR, Arranz B, San L, Dalton CF. Methylenetetrahydrofolate reductase (*MTHFR*) 677C/T polymorphism is associated with antipsychotic-induced weight gain in first-episode schizophrenia. *Int J Neuropsychopharmacol*. 2014 Mar;17(3):485-90. PMID: 24229535 (see appendix).

2. Tang H, Dalton CF, Srisawat U, Zhang ZJ, Reynolds GP. Methylation at a transcription factor-binding site on the 5-HT1A receptor gene correlates with negative symptom treatment response in first episode schizophrenia. *Int J Neuropsychopharmacol*. 2014 Apr;17(4):645-9. PMID: 24331356 (see appendix).

Conferences/Training attended /Poster presentations

1. BAP 2012 Summer Meeting, Harrogate International Centre, 22nd -25th July 2012 by the British Association for Psychopharmacology (BAP).
2. BNA 2013: Festival of Neuroscience, The Barbican Centre, London, 7th -10th April 2013 by the British Neuroscience Association's biennial meeting. (Abstract and Poster). MTHFR genotype and antipsychotic drug-induced weight gain. British Neurosci. Assoc. Abstr., Vol. 22: 772, 2013, ISSN 1345-8301
3. BAP 2013 Summer Meeting, Harrogate International Centre, 28th -31st July 2013. (Abstract and Poster). MTHFR 677C/T genotype is associated with antipsychotic drug-induced weight gain in patients with schizophrenia. The British Journal of Psychopharmacology, August 2013, 27 Suppl, (8): A69.
4. BAP 2014 Summer Meeting, Cambridge, 20th -23rd July 2014. (Abstract and Poster). The effect of DNA methylation on promoter activity of the 5-HT1A receptor gene. August 2014, 28 Suppl, (8): A109.
5. Pre-Clinical Certificate: Module 3: Experimental Design 1: Statistics and Experimental Design. Friday, 2nd March 2012 Research Beehive, Level 2, Room 2.21 Old Library Building Newcastle University, Newcastle.
6. Pre-Clinical Certificate: Module 5: Functional And Advanced Techniques? The development of pharmacological agents - Registration for Sunday, 22nd July 2012, Harrogate.
7. Pre-Clinical Certificate: Module 6: Principles Of Psychiatry For Basic Scientists, 28th January 2013, Academic Psychiatry, Campus for Ageing and Vitality, Newcastle.
8. Pre-Clinical Certificate: Module 7: Experimental Design 2; Pre-clinical models in behavioural pharmacology (Core Module), 25th March 2013, Huxley Building, University of Brighton.
9. Training session on pyrosequencing on the 20th June 2012 in 744 by Qiagen.
10. Yorkshire Immunology Group Symposium, Sheffield, June 2013.
11. BMRC/MERI Summer and Winter Poster Session Poster Conferences, Sheffield Hallam University, Sheffield, 2011, 2012, 2013, and 2014.

Abstract

Antipsychotic drug-induced weight gain is a common adverse effect of antipsychotic medication in patients with schizophrenia. Many factors including genetic and environmental factors may contribute to this adverse effect. The antipsychotic drugs and genetic factors may influence the weight gain through epigenetic mechanisms, including DNA methylation. The aim of this study was to investigate the associations of antipsychotic drug-induced weight gain with genetic polymorphisms and DNA methylation of *HTR2C*. In addition, the aim of this study was to investigate the effect of antipsychotic drug treatment on DNA methylation and mRNA expression of *HTR2C* and on leptin secretion by adipocytes.

DNA samples from both first episode drug naïve and chronic schizophrenic patients were genotyped using TaqMan® SNP Genotyping Assays and the extent of DNA methylation was measured using bisulfite pyrosequencing. The *MTHFR* rs1801133 genetic polymorphism was significantly associated with BMI change in first episode drug naïve Chinese Han and Spanish patients. In Chinese Han cohort, a significant association was also found of the *MC4R* rs489693 with BMI change. In addition, when patients were analysed as group receiving either risperidone or chlorpromazine, a significant genotype-drug interaction was observed with the *HTR2A* rs6311, and also found significant associations between the *HTR2A* rs6311 and *ADRA2A* rs1800544 with risperidone-induced weight gain.

Global DNA methylation was measured by determining methylation of *LINE-1* in chronic schizophrenia patients. Results show no significant association of *LINE-1* methylation with BMI, although the *MTHFR* rs1801133 and *FTO* rs9939609 SNPs had significant influence on *LINE-1* methylation in this cohort. DNA methylation levels of the *HTR2C* promoter sequence were also measured. The extent of DNA methylation of the *HTR2C* promoter sequences in samples taken before patients received antipsychotics was significantly higher in Chinese Han patients who subsequently had weight increase <7%; therefore, DNA methylation of the *HTR2C* promoter sequences may be a predictor for antipsychotic drug-induced weight gain in drug naïve patients. In addition, the T allele of the *HTR2C* rs3813929 polymorphism was significantly associated with higher methylation of the *HTR2C* promoter sequence. This provides a mechanistic link between the *HTR2C* rs3813929 SNP and promoter activity. The *FTO* rs9939609 SNP was significantly associated with DNA methylation of the *HTR2C* promoter sequences in Spanish male patients. In chronic patients, DNA methylation of the *HTR2C* promoter sequence was not significantly associated with BMI; however, it was significantly associated with the *HTR2C* rs3813929.

The effect of antipsychotic drugs on the *HTR2C* methylation, mRNA expression, and leptin secretion were studied in cell culture models. Clozapine and haloperidol treatment in SH-SY5Y neuroblastoma cells show no significant change DNA methylation and mRNA expression of the *HTR2C*. In 3T3-L1 adipocytes treated with clozapine, SB 242084, risperidone, and haloperidol show no significant changes in *Htr2c* mRNA expression and leptin secretion.

The associations of genetic polymorphisms and DNA methylation of the *HTR2C* with antipsychotic drug-induced weight gain may indicate the underlying mechanisms and also provide genetic and epigenetic markers for antipsychotic drug-induced weight gain.

Contents

List of Tables

List of Figures

Abbreviations

%	percentage
°C	degree Celsius
µg	microgram
µl	microlitre
µM	micromolar
18S	18S ribosomal RNA
2-ME, β-ME	2-mercaptoethanol
5-HIAA	5-hydroxyindole 3-acetic acid
5-HT	5-hydroxytryptamine, serotonin
5-HT1A	serotonin 1A
5-HT1B	serotonin 1B
5-HT2A	serotonin 2A
5-HT2C	serotonin 2C
5-HTP	5-hydroxytryptophan
5-HTT	serotonin transporter
5mC	5-methylcytosine
5-MTHF	5,10-methylenetetrehydrofolate to 5-methylenetetrahydrofolate
A	adenine
ACTB	beta-actin
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
ADRA2A	adrenergic α-2a receptor
AgRP	Agouti-related peptide
Ala	alanine
AMP	adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
APOE	apolipoprotein E
APS	adenosine 5’ phosphosulfate
ARC	arcuate nucleus
Arc	activity-regulated cytoskeletal-associated protein
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATP5B	ATP synthase subunit beta
B2M	Beta-2-microglobulin
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BH4	tetrahydrobiopterin
BMI	body mass index
bp	base pair
C	cytosine
C/EBPs	CCAAT-enhancer-binding proteins
c^2	chi-square
cAMP	cyclic adenosine monophosphate
Canx	calnexin

CART	cocaine and amphetamine-related transcript
CCAAT	cytosine-cytosine-adenosine-adenosine-thymidine
CCD	charge coupled device
CCK	cholecystokinin
cDNA	complementary DNA
Clz	clozapine
CM	complete medium
cm	centimetre
cm^2	centimetre square
CMV	cytomegalovirus
CNS	central nervous system
COMT	catechol-O-methyltransferase
CpG	cytosine-guanine dinucleotide (cytosine-phosphate-guanine)
CPU	caudate nucleus and putamen
CRH	corticotrophin releasing hormone
Ct	threshold cycle
CV	coefficient of variation
CYC1	Cytochrome c-1
Cys	cysteine
D'	a measure of linkage disequilibrium between two genetic markers
D1, DRD1	dopamine receptor 1
D2, DRD2	dopamine receptor 2
D3, DRD3	dopamine receptor 3
D4, DRD4	dopamine receptor 4
D5, DRD5	dopamine receptor 5
DA	dopamine
dATP	deoxyadenosine triphosphate
dATPαS	deoxyadenosine alpha-thio triphosphate
dBcAMP	dibutyryl cyclic adenosine monophosphate
DMEM	Dulbecco's modified Eagle's medium
DMH	dorsomedial hypothalamus
DMI	differentiation medium I
DMII	differentiation medium II
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNDP	deoxynucleoside diphosphate
dNMP	deoxynucleoside monophosphate
DNMT	DNA methyltransferases
dNTP	deoxyribonucleotide triphosphate
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
E	amplification efficiency
EIF4A2	eukaryotic translation initiation factor 4A2
ELISA	enzyme-linked immunosorbent assay
EPS	extra pyramidal syndromes
F	FAM™ dye

FAS	fatty acid synthase
FBLN1	fibulin-1
FBS	fetal bovine serum
FEP	first episode psychosis
FGA	first-generation antipsychotic drugs
FHIT	fragile histidine triad
FTO	fat mass and obesity-associated protein (human)
Fto	fat mass and obesity-associated protein (mouse/rat)
G	guanine
g	gram
GABA	gamma (γ)-Aminobutyric acid
GAD	glutamic acid decarboxylase
GAP	growth-associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCH1	GTP cyclohydrolase
gDNA	genomic DNA
GHSRs	growth hormone secretagog receptors
GLP-1	glucose-like peptides-1
Glu	glutamic acid
GNB3	G-protein beta3 subunit
GTP	guanosine-5'-triphosphate
GWAS	genome-wide association study
h	hour
H1	histamine receptor 1
H2A	histone H2A
H2B	histone H2B
H3	histone H3
H4	histone H4
Hal	haloperidol
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDL	high-density lipoprotein
HK genes	housekeeping gene
HP	high Performance
HPA	hypothalamic-pituitary-adrenal
HRP	horseradish peroxidase
Hs	*Homo sapiens*
HSL	hormone-sensitive lipase
HTR2C	serotonin 2C receptor
IBMX	3-isobutyl-1-methylxanthine
IDF	International Diabetes Federation
IGF	insulin-like growth factor
Igf2	insulin-like growth factor 2
kb	kilobase
kg	kilogram
kg/m^2	kilogram per square metre

Ki or Kd	dissociation constant
LD	linkage disequilibrium
LEP	leptin
LHA	lateral hypothalamic area
LINE-1	long interspersed nuclear element 1
lncRNAs	long-non-coding RNAs
LPL	lipoprotein lipase
LSD	lysergic acid diethylamide
M/F	male/female
M3	muscurinic receptor 3
MAO	monoamine oxidase
MAP	microtubule associated protein
MB-COMT	membrane-bound COMT
MBD	methyl-CpG-binding domain
MC	melanocortin
MC4R	melanocortin 4 receptor
MCH	melanin-concentrating hormone
mCPP	meta-chlorophenylpiperazine
ME	median eminence
MeCP1	methyl-CpG binding protein, MBD1
MECPs	methyl-CpG-binding proteins
Met	methionine
mg	milligram
min	minute
miRNAs	microRNAs
ml	millilitre
mM	millimolar
mmHg	millimetre of mercury
mmol/l	millimoles per litre
mRNA	messenger ribonucleic acid
MTHFR	methylenetetrahydrofolate reductase
MTT	3-(3, 4-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
n	numbers
NA	noradrenaline
NAs	neucleus accumbens
ncRNAs	non-coding RNAs
NeuN	neuronal nuclei
ng	nanogram
nM	nanomolar
nm	nanometre
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NSE	neuron-specific enolase
NTS	nucleus of the solitary tract
NuRD	nucleosome remodeling deacetylase
P/S	Penicillin/Streptomycin

PAH	phenylalanine hydroxylase
PBS	phosphate buffered saline
PCP	phencyclidine
PCR	polymerase chain reaction
PET	Positron Emission Tomography
pg	picogram
piRNAs	piwi-interacting RNAs
PMCo	posteromedial cortical amygdaloid nucleus
POMC	proopiomelanocortin
PPA	phenylpropanolamine
PPARγ	peroxisome proliferator-activated receptor gamma
PPi	pyrophosphate
PRAZ	α1-adrenergic antagonist prazosin
PRC	Polycomb repressive complexes
PVN	paraventricular nucleus
PYY_{3-36}	peptide YY3-36
Q	Quencher
qPCR	quantitative polymerase chain reaction
r^2	a measure of linkage disequilibrium between two genetic markers
RA	retinoic acid
RARE	retinoic acid response element
RARs	retinoic acid receptors
RELN	reelin
RFLP	restriction fragment length polymorphism
Ris	risperidone
RPL13A	Ribosomal protein L13a
Rpl13a	Ribosomal protein L13A
rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
RT-qPCR	quantitative reverse transcription polymerase chain reaction
RXRs	retinoic X receptors
s	second
S in 18S or 28S	Svedberg unit
SAM	S-adenosylmethionine
SB	SB 242084
S-COMT	soluble catechol-o-methyltransferase
SD	standard deviation
SDHA	Succinate dehydrogenase complex, subunit A
SEM	standard error of the mean
SERT	serotonin reuptake protein; or serotonin transporter
SGA	second-generation antipsychotic drugs
Sin3a	SIN3 transcription regulator family member A
siRNAs	small interfering RNAs
SN	substantia nigra

SNP	single nucleotide polymorphism
SREBP-1	sterol regulatory element-binding protein 1
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
SV40	simian virus 40
T	thymine
T2DM	type 2 diabetes mellitus
Ta	annealing temperature
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TD	tardive dyskinesia
TEs	transposable elements
TH	tyrosone-3-hydroxylase
Tm	melting temperature
TMB	tetramethylbenzidine
TOP1	Topoisomerase I
TPA	phorbol ester 12-O-tetradecanoylphorbol-13-acetate
TPH	tryptophan 5-hydroxylase
TRD	transcription repression domain
TRH	thyrotrophin releasing hormone
TrkB	tropomyosin receptor kinase B
TSS	transcription start site
U	uracil
U/mL	units per millilitre
UBC, Ubc	Ubiquitin C
UV	ultraviolet
V	VIC® dye
V	volt
v/v	volume/volume
Val	valine
VMH	ventromedial hypothalamus
vs	versus
VTA	ventral tegmental area
w/v	mass/volume
Xist	X-inactive specific transcript
Y	pyrimidine base (C or T)
y	years
Y1R	NPY Y1 receptors
Y2R	NPY Y2 receptors
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta
YY1	Yin Yang 1 (transcriptional repressor protein)
α-MSH	α-melanocyte stimulating hormone
µl	microlitre

Chapter 1: General Introduction

1.1 Schizophrenia

Schizophrenia is a chronic psychotic disorder characterized by a broad spectrum of disintegration of thought processes and emotional dysfunctions. In 1893, it was called "dementia praecox" by psychiatrist Emil Kraeplin. In 1911, schizophrenia was named by Eugen Bleuler, a Swiss psychiatrist, to describe the chronic, severe, and disabling brain illness. The term refers to the splitting of mental associations that Bleuler believed was the fundamental cause of the abnormalities in schizophrenia (Emery and Oltmanns, 2000).

About 1% of the world's general population suffer from this illness (Jablensky *et al.,* 1992). It requires a large economic burden in terms of hospitalization, chronic treatment, rehabilitation and loss of productivity (Rice, 1999). The estimated total social cost of schizophrenia in England was 11.8 billion pounds a year (Andrew *et al.*, 2012). This social cost was composed of the direct cost of treatment and care that falls on the public fund was about 7.2 billion pounds, this equates to an average social cost of £60,000 and to the public sector of £36,000 per person with schizophrenia per year. These costs come from the direct costs, including in-patient hospital costs, and support from community mental health teams, appearring as costs to both society and the plublic sector. A huge burden of indirect costs to society includes the informal care, unpaid care and private expenditures to families or friends. The costs of lost productivity of patients due to unemployment, absence from work and premature mortality represent huge costs for society and for the public sector due to the loss of tax revenue. The low employment rate for patients with schizophrenia causes loss of productive capacity of £17,200 per individual, which can give rise to an expected loss to the English economy of £3.4 billion per year. This low employment rate also affects the finances of the plublic sector through losses in tax revenue (£715 million per annum) and payments of social security benefits (£470 million per year).

1.1.1 Signs and symptoms of schizophrenia

People with schizophrenia exhibit many different symptoms. The full syndrome is characterized by positive symptoms, negative symptoms, and cognitive impairments. The symptoms typically develop during the first five to ten years and then clinical deterioration reaches a plateau (Lewis and Lieberman, 2000). Disturbance in basic cognitive functions such as attention, executive function, and specific forms of memory, particularly working memory, are thought to be central to the behavioural disturbance and functional disability of schizophrenia. Moreover, many patients have concomitant mood symptoms including depression and anxiety that may contribute to the 10% lifetime incidence of suicide in schizophrenia (Lewis and Lieberman, 2000).

Positive symptoms refer to the symptoms of schizophrenia that seem to be excesses of normal thoughts, emotions, or behaviours (Comer, 2004). Positive symptoms include delusions and hallucinations. Delusions, the first positive symptoms, are characterized by false beliefs. A delusional person believes things that could not be true (Emery and Oltmanns, 2000). For example, they believe that there is someone or a group of people trying to harm or injure them. Common delusions include the beliefs that ideas of other people are being inserted into the patients' head, and that people can read their thoughts. Sometimes the patients believe that they are being controlled by mysterious, external forces. Another positive symptom is hallucinations characterized by abnormal perceptions in the absence of actual external stimuli. For example, they may hear voices without outside stimulus which is the most common type of hallucination. Sights, sounds and other perceptions in the absence of external stimuli are also considered hallucinations.

In contrast to the active presentations of the positive symptoms of schizophrenia, the negative symptoms usually indicate the absence or insufficiency of normal behaviour (Barlow and Durand, 2005). Such negative symptoms as lack of initiative, social withdrawal, deficits in emotional response, and lack of self-care are displayed in approximately 25% of persons with schizophrenia (Malla *et al.,* 2002). These emotional disturbances exert effects on a person's social and occupational functioning. Primary negative symptoms are etilogically related to the core pathophysiology of schizophrenia including blunted or flat affect, poor rapport, emotional withdrawal,

passive social withdrawal, poor social skills, poor grooming, alogia (poverty of speech or poverty of content of speech), anergia, anhedonia, apathy, and avolition. Secondary negative symptoms are derivative of other symptoms of schizophrenia, other disease processes, medications, or environment such as the symptoms that result from positive psychotic symptoms, depression or demoralization, or medication side effects; therefore, secondary negative symptoms usually respond to treatment of the underlying cause (Lindenmayer and Khan, 2006).

The third type of schizophrenia symptom is cognitive impairments that show the manifestations of disorganized thoughts, speech and behaviour. Cognitive impairments affect multiple domains including working memory, selective attention, learning, and executive function (Konopaske and Coyle, 2015). People with schizophrenia may not be able to think logically and may display inappropriate behaviour such as laughing or crying at improper times. Sometimes they show bizarre behaviour such as accumulating objects or acting in unusual ways in public. In addition, disorganized speech is another set of symptoms of people with schizophrenia. They tend to say things that are not sensible. The verbal communication problem and disorganized thinking exert effects on patients' lives. Cognitive impairment is a core feature of the illness. Among the symptoms, the positive symptoms respond best to antipsychotic medications, whereas the negative symptoms and cognitive inpairmant do not responsd well. Therefore, cognitive inpairments and negative symptoms are the best predictors of function outcomes of schizophrenia treatment (reviewed by Konopaske and Coyle, 2015).

1.1.2 Diagnostic criteria for schizophrenia

The diagnostic criteria used to diagnose schizophrenia defined by the American Association of Psychiatry (American Psychiatric Association, 1994), a person must display signs and symptoms as follows:

The diagnostic criteria for schizophrenia from the DSM-IV

A) Characteristic symptoms: two or more of the following, each present for a significant portion of time during a one-month period (or less, if successfully treated)

- delusions
- hallucinations
- disorganized speech (e.g. frequent derailment or incoherence; speaking in abstracts)
- grossly disorganized behaviour (e.g. dressing inappropriately, crying frequently) or catatonic behaviour
- negative symptoms, i.e., affective flattening (lack or decline in emotional response), alogia (lack or decline in speech), or avolition (lack or decline in motivation)

Note: Only one criterion A symptom is required if delusions are bizarre or hallucinations consist of hearing one voice participating in a running commentary of the patient's behaviour or thoughts or of hearing two or more voices conversing with each other.

B) Social/ occupational dysfunction: for a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care, are markedly below the level achieved prior to the onset or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement.

C) Duration: continuous signs of the disturbance persist for at least six months. This six-month period must include at least one month of symptoms (or less, if successfully treated) that meet criterion A. (i.e., active-phase symptoms) and may include periods of prodromal (symptomatic of the onset) or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

Additional criteria (D, E and F) are also given that exclude a diagnosis of schizophrenia if symptoms of mood disorder or pervasive developmental disorder are present. Additionally, a diagnosis of schizophrenia is excluded if the symptoms are the direct result of a substance (e.g. drug abuse, medication) or a general medical condition.

D) Schizoaffective and mood disorder exclusion: schizoaffective disorder and mood disorder with psychotic features have been ruled out because either (1) no major depressive episode, manic episode, or mixed episode have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

E) Substance/general medical condition exclusion: The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

F) Relationship to a pervasive developmental disorder: If there is a history of autistic disorder or another pervasive developmental disorder, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

1.1.3 Subtypes of schizophrenia

Schizophrenia can be divided into five subtypes according to predominant symptoms at the time of evaluation. The five subtypes include 1) paranoid type, 2) disorganized or hebephrenic type, 3) catatonic type, 4) undifferentiated or simple type, and 5) residual type as shown in **Table 1.1**.

Table 1.1: Diagnostic criteria for schizophrenia subtypes according to DSM-IV

Paranoid type	**Catatonic type**	**Residual type**
- Preoccupation with one or more delusions or frequent auditory hallucinations - None of the following are present: disorganized speech, disorganized or catatonic behaviour, flat or inappropriate affect **Disorganized type** - Disorganized speech, disorganized behaviour, and flat or inappropriate affect are prominent	- Dominated by at least 2 of the following • Motoric immobility as evidenced by catalepsy (including waxy flexibility) or stupor • Excessive motor activity • Extreme negativism (motiveless resistance to instruction or maintenance of rigid posture) or mutism • Peculiarities of voluntary movement • Echolalia or echopraxia	- Absence of prominent delusions, hallucination, disorganized speech, and grossly disorganized or catatonic behaviour - Continuing evidence of the disturbance as indicated by the presence of negative symptoms or 2 or more symptoms in an attenuated form **Undifferentiated type** A type of schizophrenia in which its symptoms meet the criteria for schizophrenia, but do not meet the criteria for the paranoid, disorganized or catatonic type

1.1.4 Biology of schizophrenia

The study of the aetiology of schizophrenia is ongoing. It is a multifactorial disease. There are several causes and risk factors that might contribute to the development of schizophrenia, including both environmental and genetic influences. Genetic factors play an important role in the development of schizophrenia. Obviously, schizophrenia is an inherited disorder. Waddington *et al.* (2007) stated that;

> "Schizophrenia is an inherited, likely complex genetic disorder that runs in families and the single best predictor for developing the illness is having an affected first-degree relative".

Nevertheless, many people are affected despite lacking the family history. In addition, it has also been pointed out that the general population had an average risk of developing schizophrenia of 1%; in other words, the lifetime prevalence of schizophrenia is 1% worldwide (American Psychiatric Association, 1994), whereas the first-degree relatives of two parents with schizophrenia and monozygotic twins had risks of 46 and 48%, respectively (Gottesman, 1991). Although these figures show a high risk of developing schizophrenia in the first-degree relatives, none of the schizophrenia twin studies has found that the risk reaches 100%. It is suggested that environmental factors also play an important role in developing schizophrenia.

There are many environmental influences that contribute to schizophrenia risk. The lack of oxygen during pregnancy is an important factor that may lead to the disorder. For example, the situation of the umbilical cord wrapping around the baby's neck results in the impairment of blood circulation and reduction of an oxygen delivery to the developing brain regions (McNeil *et al.*, 1994). Maternal malnutrition in the early months of pregnancy can also lead to an increased risk of schizophrenia in the offspring. It is similarly related to oxygen availability, because malnutrition can cause a lack of nutrient supply to the fetus's brain tissue. Normally, both oxygen and nutrients are important to normal development of the fetus's nervous system. As a result, impairment of these factors may be a leading cause of the brain-related disorders such as schizophrenia. Other environmental factors playing a role in developing schizophrenia are infection and drug treatment during pregnancy (Emery and Oltmanns, 2000).

1.1.4.1 Neurodevelopmental hypothesis in schizophrenia

In normal neurodevelopment, the different brain regions are formed at different times during development. Therefore, the timing of the insult to the developing fetus is a major determinant of the subsequent abnormality. Additionally, small abnormalities in early events can generate a large difference in subsequent stages. Beside these, specific molecular signals play specific roles at various stages of neurodevelopment, for example, noggin and follistatin play an important role in induction of the central nervous system; brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF) are the major signals for proliferation. Many proteins are also involved in neurodevelopment such as reelin and astrotactin which cause appropriate migration of the growing neurons to the specific location in the brain. Signals from interneurons are thought to be important in the trimming process that occurs in adolescence. (Reviewed by Gupta and Kulhara, 2010). However, all of these proteins are controlled by specific genes. Abnormal regulation of fundamental neurodevelopmental processes may occur, or there may be disruption by various forms of insult, this is referred to as a neurodevelopmental disorder.

The hypothesis that schizophrenia might be a neurodevelopmental disorder was first proposed by Thomas Clouston who called itdevelopmental insanity (Murray and Bramon, 2005). The neurodevelopmental hypothesis was proposed in response to research findings of an association of schizophrenia with complications of pregnancy and delivery. This association was proposed by Rosanoff as early as 1934 (reviewed by Gupta and Kulhara, 2010). In addition, several studies showed an increase in risk of developing schizophrenia when the rate of obstetric complications was higher (Cannon *et al.,* 2002; Zornberg *et al.,* 2000; Geddes *et al.,* 1999). Some studies reported an association between development schizophrenia in the offspring and maternal influenza infections, especially during the second trimester (Mednick *et al.,* 1988; Kendell and Kemp, 1989; Kunugi *et al.,* 1995). However, several studies show no relation between maternal influenza infection and risk of schizophrenia (Crow and Done, 1992; Selten and Slaets, 1994; Susser *et al.,* 1994). Furthermore, an increased risk for schizophrenia, about two-fold, has been reported in the offspring of mothers exposed to famine in the Dutch Hunger Winter of 1944-1945 (Susser *et al.,* 1996). This evidence shows an association of schizophrenia in offspring with obstetric

complications and prenatal exposure to infectious agents or toxins as well as maternal malnutrition.

1.1.4.2 Neurotransmitter involvement in schizophrenia

1.1.4.2.1 Dopamine

Dopamine is a catecholamine neurotransmitter that is synthesized from the amino acid tyrosine by dopaminergic neurons. The neuron stores dopamine in small compartments called vesicles in the axon terminals. Dopamine is released into the synaptic cleft through a process called exocytosis. The released dopamine in the synaptic cleft may undergo reuptake into the presynaptic terminal by the dopamine transporter on the presynaptic membrane.

To affect target cells, dopamine interacts with its receptors on the target cell membrane. Dopamine receptors are divided into two subfamilies, D1-like receptor subfamily which includes the receptor subtypes D1 and D5, and D2-like receptor subfamily which include the receptor subtypes D2, D3 and D4 (Neve and Neve, 1997). Upon agonist binding, dopamine receptor signalling is mediated by the heterotrimeric G proteins. D1-like receptor binding activates adenylyl cyclase through coupling to stimulatory G protein $G_s\alpha/G_{olf}\alpha$ subunits, resulting in an increase in the intracellular cAMP level. By contrast, D2-like receptors couple to $G_i\alpha/G_o\alpha$ subunits to inhibit the activation of adenylyl cyclase (reviewed by Neve *et al.*, 2004).

Dopaminergic neurons originate in three cell groups located in the mesencephalon and diencephalon of the brain. The axons of dopaminergic neurons from these cell groups provide widespread projections to regions of the forebrain, forming the following three dopamine pathways in the brain.

The major pathway is the nigrostriatal pathway, where dopaminergic neurons that originate in the substantia nigra send axons projecting to the dorsal striatum which includes the caudate nucleus and putamen (CPU). This region is involved in learning to automatically execute complex movement triggered by a voluntary command. The degeneration of dopaminergic neurons in this brain region causes the motor disturbances that are found in Parkinson's disease. Both D_1 and D_2 receptors are found in the striatum.

The second dopamine pathway: neurons originating in the ventral tegmental area (VTA) send axons projecting to the limbic areas of the brain (nucleus accumbens (NAs), ventral striatum and amygdala), known as the mesolimbic pathway, and to the cortex (medial, prefrontal, cingulate and entorhinal cortex), known as the mesocortical pathway. These pathways are believed to be associated with schizophrenia. D1-D4 receptors are localized in the limbic areas and associated with these pathways.

The final pathway is the tuberoinfundibular pathway, where neurons in the hypothalamus secrete dopamine into the hypophysial portal blood to have effects on dopamine receptors of the anterior pituitary gland, where it acts as prolactin inhibitory factor. This pathway plays an important role in the inhibitory regulation of prolactin release mediated by the D2 receptor. An increased prolactin release is one of the side effects of typical antipsychotic drugs, this can lead to problems with reproductive dysfunction in both men and women.

The classical "dopamine hypothesis of schizophrenia" proposed that a hyperactivity of dopaminergic transmission leads to the symptoms of schizophrenia (Carlsson, 1988). It has been found that drugs that are effective at decreasing psychotic symptoms are dopamine receptor antagonists (Carlsson and Lindqvist, 1963) which block dopamine D2 receptors in the mesencephalic projections to the limbic striatum especially in the etiology of positive symptoms (Creese *et al.*, 1976; Seeman and Lee, 1975). Additionally, amphetamine, a dopamine agonist which increases synaptic monoamine levels, can induce psychotic symptoms (reviewed in Lieberman *et al.*, 1987). In 1991, the dopamine hypothesis was reconceptualized to subcortical hyperdopaminergia with prefrontal hypodopaminergia (Davis *et al.,* 1991).

The dopaminergic hypothesis of schizophrenia is founded on the major following facts: the therapeutic efficiency of neuroleptics (dopaminergic antagonists); a positive correlation between plasma homovanillic acid (metabolite of dopamine) concentration and the severity of schizophrenic illness; a higher density of dopaminergic D2-receptors revealed by Positron Emission Tomography (PET), particularly in the striatum; and an abnormal growth-hormone response to apomorphine (dopaminergic agonist) (Duncan *et al.*, 1999; Lembreghts and Ansseau, 1993).

1.1.4.2.2 Glutamate and Gamma (γ)-Aminobutyric acid (GABA)

Besides the dysfunction in the dopamine system in the brain of schizophrenic patients, the dysfunction in glutamate and Gamma (γ)-Aminobutyric acid (GABA) also plays an important role in the pathophysiology of schizophrenia.

Glutamate is a major excitatory neurotransmitter in the central nervous system (CNS) which acts through multiple excitatory receptors. It is synthesized in the nerve terminals from two sources including glucose and glutamine. The N-methyl-D-aspartate (NMDA) receptor is one of glutamate receptors associated with schizophrenia. The NMDA receptor hypofunction hypothesis of schizophrenia (Olney and Farber, 1995; Olney *et al.,* 1999) was proposed from the observation that drugs such as phencyclidine (PCP) and ketamine, which are NMDA receptor antagonists, have effects that mimic schizophrenia-like psychotic symptoms including delusions, hallucinations, thought disorder and negative symptoms (Krystal *et al.,* 1994). In addition, repeated subcutaneous injections of NMDA channel blockers caused neurodegenerative changes in rat cortex which included posterior cingulate retrosplenial cortex, anterior cingulate, hippocampus, and amygdala. Changes in these regions coincide with the structural changes seen in schizophrenia (Olney and Farber, 1995). The primary sites of action for ketamine and PCP in inducing schizophrenia-like psychotic symptoms and neurotoxicity were NMDA receptors expressed on the GABAergic interneurons in the thalamus and basal forebrain (Olney and Farber, 1995).

Gamma (γ)-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the CNS. It is converted from glutamic acid by the action of glutamic acid decarboxylase (GAD). Many studies indicated that neurochemical abnormalities in the GABAergic system are implicated in the pathophysiology of schizophrenia. Dopamine production in dopaminergic cells is under the direct control of GABAergic neurons. An abnormally low concentration of GAD leads to a low effective concentration of GABA, and results in promotion of dopamine production (Kaplan and Sadock, 1995). A decreased activity of GAD has been reported in schizophrenics. This decreased activity has been found in the nucleus accumbens, putamen, amygdala and the hippocampus. In addition, a decrease in numbers and abnormalities in the distribution of GABAergic neurons in the cortex have been associated with schizophrenia. A decrease in GABAergic interneurons

(parvalbumin staining cells) in the frontal cortex and hippocampal regions have been reported in schizophrenia (Nestler, 1997; Benes, 2000; Reynolds *et al.,* 2004). This suggests that loss of neuroinhibitory control of GABA, in specific regions of the brain, may be responsible for some symptoms of schizophrenia.

Dysregulation in GABAergic neurotransmission can cause cognitive impairment in patients with schizophrenia. The findings from post-mortem brain studies suggest an association of schizophrenia with the impairment of GABA-mediated synaptic transmission (Gonzalez-Burgos *et al.,* 2011). Alterations in cortical GABA neurotransmission and decreased mRNA expression of GAD67 in PFC have been reported in post-mortem studies (reviewed by Volh and Lewis, 2005). Injection of GABA receptor antagonists into monkey PFC impairs performance on working memory tasks (Sawaguchi *et al.*, 1989). The role of GABA in mediating synaptic transmission, which facilitates the flow and processing of information within and between brain regions, may be essential for normal cognitive function (Fries, 2009). It has been suggested that selective GABA-A receptor agonists may be novel therapeutic strategies for treatment cognitive dysfunction in patients with schizophrenia (Stan and Lewis, 2012).

1.1.4.2.3 Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter synthesized in serotonergic neurons in the CNS and enterochromaffin cells in the gastrointestinal tract. 5-HT is synthesized from essential amino acid L-tryptophan. In the brain, the enzyme tryptophan hydroxylase catalyzes the hydroxylation reaction to convert tryptophan to 5-hydroxytryptophan (5-HTP), which is immediately decarboxylated to yield 5-hydroxytryptamine (5-HT) by enzyme amino acid decarboxylase. The principle route of continued metabolism for 5-HT is deamination of the side chain by monoamine oxidase (MAO) yielding a 5-hydroxyindole acid aldehyde, which can be further oxidized to 5-hydroxyindole 3-acetic acid (5-HIAA), a primary metabolite of 5-HT (**Figure 1.1**).

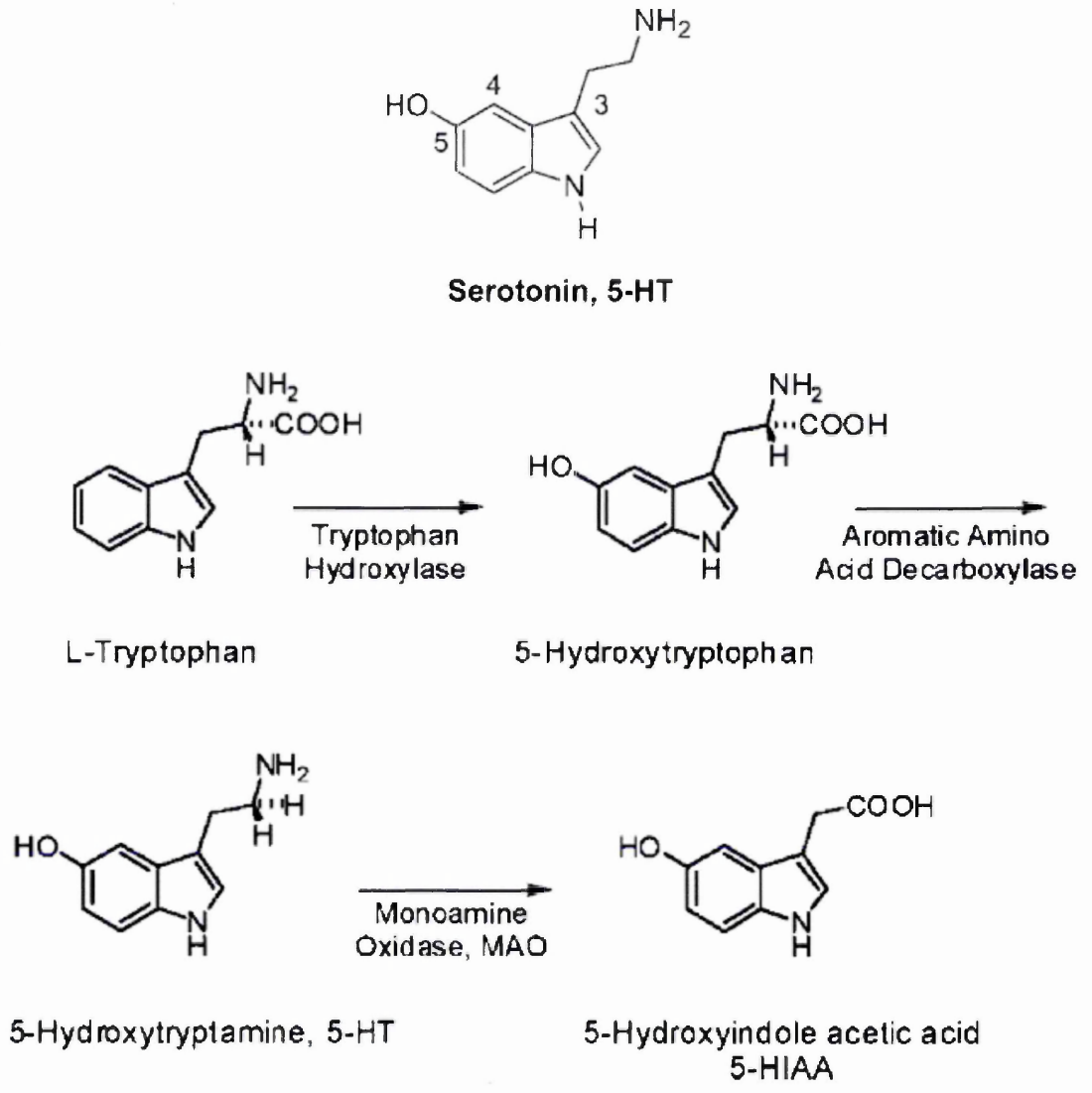


Figure 1.1: 5-HT, biosynthesis and metabolism of 5-HT

The rate-limiting step in 5-HT synthesis is the conversion of L-tryptophan to 5-hydroxytryptophan (5-HTP) which is catalysed by tryptophan hydroxylase. Then the 5-HTP is converted to 5-HT by decarboxylation catalysed by aromatic amino acid decarboxylase. 5-HT degradation is the side chain deamination by monoamine oxidase (MAO) producing 5-hydroxyindole-3-acetic acid (5-HIAA).
Source: From (Nichols and Nichols, 2008)

Synthesized 5-HT is packaged into vesicles. The action potential of the axon causes membrane depolarization and calcium influx, and subsequently 5-HT is released into the synaptic cleft by membrane fusion of the vesicles. 5-HT diffuses across the cleft to activate receptors at post-synaptic neurons to initiate intracellular signalling cascades. 5-HT is removed from the synaptic cleft by the serotonin reuptake transporter (SERT)

which pumps the free 5-HT back to the neuronal terminal where it is repackaged again into neurotransmitter vesicles. Free 5-HT in the cytoplasm is degraded by MAO in the mitochondrial membrane producing the biologically inert metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) as illustrated in **Figure 1.2**.

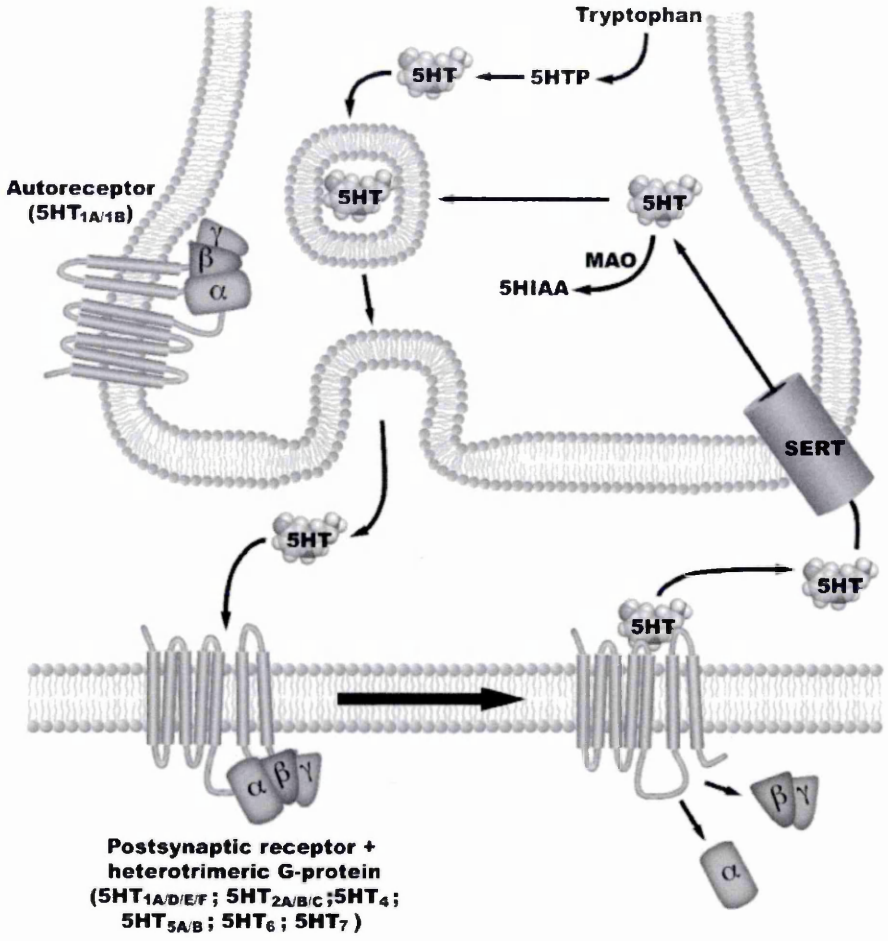


Figure 1.2: Serotonin synapse model

5-HT is packaged into the vesicles after synthesized. When calcium influx induced by membrane depolarization, vesicles fuse with the presynaptic membrane to release 5-HT into synaptic cleft. 5-HT diffuses across the cleft to activate post-synaptic receptors inducing signalling cascades within the cells. Free 5-HT in the cleft is pumped back to presynaptic neuron by SERT to recycle. SERT, serotonin reuptake protein; 5-HTP, 5-hydroxytryptophan; MAO, monoamine oxidase; 5-HIAA, 5-hydroxyindole-3-acetic acid.
Source: From (Nichols and Nichols, 2008)

There are seven 5-HT receptor families, 5-HT1-7. The 5-HT receptor families are mostly seven putative transmembrane domains, G-protein coupled receptors, but one member, the 5-HT3 receptor, is a ligand-gated ion channel. Concentrating on the major human 5-HT receptors implicated in schizophrenia and its treatment, 5-HT1A is highly localized to the hippocampus, cortical regions (particularly cingulate and entorhinal cortex) and mesencephalic raphe nuclei. It has been found in both postsynaptically and somatodendritic sites of the 5-HT neuron. A high expression of 5-HT2A receptors is found in many forebrain regions, but particularly cortical regions (neocortex, entorhinal and piriform cortex, claustrum), caudate nucleus, nucleus accumbens, olfactory tubercle and hippocampus. 5-HT2C binding sites are widely distributed and present in areas of cortex (olfactory nucleus, piriform, cingulate and retrosplenial), limbic system (nucleus accumbens, hippocampus, amygdala) and the basal ganglia (caudate nucleus, substantia nigra) (Barnes and Sharp, 1999).

A hyperserotonin hypothesis for schizophrenia was first proposed in 1954 when researchers found that a serotonin agonist, lysergic acid diethylamide (LSD), could induce hallucinations, a symptom associated with schizophrenia (Woolley and Shaw, 1954). LSD competes for and occupies serotonin receptor sites with very high potency. Several post-mortem studies showed 5-HT dysfunction in cortical areas in patients with schizophrenia, for example, there is a decrease in 5-HT2A/C receptor density (Gurevich and Joyce, 1997) but an increase in 5-HT1A receptor density (Simpson *et al.,* 1996) in schizophrenic patients compared to controls.

1.1.5 Treatment of schizophrenia

Patients with schizophrenia are treated in psychosocial aspects. To relieve the symptoms and improve patients' quality of life, they require close care and both physical and mental support from clinicians, psychiatrists, and their families. Schizophrenia is a complex disorder that must be treated over an extended period of time. Most people suffering from the disorder have to deal with symptoms throughout their lives. Therefore, the clinicians and psychiatrists must be concerned about the treatment not only in the acute period, but also in the long run. In other words, the prevention of the future psychotic symptoms is essential care for schizophrenia patients. Furthermore, the patients and family members should seek advice from a

psychiatrist to understand and accept their condition. The patients as well as their families will gain real advantages from this and can sometimes live happily in their own communities (Buckley *et al.*, 2015; Okpokoro and Sampson, 2014).

1.2 Antipsychotic drug treatment in schizophrenia

1.2.1 Typical antipsychotic drugs

It is known that all typical antipsychotics are dopamine D2 receptor antagonists which remain the main pharmacological treatment of schizophrenia. However, dopamine D2 antagonists are generally effective in the relief of positive symptoms which include hallucinations, delusions and thought disturbances, but do not fully relieve negative symptoms. They also have risks of side effects including extra pyramidal syndromes (EPS) including akathesia, dystonia, parkinsonism and tardive dyskinesia (TD) or tardive dystonia. Tardive dyskinesia affects up to 20-40% of patients receiving chronic first-generation antipsychotic drugs.

An example of typical antipsychotic drugs is haloperidol which has a high affinity to dopamine D2 receptor and is associated with motor side effects. It has been found that long term treatment with antipsychotic drugs with high affinity for DRD2 like haloperidol induced DRD2 receptor upregulation in human (Silvestri *et al.,* 2000) and rat striatal neurons (Bernard *et al.*, 1991). In addition, it has been found that 5-HT2C receptor antagonism reduced motor side effects induced by both acute and chronic haloperidol treatment suggesting that the 5-HT2C receptor is involved in motor side effects induced by typical antipsychotic drug treatment and also that 5-HT2C receptor antagonism could be a potential target of new antipsychotic medications due to the capability to reduce this side effects (Creed-Carson *et al.*, 2011).

1.2.2 Atypical antipsychotic drugs

Following the proposed role of serotonin in schizophrenia, the newer or second generation, "atypical", antipsychotic drugs were first developed. Atypical antipsychotic drugs block both dopamine and serotonin receptors and can reduce problematic side effect of the first generation drugs. This growing list of drugs including: for example; clozapine, olanzapine, risperidone, quetiapine, ziprasidone, aripiprazole and

amisulpride. Clozapine was the first atypical antipsychotic drug that was clinically introduced in 1970s. Atypical antipsychotic drugs can reduce EPS side effects of typical antipsychotic drugs but some of them can induce weight gain with varying degrees.

Dopamine receptors and 5-HT receptors are the targets of these atypical antipsychotic drugs.However, other neurotransmitter receptors are also affected including histamine H1, adrenergic, and muscarinic receptors. The drugs have different metabolic effects and also their affinities to these receptors as shown in **Table 1.2**. The difference of potential to cause metabolic side effects and differential receptor affinities may contribute to variability of weight gain among patients with schizophrenia.

Recently, the NIMH-funded Clinical Antipsychotic Trials in Intervention Effectiveness (CATIE) project found that treatment of psychosis with atypical antipsychotic drugs is not significantly more effective than with typical antipsychotic medications, although atypical antipsychotic drugs showed little benefit for improving cognitive symptoms (Lieberman *et al.*, 2005; Carpenter and Buchanan, 2008; Crossley *et al.*, 2010). Atypical antipsychotic drugs may have advantages over typical antipsychotic drugs through their better safety profile that results in better adherence to treatment (Melnik *et al.*, 2010); the differences in side effects between atypical (e.g. weight gain) and typical (e.g. EPS) antipsychotic medications mean that atypical drugs are easier to live with for patients with schizophrenia.

Table 1.2: Antipsychotic drug affinities for receptors relative to dopamine D2 receptor affinity

Dopamine D2 Ki (nM)	Haloperidol 2.0	Clozapine 431	Olanzapine 72	Risperidone 4.9	Paliperidone 9.4	Quetiapine 567	Ziprasidone 4.0	Aripiprazole 0.95	Asenapine 1.0
α1A-adrenergic	0.17 [12]	**270** [1.6]	**0.66** [109]	**0.98** [5.0]	**3.8** [2.5]	**25** [22]	0.22 [18]	0.038 [25]	**1.1** [1.2]
α2A-adrenergic	$<10^{-2}$ [>1000]	**3.0** [142]	0.24 [314]	0.032 [151]	**2.0** [4.7]	0.16 [3600]	0.025 [160]	0.012 [74]	[1.3]
Histamine H1	$<10^{-2}$ [>1000]	220 [2.0]	15 [4.9]	0.96 [5.2]	1.7 [5.6]	76 [7.5]	0.031 [130]	0.032 [28]	**1.3** [1.0]
Muscarinic M3	$<10^{-2}$ [>1000]	**17** [25]	**1.4** [51]	$<10^{-2}$ [$>10^4$]	$<10^{-2}$ [$>10^4$]	0.29 [1943]	$<10^{-2}$ [>1000]	$<10^{-2}$ [>1000]	$<10^{-2}$ [>1000]
5-HT1A	$<10^{-2}$ [>1000]	**4.1(a)** [105]	0.036 [2063]	0.011 [427]	0.015 [640]	**1.3(a)** [430]	0.053**(a)** [76]	**0.17*(a)** [5.6]	**0.52 (a)** [2.5]
5-HT1B	0.012 [165]	**1.1** [398]	0.14 [509]	0.091 [53.6]	0.087 [109]	**0.52** [1109]	**1.0(a)** [4.0]	$<10^{-2}$ [830]	0.33 [4.0]
5-HT2A	0.035 [57]	81 [5.35]	30 [3.73]	29 [0.17]	4.9 [1.9]	2.8 [200]	13 [0.30]	0.11* [8.7]	18 [0.071]
5-HT2C	$<10^{-2}$ [>1000]	46 [9.44]	7.2 [10.2]	0.41 [12]	0.2 [48]	0.22 [2500]	0.31 [13]	0.043*(a) [22.4]	37 [0.035]
5-HT6	$<10^{-2}$ [>1000]	25 [17]	12 [6.0]	$<10^{-2}$ [>1000]	$<10^{-2}$ [>1000]	0.30 [1864]	0.066 [61]	$<10^{-2}$ [642]	5.2 [0.25]

Values indicate drug affinity for the receptor expressed relative to dopamine D2 receptor affinity, calculated (D2 Ki÷receptor Ki) from Ki data provided in the PDSP Ki Database: http://pdsp.med.unc.edu/pdsp.php and, for asenapine, Shahid *et al.*,(2009). Receptor Ki [nM] is included in square brackets below the relative affinity value.

Affinity values approaching unity and above (shown in **bold** type) indicate the likelihood of substantial receptor occupancy at normal clinical doses. These relative affinities generally reflect antagonist or inverse agonist effects; known agonist or partial agonist effects are indicated **(a)**

The partial agonist action of aripiprazole at D2 receptors permits high D2 receptor occupancy by drug without the emergence of dopaminergic side effects; therefore there may be significant binding to some lower affinity receptors (indicated with *) due to the high relative concentration of available drug.

Source: From (Reynolds and Kirk, 2010)

1.3 Antipsychotic drug-induced weight gain in patients with schizophrenia

Weight gain is a common side effect of many second-generation antipsychotic drugs and usually results from increased appetite that brings about the subsequent excessive food intake. Antipsychotic drugs have differed potential to cause weight gain and metabolic phenotypes. As shown in **Table 1.3**, clozapine and olanzapine have the greatest risk for weight gain and ziprasidone and aripiprazole have the least risk.

Table 1.3: Relative likelihood of weight gain and metabolic disturbances of antipsychotic drugs

Medication	Weight Gain	Glucose Metabolism Abnormalities	Dyslipidemia	Metabolic Syndrome
Aripiprazole	Low	Low	Low	Low
Clozapine	High	High	High	High
Haloperidol	Low to mild	Low to mild	Low	Low
Olanzapine	High	High	High	High
Perphenazine	Low to mild	Low to mild	Low	Low
Risperidone	Mild to moderate	Mild	Mild	Mild
Quetiapine	Moderate	Moderate	High	Moderate
Ziprasidone	Low	Low	Low	Low

Reference source: (Hasnain *et al.,* 2009; Hasnain *et al.,* 2010; Patel *et al.,* 2009; Baker *et al.,* 2009; Duncan *et al.,* 2009; Lambert *et al.,* 2006; Sikich *et al.,* 2008)

The 5-HT receptor is a target of atypical antipsychotics. It has been suggested that 5-HT2 receptor antagonists can reduce EPS side effect problems induced by typical antipsychotics and the 5-HT2 receptor is a site at which antipsychotic drugs may relieve the negative symptoms of schizophrenia. Additionally, clinical and preclinical investigations indicate that several new atypical antipsychotic drugs improve cognition in schizophrenic patients and some of them display affinity for several 5-HT receptors, including 5-HT1A, 5-HT4, 5-HT6 and 5-HT7 receptors in addition to the 5-HT2A site (Millan, 2000; Meltzer and Sumiyoshi, 2003). Thus, the 5-HT system has been strongly suggested to be involved in pharmacological treatment of schizophrenia. However, treatment with both typical and atypical antipsychotic drugs may be associated with weight gain.

Among the second generation antipsychotic drugs, clozapine was found to be associated with the largest mean increase in body weight (4.45 kg over 10 weeks) studied by meta-analysis (Allison *et al.,* 1999). The weight gain tended to occur mainly in the first year of treatment. Obesity and weight gain in adulthood have been associated with significant health complications including hypertension, type II diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis, sleep apnea, respiratory problems and some types of cancers (Rubenstein, 2005; Pi-Sunyer, 2009).

It has been suggested that the serotonin system may be involved in regulating feeding behavior and weight gain. Both animal and human studies have shown that increasing serotonin causes decreased feeding and decreasing serotonin results in increased feeding (Comuzzie and Allison, 1998). Many studies have focused on the 5-HT2C receptors regarding the control of feeding and weight gain. The evidence supporting the role of the 5-HT2C receptor in feeding behavior was studied in 5-HT2C receptor knockout mice which found that the knockout mice that lack the 5-HT2C receptor were overweight compared to wild-type mice (Tecott *et al.,* 1995). The 5-HT2C receptor gene is located on the X chromosome at q24 (Milatovich *et al.,* 1992). It has a promoter region which contains a polymorphism at position -759 in the 5' flanking region consisting of a C to T transversion. This 5' flanking region contains regulatory regions and a putative transcription factor binding region that may affect gene expression. This 5' flanking region contains many potential binding sites for various transcription factors including AP1, AP2, bHLH, GCF, HNF-5, LF-A1, NF-E1, NF-IL6, NF-kB, and TCF-1 (Xie *et al.,* 1996). Among these transcription factor binding sites, the sites nearby the -759C/T and -697G/C SNPs are TCF-1, LF-A1, and bHLH, the SNPs may exert influence through their effect on the conformation of DNA that affects the affinity for these transcription factors (Shastry, 2009). In humans, this 5-HT2C receptor polymorphism has been associated with the development of obesity and type II diabetes in normal subjects; the researchers found that the variant T allele showed a higher frequency in the non-obese indicating a protective effect of this allele (Yuan *et al.,* 2000). In one of the most consistently replicated pharmacogenetic findings, Reynolds *et al* has identified a common SNP (-759C/T) in the 5-HT2C receptor gene (*HTR2C*) that contributes substantially to initial drug-induced weight gain (Reynolds *et al.,* 2002; Templeman *et al.,* 2005; Miller *et al.,* 2005). Further studies have reported that this SNP has effects on promoter activity (Hill and Reynolds, 2007, 2011); it seems likely that it is this effect on gene expression that mediates the association between *HTR2C* genotype and antipsychotic drug-induced weight gain.

1.3.1 Normal body weight control

1.3.1.1 Regulation of food intake (Appetite)

Short-term regulation of appetite and satiety involves nutrient concentrations and hormonal signals to the CNS. During the inter-meal intervals, hunger develops in response to decreasing nutrients in blood circulation such as glucose, fatty acids, and amino acids and also increasing ghrelin hormone which is secreted from the stomach during gastric emptying. After a meal, there are increasing nutrient concentrations and satiety hormones including 5-HT, cholecystokinin (CCK), PYY_{3-36}, insulin, glucose-like peptides-1 (GLP-1) and decreasing hunger signals; these factors act on the CNS to inhibit hunger and stimulate a feeling of fullness (Wilding, 2010).

Long-term regulation of energy balance depends on the magnitude of energy stores and involves leptin which is secreted from adipocytes. Leptin receptors and downstream signalling pathways are in the hypothalamus. When fat mass is low and leptin decreases below a critical level, hunger signals are stimulated in hypothalamus such as neuropeptide Y (NPY) while pro-opiomelanocortin (POMC) is inhibited, resulting in stimulated food intake and inhibited thermogenesis (Wilding, 2010).

Energy balance is under the control of the CNS, and the most important region is the hypothalamus. There are several hypothalamic nuclei involved in energy metabolism, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and lateral hypothalamic area (LHA) (Hetherington and Ranson, 1940; Schwartz *et al.,* 2000). Nucleus accumbens, amygdala, nucleus of the solitary tract and the area postrema are also involved in energy homeostasis (Schwartz *et al.,* 2000). The first order neurons are located in ARC while the second order neurons are in other hypothalamic regions such as PVN, DMH, VMH, and LHA as illustrated in **Figure 1.3** (Reynolds and Kirk, 2010). These hypothalamic nuclei receive signals from both peripheral tissue hormones such as leptin, insulin, and ghrelin, and also signals from extra-hypothalamic brain areas such as dopamine, serotonin, and noradrenaline.

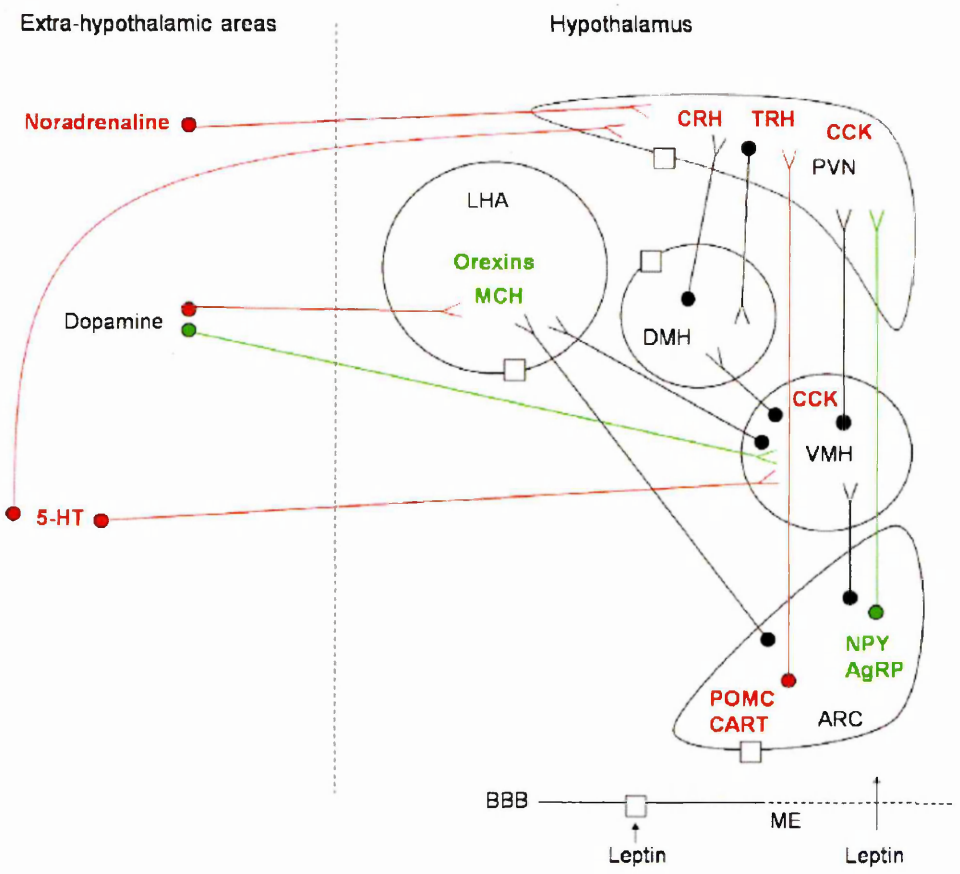


Figure 1.3: Relationship between neuropeptides, neurotransmitters and pathways in appetite regulation in rat hypothalamus

Diagram illustrating the hypothalamic nuclei and the relationship between the appetite regulation neuropeptides, neurotransmitter, and pathways involved in appetite regulation. Orexigenic neuropeptides, neurotransmitters, and pathways are presented in green, anorexigenic are shown in red. □, leptin receptors; ARC, arcuate nucleus; AgRP, agouti-related peptide; BBB, blood brain barrier, CART, cocaine and amphetamine-related transcript; CCK, cholecystokinin; CRH, corticotrophin releasing hormone; DMH, dorsomedial nucleus; LHA, lateral hypothalamus; ME, median eminence; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus; TRH, thyrotrophin releasing hormone; VMH, ventromedial hypothalamus.

Source: From (Reynolds and Kirk, 2010)

The neurons in the ARC play an important role in regulating energy balance between food intake and energy expenditure. There are two neuronal groups in the ARC: the neuropeptide Y (NPY)/agouti-related protein (AgRP) neuron (orexigenic effect) and the pro-opiomelanocortin (POMC)/cocaine- and amphetamine-related transcript (CART) neurons (anorexigenic effect) as shown in **Figure 1.4**. They receive peripheral hormonal signals including satiety factors such as leptin, insulin, CCK, bombesin, enterostatin, and GLP-1, and peripheral orexigenic factors such as ghrelin and adiponectin. These peripheral hormones have a direct influence on the neuropeptides in the hypothalamus.

The neuropeptides in ARC are affected by these peripheral signals which have different pathways in regulating food intake and energy expenditure as shown in **Figure 1.4**; for example: leptin and insulin stimulate POMC/CART and inhibit NPY/AgRP neurons leading to increased POMC/CART neuropeptides and decreased NPY/AgRP neuropeptides, and ultimately inhibit food intake (Bell *et al.,* 2005). Whereas, ghrelin secreted from the stomach during gastric emptying directly stimulates its receptors, growth hormone secretagog receptors (GHSRs) on NPY/AgRP neurons (Meier and Gressner, 2004) which results in increased food intake.

Neurons in ARC have interaction with each other such as GABA secreted from NPY/AgRP neurons inhibits POMC neurons (Cowley *et al.,* 2001). They project to second-order neurons including PVN and LHA. The NPY/AgRP neurons release NPY which bind to NPY 1 receptor (Y1R) while POMC/CART neurons release α-melanocyte stimulating hormone (α-MSH) which bind to MC4R in PVN neurons. Second-order neurons project to the nucleus of the solitary tract (NTS) where satiety signals are processed.

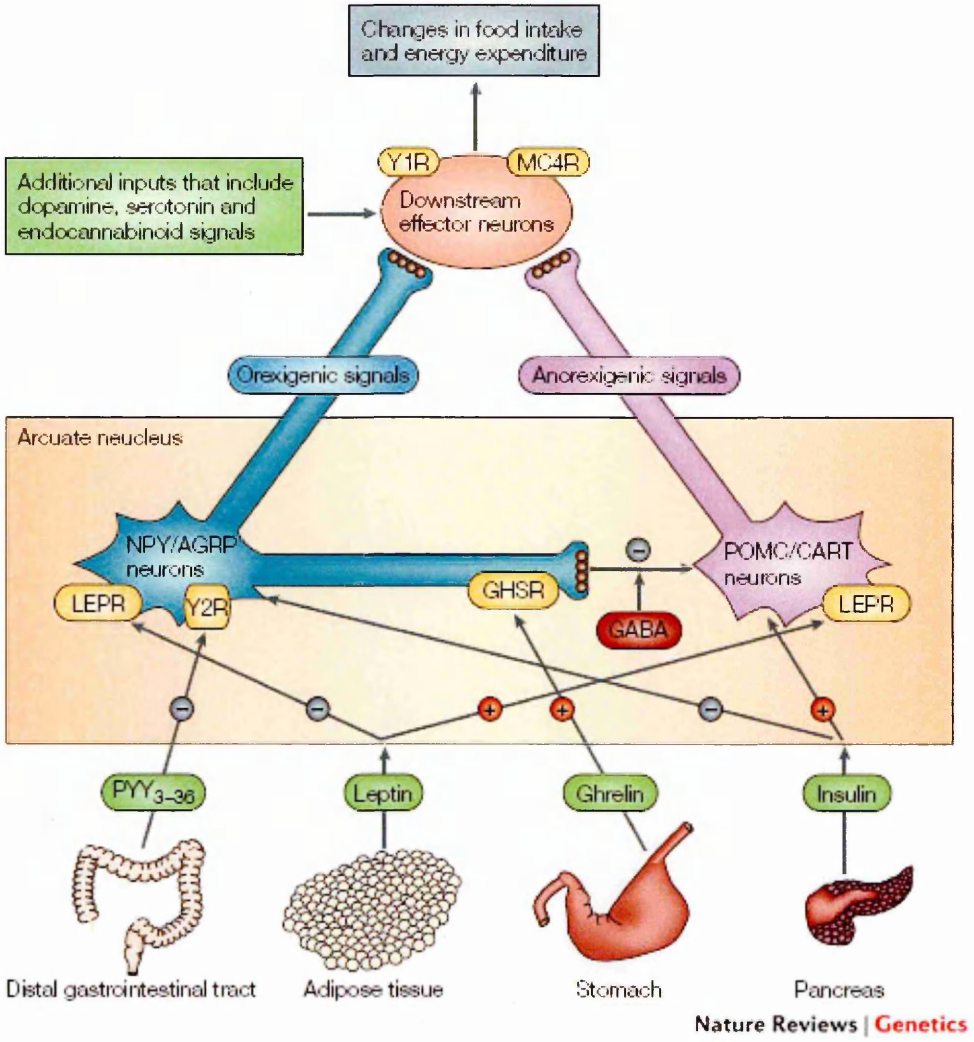


Figure 1.4: Hypothalamic control of central energy balance

The ARC neurons, NPY/AgRP (orexigenic) and POMC/CART (anorexigenic), are influenced by peripheral hormones including; PYY_{3-36} secreted from colon inhibits NPY/AgRP via NPY Y2 receptor (Y2R); leptin from adipocytes and insulin from pancreas inhibit NPY/AgRP but stimulate POMC/CART via leptin receptor (LEPR) and insulin receptors, respectively; ghrelin from stomach stimulates NPY/AgRP via growth hormone secretagog receptors (GHSRs). The NPY/AgRP neurons can inhibit POMC/CART through GABA release. The downstream second-order neurons are influenced by the ARC neurons via NPY Y1 receptor (Y1R) and melanocortin receptor (MC4R) and also received modifying signals from neurotransmitters such as dopamine, serotonin, and endocannabinoids. *Source: From* (Bell *et al.*, 2005)

1.3.1.2 Regulation of metabolism

Body weight maintenance requires the balance between energy intake and energy expenditure and also balance between macronutrient intake and macronutrient oxidation. Individuals who have a tendency to gain weight seem to have low energy expenditure, low spontaneous physical activity, low fat oxidation, or low sympathetic activity in addition to excess energy intake (Galgani and Ravussin, 2008).

There are three components of energy expenditure including the basal metabolic rate, the thermic effect of food (dietary thermogenesis), and the energy consumed during physical work, of which the latter part accounts for a variable amount of total energy expenditure (Ravussin *et al.,* 1986). The basal metabolic rate is the energy required to maintain normal metabolism for example during sleep, normal breathing, and at rest. Although basal metabolic rate is not a large portion of total energy expenditure, it may help determine the ability to resist weight gain in response to overfeeding. The thermic effect of food is the energy used in food digestion and storage. The energy used for protein is the greatest, intermediate for carbohydrate, and very low for fats. This may partly explain why a high fat intake is more likely to develop weight gain (Wilding, 2010).

Low energy expenditure due to relative low resting metabolic rate and 24-hour energy expenditure is a risk factor for body weight gain (Ravussin *et al.,* 1988; Tataranni *et al.,* 2003). Spontaneous physical activity accounts for 8-15% of total daily energy expenditure (Ravussin *et al.,* 1986), and low spontaneous energy expenditure is related to weight gain and fat mass gain (Zurlo *et al.,* 1992; Levine *et al.,* 1999).

Sympathetic activity is related to energy expenditure in all components including resting metabolic rate (Spraul *et al.,* 1993), thermic effect of food (Schwartz *et al.,* 1988), and spontaneous physical activity (Christin *et al.,* 1993). Spraul *et al.* (1993) found that individuals (Pima Indians) prone to obesity have lower rates of muscle sympathetic activity compared to weight-matched Caucasians.

1.3.2 Weight gain in patients with schizophrenia

Weight gain in patients with schizophrenia and other mental disorders is one common physical health problem which can result in progression towards the development of metabolic syndrome, diabetes, dyslipidemia, hypertension, and cardiovascular disease. The prevalence of obesity and metabolic syndrome in schizophrenia patients (particularly women) is about two-fold comparing to general population (Dickerson *et al.,* 2006; De Hert *et al.,* 2006). Weight gain in patients with schizophrenia receiving antipsychotic drugs is likely to be due to an accumulation of fat mass, particularly abdominal fat reflected by an increase in waist-hip ratio (Stedman and Welham, 1993). Using magnetic resonance imaging an increased subcutaneous and intra-abdominal fat deposition was found in first episode drug naïve schizophrenia patients who had received antipsychotic drugs for 10 weeks (Zhang *et al.,* 2004).

There are many factors contributing to weight gain in patients with schizophrenia. In addition to antipsychotic drug treatment, the disease conditions of schizophrenia and its consequences, the dietary, lifestyle, physical activity, and the psychological and motivational factors; these may partly contribute to weight gain. The weight gain among patients with schizophrenia receiving antipsychotic drug treatment has high variability which may be the result of the contribution of the genetic, environmental, behavioural, and neurochemical factors.

The genetic factors which contribute to differences in the inherent biological processes between individuals are likely to contribute to the variability of weight gain. The initial body weight or body mass index (BMI) before treatment is associated with greater weight gain following treatment with antipsychotic drugs (Lambert *et al.,* 2005). The genetic variation may also determine the different degree of antipsychotic drug-induced weight gain. Genetic polymorphisms of other candidate genes are also implicated in antipsychotic drug-induced weight gain (Balt *et al.,* 2011) and are described in the next section (**see section 1.4**).

Other environmental, life style, and behavioural factors that influence energy balance are also likely to contribute to the variability of weight gain. For example, the negative symptoms such as apathy and social withdrawal can lead to reduced propensity to

exercise, or because of weight gain, patients feel resistant to exercise socially. The side effect of antipsychotic drugs such as sedation may cause decreased physical activity.

Exposure to antipsychotic drugs, particularly second-generation drugs, are likely to cause weight gain (Newcomer, 2005). Although clozapine and olanzapine are the greatest risk factors for weight gain, other drugs also have influence on weight gain in various degrees, these may be due to each drug exerting its action to a different degree on different neurotransmitter systems including serotonergic, dopaminergic, adrenergic, histaminergic and others (Reynolds and Kirk, 2010; Panariello *et al.*, 2011) as shown in **Table 1.2** (**see section 1.2.2**).

1.3.3 Mechanisms of atypical antipsychotic drug-induced weight gain

The mechanisms underlying antipsychotic drug-induced weight gain are not completely understood. Weight gain results from excess energy intake over energy expenditure and antipsychotic drugs can affect these parameters in many ways; for example: by increasing appetite or decreasing satiety, affecting food preferences, changing blood circulating hormones, changing metabolic rate, inducing sedation. Therefore, antipsychotic drugs can induce weight gain through an influence on many interacting systems as shown in **Figure 1.5**.

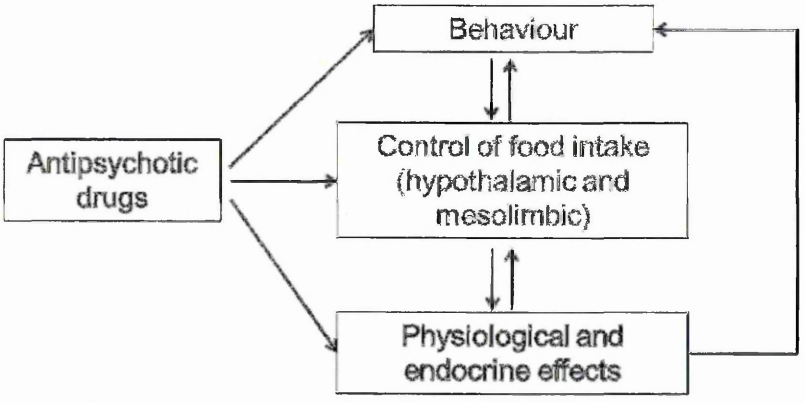


Figure 1.5: Overview of mechanisms involved in antipsychotic drug-induced weight gain.
The mechanisms may involve many levels including behavioural, neural, and endocrine systems where antipsychotic drugs can directly or indirectly act at all these sites.
Source: Adapted from (Goudie *et al.*, 2005)

The mechanisms underlying antipsychotic drug-induced weight gain that have been investigated in most detail to date are drug effects on receptors because many receptors that are the targets of antipsychotic drugs are involved in food intake and body weight regulation.

1.3.3.1 Disruption of hypothalamic control of energy balance

The disruption of hypothalamic control of energy homeostasis may be a mechanism of antipsychotic drug-induced weight gain. The role of the circulating hormone from adipose tissue, leptin, which acts at its receptor in the hypothalamus in the regulation of food intake and energy expenditure, provides clues as to the mechanism. Several studies have reported an increase in plasma leptin in patients receiving antipsychotic drugs both for 10 weeks treatment in first episode drug naïve patients (Zhang *et al.,* 2004) and 1 year treatment (Perez-Iglesias *et al.,* 2008). Increased leptin levels are also related to antipsychotic drugs induced weight gain; the magnitude of increased plasma leptin is positively correlated with the magnitude of increase in BMI (Venkatasubramanian *et al.,* 2010). This is a normal response when body weight increases which increases fat deposition and results in increased leptin secretion from adipocytes. But the increased plasma leptin levels fail to suppress food intake in patients receiving antipsychotic drugs. Therefore, it has been suggested that there may be disruptions of leptin signaling in the hypothalamus by the antipsychotic drugs (Reynolds and Kirk, 2010). However, the mechanisms by which plasma leptin levels increase in patients receiving antipsychotic drugs still unclear. It could be a normal response with body weight gain which increases fat deposition results in increased leptin secretion from adipocytes; however, a study reported that atypical antipsychotic treatment increased leptin secretion in human adipocytes *in vitro* (Sarvari *et al.,* 2014). Therefore, peripheral drug effect on adipocyte secreting leptin cannot be excluded.

In addition, the genetic polymorphism of leptin gene (*LEP*), -2548A/G is associated with antipsychotic drug-induced weight gain. The G allele of the SNP is associated with greater weight increase following antipsychotic drug treatment in first episode schizophrenia patients (Templeman *et al.,* 2005). The *LEP* -2548A/G also has a reported interaction with the *HTR2C* -759C/T polymorphism on BMI and waist circumference in chronic schizophrenia patients (Yevtushenko *et al.,* 2008). Presence of the *LEP* -2548G

allele in patients with the *HTR2C* -759C allele is associated with higher BMI and waist circumference (Yevtushenko *et al.,* 2008) and obesity (Gregoor *et al.,* 2010).

Several neurotransmitter systems are involved in food intake and body weight regulation and many receptors in these neurotransmitter systems are the targets of the antipsychotic drugs. These neurotransmitter systems include serotonin, histamine, noradrenaline and dopamine.

1.3.3.1.1 Serotonin receptors

It is well known that the 5-HT plays an important role in regulating food intake. Considering the second-generation of antipsychotic drugs have differences in 5-HT receptor affinity compared to first-generation antipsychotic drugs, 5-HT receptors therefore have been of interest to search for molecular mechanisms of antipsychotic drug-induced weight gain. 5-HT is a potent satiety signal illustrated by 5-HT administration decreasing food intake in rodents (Blundell and Leshem, 1975). The 5-HT1A and 5-HT2C receptors are related to food intake regulation. 5-HT1A agonists increase food intake (Dourish *et al.,* 1985) while 5-HT2C agonists decrease food intake (Clifton *et al.,* 2000).

5-HT1A receptor agonists increase food intake in animals (Voigt *et al.,* 2002). Clozapine which causes weight gain is a 5-HT1A partial agonist and has high affinity for the receptor, whereas olanzapine which also cause weight gain has low affinity for this receptor (Richelson and Souder, 2000). Moreover, ziprasidone and aripiprazole which do not cause weight gain are 5-HT1A partial agonists (Reynolds and Kirk, 2010).

5-HT2C antagonists can increase food intake and weight gain in rats (Bonhaus *et al.,* 1997) and also attenuate the decreased food intake which is induced by 5-HT2C agonists (Clifton *et al.,* 2000; Hayashi *et al.,* 2005). Another study in mice, knockout of the 5-HT2C receptor results in increased feeding and obesity (Tecott *et al.,* 1995). Antipsychotic drugs such as clozapine and olanzapine which are related to the greatest weight gain have high affinities for the 5-HT2C receptors.

It has been suggested that 5-HT regulates NPY in mice (Redrobe *et al.,* 2005). The 5-HT 1B/2C receptor agonist meta-Chlorophenylpiperazine (mCPP) results in the rat in

decreased food intake and NPY levels in the PVN indicating that the NPY may mediate the effect of 5-HT on food intake (Dryden *et al.,* 1996). The PVN is an important region involving food intake and body weight regulation and it is rich in the 5-HT2C receptor (Abramowski *et al.,* 1995; Clemett *et al.,* 2000). In addition, 5-HT2C also has an interaction with leptin; 5-HT2C antagonists attenuate the decreased food intake which is induced by leptin in rats (Hay-Schmidt *et al.,* 2001). Antipsychotic drug treatment can affect NPY neuron expression; chronic treatment with clozapine results in an increase in NPY-immunoreactive cell density in rat arcuate nucleus (Kirk *et al.,* 2006), indicating that antagonism of the 5-HT2C receptors may, in part, result in disinhibition of the NPY neurons and also indicates the role of NPY in clozapine-induced weight gain.

The role of the 5-HT2C receptor in antipsychotic drug-induced weight gain is also demonstrated by pharmacogenetic studies. For example, Yuan *et al.* (2000) have identified several haplotypes of genetic polymorphisms (-995G/A, -759C/T and -697G/C) in the promoter region of the 5-HT2C receptor gene which are associated with obesity and diabetes in which the frequency of -995/-759/ and -697/ variants (A-T-C) was higher in non-obese subjects and non-diabetic subjects. The association of the *HTR2C* -759C/T with antipsychotic drug-induced weight gain is mentioned previously in **section 1.3**. In addition, the T allele of the *HTR2C* is associated with higher leptin levels (Templeman *et al.,* 2005).

1.3.3.1.2 Histamine receptors

Histamine is involved in food intake regulation. Antihistamine drugs enhance food intake and increase appetite (Orthen-Gambill, 1988). Histamine H1 (H1) receptors may be involved in antipsychotic drug-induced weight gain through influence on food intake regulation and lower physical acitivity (enery expenditure) because H1 antagonism also cause sedation (Richelson and Souder, 2000). Many antipsychotic drugs have high histamine H1 receptor affinities which are closely correlated with antipsychotic drug-induced weight gain such as that with clozapine and olanzapine. The H1 receptor affinity is suggested to be the most reliable predictor of weight gain (Wirshing *et al.,* 1999). Consistent studies in animals show that H1 receptor antagonism increases feeding in rodents and H1 knockout mice are prone to weight

gain (Masaki *et al.,* 2001; Masaki *et al.,* 2004). In these H1 knockout mice exhibit partially attenuated leptin-induced feeding suppression (Masaki *et al.,* 2001; Masaki *et al.,* 2004). These findings indicate the involvement of histamine neurons and the H1 receptors regulating food intake in affecting downstream signaling of leptin in the brain.

Olanzapine treatment, but not aripiprazole or haloperidol treatment, reduces H1 receptor mRNA expression in the ARC and downstream regions (Han *et al.,* 2008). In addition, olanzapine treatment increases histamine neurotransmission which may be related to the change in receptor expression (Davoodi *et al.,* 2008). Furthermore, olanzapine, clozapine, and quetiapine, but not risperidone, enhance H1-receptor-mediated 5' adenosine monophosphate-activated protein kinase (AMPK) in the hypothalamus (Kim *et al.,* 2007). Risperidone and quetiapine have relative similar effects on weight gain but exhibit different effect on AMPK in the hypothalamus suggesting that there are other receptors such as 5-HT2C receptors in conjunction with dopamine D2 receptor that may also contribute to weight gain in different degrees with different drugs.

1.3.3.1.3 Adrenergic receptors

The adrenergic system plays a role in food intake regulation. Acute injections of noradrenaline (norepinephrine) into rat PVN cause hyperphagia and chronic injections result in body weight gain (Leibowitz *et al.,* 1984). Two subtypes of alpha-adrenergic receptors (α1- and α2-adrenoceptors) within the hypothalamic PVN exert opposing effects on food intake regulation. Injection of the α1-adrenoceptor agonists, phenylpropanolamine (PPA), cirazoline, methoxamine, or 1-phenylephrine into the rat PVN decrease food intake (Wellman and Davies, 1992), and systemic injections of the α1-adrenergic antagonist prazosin (PRAZ) can effectively reverse the anorexia induced by systemic injections of PPA as well as cirazoline (Wellman and Davies, 1992). Administration of the α2-adrenoceptor agonist, clonidine, stimulates food intake when injection either into PVN (Goldman *et al.,* 1985) or periphery (McCabe *et al.,* 1984). Antagonist actions at α1-adrenoceptors of antipsychotic drugs could account for weight gain (Richelson and Souder, 2000; Reynolds and Kirk, 2010). In addition, the

genetic polymorphism of the α2A-adrenoceptors may also be involved in antipsychotic drug-induced weight gain (Park *et al.,* 2006).

Beta-adrenoceptors (β-adrenoceptors) also play a role in body weight regulation. Knockout mice for all β-adrenoceptors (β1/β2/β3) develop a progressive obesity in adulthood (Bachman *et al.,* 2002) indicating the role of these receptors in resistance to obesity. In addition, implication of β-adrenoceptors in weight gain is suggested by pharmacogenetic studies. Meta-analysis of human studies found that the genetic polymorphism of β3-adrenoceptor (Trp64Arg) is associated with obesity (Fujisawa *et al.,* 1998; Hoffstedt *et al.,* 1999; Thomas *et al.,* 2000); with the presence of the arginine allele exhibiting higher BMI and predicting weight gain. One study in schizophrenia patients found a trend (p=0.1) between the Trp64Arg polymorphism and clozapine-induced weight gain (Basile *et al.,* 2001). However, others studies fail to observe the association (Allison and Heo, 1998). The central β-adrenoceptors are not the sites of antipsychotic drugs. And the two drugs with the highest liability for weight gain, clozapine and olanzapine do not have significant affinity for β-adrenoceptors.

1.3.3.1.4 Dopamine receptors

Dopamine plays an important role in determining food intake through action in many areas of the brain including hypothalamus. The effects of dopamine can vary depending on brain areas and its concentrations. Dopamine release in nucleus accumbens is associated with the reinforcement effect in feeding (Hajnal *et al.,* 1997). In the hypothalamus, dopamine release in hypothalamic VMN and LHA affects the feeding pattern by influencing duration of meal consumption (the size of a meal) and the frequency of meals (Meguid *et al.,* 2000). Amphetamine-induced anorexia is completely reversed by receiving the selective dopamine D1 receptor antagonist, but it is not affected by treatment with the selective D2 receptor antagonist (Gilbert and Cooper, 1985). However, intrahypothalamic injections of sulpiride, a specific D2 receptor blocker, can attenuate amphetamine-induced anorexia in food-deprived rats and increase feeding and water drinking in satiated rats (Parada *et al.,* 1988). In addition, D2 receptor antagonists illustrate their influence on feeding behaviour (Clifton *et al.,* 1991). These studies suggest a role of dopamine and its receptors in feeding regulation. Measurement by using PET and [C-11]raclopride (a radioligand for

the dopamine D2 receptor), brain dopamine D2 receptor availability found to be lower in obese individuals compared to controls; this decreased availability of dopamine D2 receptor in obese subjects is correlated with increased BMI (Wang *et al.,* 2001). Thus, it has been suggested that obese individuals have a dopamine deficiency which cause an increase in food intake leading to obesity. Treatment with antipsychotic drugs may decrease dopamine and induced food intake. However, atypical antipsychotic drugs which are associated with higher incidence of weight gain have lower D2 receptor occupancy at effective doses compared to typical antipsychotic drugs (Kapur *et al.,* 2000; Pilowsky 2001).

1.4 Genetic variants associated with antipsychotic drug-induced weight gain in patients with schizophrenia

In addition to the *HTR2C* -759C/T polymorphism that has influence on antipsychotic drug-induced weight gain as mentioned above (**section 1.3**), there are many other genetic polymorphisms that have been proposed or reported to be associated with antipsychotic drug-induced weight gain which are shown in **Table 1.4**.

Table 1.4: Candidate genes associated with antipsychotic drug-induced weight gain

Gene	Name	Location	Function(s)*	SNP related AIWG	Reference
ADIPOQ	Adiponectin	3q27	fat metabolism, insulin sensitivity, direct anti-diabetic, anti-atherogenic and anti-inflammatory activities	rs1501299	(Wu *et al.,* 2011)
ADRA2A	Adrenoceptor alpha 2A	10q25.2	modulation of neurotransmission, smooth muscle contraction, and thermoregulation	rs1800544	(Park *et al.,* 2006; Wang *et al.,* 2005b; Sickert *et al.,* 2009)
ADRB3	Adrenoceptor beta 3	8p12	lipolysis and thermogenesis	rs4994	(Ujike *et al.,* 2008)
BDNF	Brain-derived neurotrophic factor	11p14.1	survival and differentiation of neurons, synaptic transmission and neuroplasticity	rs6265	(Zhang *et al.,* 200;, Zai *et al.,* 2012; Tsai *et al.,* 2011)
CCKBR	Cholecystokinin B receptor	11p15.4	anxiety, nociception, neuroleptic activity, gastric acid release, gastric	rs2929183	(Tiwari *et al.,* 2010b)

Gene	Name	Location	Function(s)*	SNP related AIWG	Reference
			mucosal cell growth and histamine release		
CNR1	Cannabinoid receptor 1	6q14-q15	is involved in the cannabinoid-induced CNS effects (including alterations in mood and cognition)	rs806378	(Tiwari *et al.*, 2010a)
DRD2	Dopamine D2 receptor	11q23.2	modulation of locomotion, reward, reinforcement and memory and learning	rs6277, rs1079598, rs1800497, rs4436578, rs1799732	(Muller *et al.*, 2012; Hong *et al.*, 2010; Lencz *et al.*, 2010)
FTO	Fat mass and obesity-associated protein	16q12.2	Dioxygenase (repairs alkylated DNA and RNA by oxidative demethylation), regulation of energy homeostasis, body size and body fat accumulation	rs9939609, rs9922047	(Reynolds *et al.*, 2013; Song *et al.*, 2014; Tiwari *et al.*, 2011)
GHRL	Ghrelin	3p26-p25	stimulation of hunger, appetite, gastric acid secretion and gastrointestinal motility, and adiposity	rs27647	(Yang *et al.*, 2012)
GNB3	Guanine nucleotide binding protein (G protein), beta polypeptide 3	12p13	modulator or transducer in various transmembrane signaling systems	rs5443	(Ujike *et al.*, 2008; Wang *et al.*, 2005a)
HTR2A	Serotonin 2A receptor	13q14-q21	appetite control, regulation of neural activity, perception, cognition, mood, behavior, sleep, and thermoregulation,	rs6311 rs6313	(Mou *et al.*, 2005; Ujike *et al.*, 2008)
LEP	Leptin	7q31.3	regulation of energy homeostasis, obesity, reproduction, glucose homeostasis, bone formation, wound healing and immune system	rs7799039, rs4731426	(Templeman *et al.*, 2005; Wu *et al.*, 2011; Kuo *et al.*, 2011; Srivastava *et al.*, 2008)
LEPR	Leptin receptor	1p31	receptor for mediating leptin effects	rs1137101	(Gregoor *et al.*, 2009; Gregoor *et al.*, 2011)
MC4R	Melanocortin 4 receptor	18q22	energy homeostasis, somatic growth,	rs489693, rs17782313,	(Malhotra *et al.*, 2012;

Gene	Name	Location	Function(s)*	SNP related AIWG	Reference
			pigmentation, inflammation, immunomodulation, steroidogenesis, and temperature control	rs8087522, rs2229616	Czerwensky *et al.*, 2013a, 2013b, Chowdhury *et al.*, 2013; Kuo *et al.*, 2011)
MTHFR	Methylene-tetrahydrofolate reductase	1p36.3	key enzyme in folate metabolism, important in epigenetic modification	rs1801131, rs1801133	(Ellingrod *et al.*, 2008; van Winkel *et al.*, 2010a, 2010b)
NPY	Neuropeptide Y	7p15.3	control of feeding, circadian rhytm, pituitary hormone release, anxiolysis	rs16147, rs5573, rs5574	(Tiwari *et al.*, 2013)
PPARG	Peroxisome proliferator-activated receptor gamma	3p25	regulation of adipocyte differentiation	rs1801282	(Herken *et al.*, 2009)
SNAP-25	Synaptosomal-associated protein, 25kDa	20p12-p11.2	regulation of neurotransmission release	rs1051312, rs3746544, rs8636	(Musil *et al.*, 2008)

AIWG, antipsychotic drug-induced weight gain; *data from database (www.genecards.org)

From **Table 1.4**, some of the genetic polymorphisms which are strong candidate polymorphisms associated with antipsychotic drug-induced weight gain include those in: *MTHFR* which is a strong candidate gene associated with epigenetic modification and metabolic syndrome; *HTR2A* and *ADRA2A*, which are antipsychotic drug target receptors and involved in appetite control; *FTO*, *MC4R*, and *BDNF* which are the strong candidate genes associated with BMI in GWAS; *GNB3* which is involved in mediating the serotonergic effect. These strong candidate polymorphisms associated with antipsychotic drug-induced weight gain on which this work has focused will be described in the following section.

1.4.1 *MTHFR*

Recently, genetic variants of the methylenetetrahydrofolate reductase (*MTHFR*) gene have been proposed as potential predictors for antipsychotic induced metabolic side effects (Kuzman and Muller, 2012). MTHFR exerts an important role in folate and homocysteine metabolism by catalysing the reduction of 5,10-

methylenetetrehydrofolate to 5-methylenetetrahydrofolate (5-MTHF), which is used in methionine synthesis from homocysteine. The methionine is further converted to S-adenosylmethionine (SAM), which is a major methyl donor in a wide variety of enzymatic processes including the methylation of DNA (Fox and Stover, 2008). MTHFR deficiency can increase serum homocysteine while the decrease in 5-MTHF and SAM causes deficits in DNA methylation, DNA synthesis and repair, and may predispose to neurodevelopmental and oncogenic processes resulting in the development of many disorders including cardiovascular disease, renal failure, cancer and congenital abnormalities (Ueland *et al.,* 2001).

High plasma homocysteine is a risk factor for metabolic syndrome (Yakub *et al.,* 2014), although the link between homocysteine and metabolic syndrome is not well established. Elevated homocysteine also is an independent risk factor for cardiovascular diseases such as coronary heart disease and stroke (Selhub 2008; Wang *et al.,* 2007a). Homocysteine has been shown to be a thrombogenic and atherogenic substrate that may be lead to atherosclerotic phenomena and adverse cardiometabolic events (Guilland *et al.,* 2003). Hyperhomocysteinemia also has been linked to hypertension (Yakub *et al.,* 2014; Lim and Cassano, 2002; Wang *et al.,* 2014) via homocysteine-induced arteriolar vasoconstriction (Vermeulen *et al.,* 2001; Mujumdar *et al.,* 2002), vascular endothelia injury (Harker *et al.,* 1983), and decreased vasodilator responsiveness (Cheng *et al.,* 2011). On the other hand, homocysteine has been linked to lower high-density lipoproteins (HDL) (Yakub *et al.,* 2014; Baszczuk *et al.,* 2014). Global DNA hypomethylation in vascular smooth muscle cells has been suggested as a mechanism linking homocysteine to atherosclerosis (Obeid and Herrmann, 2009) as well as lipid accumulation in tissues (Yideng *et al.,* 2007).

The functional polymorphisms of *MTHFR* located within the coding region include 677C/T (rs1801133) and 1298A/C (rs1801131). The T allele of the 677C/T SNP causes an amino acid substitution from an alanine to a valine at codon position 222 (exon 4) (Frosst *et al.,* 1995). The C allele of the 1298A/C results in a change from a glutamine to alanine at codon position 429 (exon 7) and is found in the regulatory region of the MTHFR enzyme (van der Put *et al.,* 1998). The variant alleles of these SNPs (T allele of 677C/T and C allele of the 1298A/C) are associated with reduction in MTHFR enzyme activity (Le Marchand *et al.*, 2002). Therefore, the SNPs located in coding regions that

alter amino acids may affect protein funtion throught alteration of conformation or stability of protein especially at the catalytic binding site. In addition to a change in the encoded amino acids (nonsynonymous), SNPs can be silent (synonymous) or occur in the noncoding regions. They may influence promoter activity (and so gene expression), messenger RNA (mRNA) conformation (stability), and subcellular localization of mRNAs and/or proteins and hence may produce disease (Shastry, 2009), for example the T allele of the *MTHFR* 677C/T is significantly associated with schizophrenia (Lochman *et al.*, 2013).

The association of *MTHFR* polymorphisms with metabolic syndrome has been reported in the general population. Obesity has been associated with *MTHFR* 1298A/C (Terruzzi *et al.,* 2007) and 677C/T genotypes (Lewis *et al.,* 2006). Carriage of the 677T allele is associated with insulin resistance (Chen *et al.,* 2010; Lunegova *et al.,* 2011). Association of the 677T allele with central obesity, hypertriglyceridemia and low levels of HDL cholesterol was also reported in the latter study (Lunegova *et al.,* 2011). A replicated study reported that the 677T allele but not the 1298A/C polymorphism of *MTHFR* was associated with a greater risk of developing metabolic syndrome and the TT genotype was associated with risk of insulin resistance with greater central adiposity induced by antipsychotic treatment (Ellingrod *et al.,* 2008, 2012). Others have reported the association of metabolic syndrome in schizophrenia with the 1298A/C polymorphism in 518 Caucasian patients (van Winkel *et al.,* 2010a). These authors also reported that the 1298C variant was associated with an increased weight and impaired glucose tolerance in 104 Caucasian patients who had received antipsychotic treatment for 3 months (van Winkel *et al.,* 2010b).

1.4.2 *ADRA2A*

Genetic variants of the adrenergic α-2a receptor (*ADRA2A*) gene have been proposed as a predictor for antipsychotic drug-induced weight gain. ADRA2A receptor has a critical role in regulating hypothalamic-pituitary-adrenal (HPA) axis and in regulating neurotransmitter release from sympathetic and adrenergic nerves in the brain (Delitala *et al.,* 1994; Langer, 1997; Hein *et al.,* 1999). This receptor plays an important role in lipolysis, thermogenesis and also regulation of food intake (Park *et al.,* 2006; Arner, 1992). Activation at alpha-2 adrenergic receptors decreases heat production and

lipolysis rate (Arner, 1992). In addition, direct injection of a selective alpha-2 adrenergic receptor agonist into the PVN of rats resulted in increased food intake (Wellman *et al.,* 1993). Obesity-prone rats showed lack of ability to alter brain alpha-2 adrenergic receptors in regulating glucose levels (Levin and Planas, 1993). In addition, a direct overexpression of *ADRA2A* in pancreatic beta cells of transgenic mice may alter the regulation of insulin secretion and glucose metabolism (Devedjian *et al.,* 2000).

The -1291C/G polymorphism is located in the promoter region or regulatory region of the *ADRA2A* gene and could therefore affect the gene expression and so alter receptor density. Previous findings found that the -1291C/G polymorphism was significantly associated with receptor binding and density (Deupree *et al.,* 2006). The C allele of this SNP is significantly associated with a protective effect for schizophrenia risk (Lochman *et al.*, 2013). The G allele shows a better response to methamphetamine treatment (Cheon *et al.,* 2009; Polanczyk *et al.*, 2007). This SNP has been reported to be associated with body fat accumulation (Garenc *et al.,* 2002) and sympathetic-HPA system in the hypothalamus (Rosmond *et al.,* 2002a).

The ADRA2A -1291C/G polymorphism has been associated with olanzapine-/clozapine-induced weight gain in Asian schizophrenia patients (Park *et al.,* 2006; Wang *et al.,* 2005b); G allele carriers showed more weight gain. However, European samples have shown the opposite; the G allele was reported to be protective against weight gain following antipsychotic treatment (Sickert *et al.,* 2009); some studies did not observe an association of the SNP with weight gain or metabolic syndrome in schizophrenia patients (De Luca *et al.,* 2011; Risselada *et al.,* 2010).

1.4.3 *HTR2A*

The 5-HT2A receptor is an important site of action of atypical antipsychotic agents (Kane, 1994). Several post-mortem studies have shown a decrease in 5-HT2A receptor density in the frontal cortex of schizophrenic patients (Mita *et al.,* 1986; Laruelle *et al.,* 1993). In addition, association between the *HTR2A* 102T/C polymorphism and schizophrenia has been reported (Arranz *et al.,* 1995; Williams *et al.,* 1996).

The 5-HT2A receptor is also involved in food intake and cortisol secretion (Currie and Coscina, 1998; Rittenhouse *et al.,* 1994). There is evidence illustrating the role of cortisol in abdominal obesity (Bjorntorp and Rosmond, 2000). It has long been recognized that the serotonin is involved in regulating cortisol secretion or HPA axis function (Dinan, 1996), and it has been suggested that cortisol secretion is regulated by central 5-HT2A (Rittenhouse *et al.,* 1994). Furthermore, administration of 5-HT2A receptor agonists decreased the NPY stimulated food intake in rats (Currie and Coscina, 1998).

The *HTR2A* -1438G/A polymorphism is located in the promoter region and is associated with promoter activity; the presence of an A allele has greater promoter activity relative to the G allele (Parsons *et al.,* 2004), although an earlier study failed to find the difference between the G and A alleles in luciferase reporter gene expression (Spurlock *et al.,* 1998). Therefore, the effect of genetic polymorphism on transcriptional activity of the *HTR2A* gene to bring about a difference in receptor expression is still unknown.

In the general population, the *HTR2A* -1438 GG genotype is associated with greater BMI, waist-to-hip ratio, and abdominal sagittal diameter along with less suppression of cortisol suggesting the involvement of the HPA-axis and serotonergic system in the pathology of abdominal obesity (Rosmond *et al.,* 2002b). In addition, the *HTR2A* -1438A allele is associated with lower energy intake and alcohol consumption in a French sample of overweight subjects (Aubert *et al.,* 2000); however, the association was not significant after correction for the number of comparisons. Some studies found that the *HTR2A* -1438G/A was not related to BMI or obesity (Aubert *et al.,* 2000; Hinney *et al.,* 1997). The *HTR2A* -1438G/A was associated with clinical response to clozapine in which homozygosity for the G allele was more frequent among non-responders than in responders (Arranz *et al.,* 1998). Ujike *et al.* (2008) reported the association between multiple genetic polymorphisms of the 102T allele of *HTR2A* rs6313, the 825T allele of G-protein beta3 subunit (*GNB3*), the 23Cys allele of *HTR2C* with olanzapine-induced weight gain in schizophrenia patients in Japan). Other studies reported the association between the 102T allele and risperidone-induced weight gain in Chinese patients, and the association between TT genotype and weight gain in Caucasians with multiple antipsychotic drug treatments (review by (Balt *et al.,* 2011).

Another study found no significant association of the *HTR2A* -1438G/A polymorphism with antipsychotic drug-induced weight gain in Chinese Han patients with schizophrenia (Mou *et al.,* 2005). The *HTR2A* -1438G/A is in complete linkage disequilibrium (LD) with the *HTR2A* 102C/T polymorphism (A with T) (McMahon *et al.,* 2006).

1.4.4 *MC4R*

The melanocortin-4-receptor (MC4R) is a key in regulating food intake, body weight, and glucose homeostasis (Cone, 2006). MC4R is a G protein-coupled receptor activated by α-Melanocyte-stimulating hormone (α-MSH) and blocked by agouti-related protein (AgRP) (Hinney *et al.,* 2013; Tao 2010). MC4R is not only associated with regulation of energy balance by decreasing food intake and increasing energy expenditure (Fan *et al.,* 1997), it has also been associated with nutrient absorption, lipid metabolism, adiposity, thermogenesis, insulin secretion, and appetite (Adan *et al.,* 2006; Malhotra *et al.,* 2012). In addition, the melanocortin pathway has interactions with other pathways or hormones such as leptin, 5-HT (Zhou *et al.,* 2007), NPY, AgRP, POMC (Biebermann *et al.,* 2012), and autonomic nervous system (Rossi *et al.,* 2011; Sohn *et al.,* 2013), glucagon-like peptides (Guan *et al.,* 2012; Ma *et al.,* 2007), cholecystokinin (Fan *et al.,* 2004), and vagal afferent fibers (Gautron *et al.,* 2010).

The *MC4R* is a very strong candidate gene influencing antipsychotic drug-induced weight gain. The rs17782313, a common genetic variant is located 188 kb downstream from *MC4R* gene has no known functional relevance but it has been recently identified as a gene for obesity susceptibility in a genome-wide association study (GWAS) of 249,796 individuals (Speliotes *et al.,* 2010) in which the C allele has been associated with higher BMI, food intake, body fat mass, weight, and risk of obesity (Loos *et al.,* 2008; Stutzmann *et al.,* 2009; Xi *et al.,* 2012; Loos 2011). The mechanistic link may be due to the risk allele of this polymorphism influencing reward mechanisms, particularly in females because a recently study reported that female homozygous risk allele carriers showed significant increase in grey matter volume in the right amygdala, which is a region known to influence eating behaviour, and in the right hippocampus (Horstmann *et al.,* 2013). In addition, the obesity effect of the risk allele may be due cerebral insulin resistance (Tschritter *et al.,* 2011). It has been hypothesized that the

polymorphism may down-regulate the *MC4R* function or *MC4R* expression but further study is required to address this question.

There have been two studies investigating the association of the rs17782313 C/T and antipsychotic drug-induced weight gain; the first study found the significant association between the C allele and weight gain after a 4-week second generation antipsychotic drug treatment in Caucasian patients (Czerwensky *et al.,* 2013a). However another study did not observe any association between rs17782313 and antipsychotic drug-induced weight gain after a 14-week treatment in European-ancestry patients (Chowdhury *et al.,* 2013). Therefore, further studies are needed to confirm the association of this polymorphism with antipsychotic drug-induced weight gain.

Another SNP, the rs489693 A/C polymorphism located approximately 190 kb downstream from the *MC4R* gene has no known functional relevance but it has been identified in GWAS to be associated with second generation antipsychotic drug-induced weight gain in 4 cohorts (Malhotra *et al.,* 2012) in which the AA genotype was associated with greater weight gain. This finding has been replicated in another study of Caucasian schizophrenia patients (Czerwensky *et al.,* 2013b).

1.4.5 *GNB3*

The G-protein beta3 subunit (GNB3) protein plays important roles in intracellular signal transduction and adipogenesis (Allison *et al.,* 1999; Comuzzie and Allison, 1998; Malbon, 1997). The *GNB3* 825C/T polymorphism is located on exon 10 of the *GNB3* gene; the T allele was found to be associated with the alternative splicing with a deletion of 41 amino acids, this splicing variant is active (enhances G-protein activation) and increases cellular responses, and thus increased *in vitro* cell proliferation and increased intracellular signal transduction (Siffert *et al.,* 1998). The polymorphism has been associated with many pathophysiological conditions, for example: the T allele is associated with an increased risk for hypertension (Siffert *et al.,* 1998), BMI and obesity (Siffert *et al.,* 1999), type II diabetes (Kiani *et al.,* 2005), and depression (Klenke *et al.,* 2011). The influences of *GNB3* 825C/T polymorphism on lipolysis has been also reported that the T allele results in decreased GNB3 production

in fat cells and thus inhibit lipolysis via β1-, β2-, and β2a-adrenergic receptor signalling (Ryden *et al.,* 2002).

The TT genotype of the *GNB3* 825C/T polymorphism has been associated with antipsychotic drug-induced weight gain in Japanese schizophrenia patients who are receiving olanzapine for 8-24 weeks (Ujike *et al.,* 2008) and also in Chinese schizophrenia patients who are receiving clozapine for 13 months (Wang *et al.,* 2005a). However, several studies and meta-analyses failed to observe a significant association (Tsai *et al.,* 2004; Park *et al.,* 2009; Bishop *et al.,* 2006; Souza *et al.,* 2008). These controversies suggest that more studies are needed to elucidate this association. Interestingly, the C allele of the *GNB3* 825C/T polymorphism which exhibits lower signal transduction has been associated with clinical improvement with antipsychotic treatment in schizophrenia patients (Muller *et al.,* 2005; Kohlrausch *et al.,* 2008). This finding suggesting that the genetic susceptibility for decreased signal transduction may enhance antipsychotic efficacy as well as antipsychotic adverse effects.

1.4.6 *BDNF*

Brain-derived neurotrophic factor (BDNF) plays critical roles in the nervous system including neuronal cell growth and maintenance, differentiation, activity-dependent plasticity and survival of neurons in the central nervous system (Noble *et al.,* 2011). BDNF influences many neurotransmitters such as noradrenergic, dopaminergic, serotonergic, glutamatergic, and cholinergic neurotransmitters (Gratacos *et al.,* 2007; Russo-Neustadt, 2003; Tapia-Arancibia *et al.,* 2004). Therefore, BDNF is implicated in many mental disorders and disturbed behaviors (Russo-Neustadt, 2003). Serum BDNF was found to be decreased in schizophrenia patients (Zakharyan and Boyajyan, 2014). In addition, the antipsychotic drugs may also affect the synthesis of BDNF in the brain; chronic olanzapine or risperidone treatment resulted in decrease BDNF in rat brains (Angelucci *et al.,* 2000; Angelucci *et al.,* 2005).

It is reported that BDNF is important in food intake and body weight regulation (Lebrun *et al.,* 2006). Studies in animals and humans indicated that hypothalamic BDNF and its receptor, tropomyosin-related kinase B (TrkB) appear to inhibit food intake and increase energy expenditure, being therefore related to body weight gain and obesity (Unger *et al.,* 2007; Toriya *et al.,* 2010; Zhang *et al.,* 2007). On the other hand, brain

infusion of BDNF in rats resulted in appetite suppression, weight loss, and increases in 5-HT (Pelleymounter *et al.,* 1995). BDNF is highly expressed in the VMN hypothalamus (Unger *et al.,* 2007) which is involved in regulation of energy balance. Nicholson *et al.* (2007) demonstrated that activation of MC4R causes an increase in BDNF release from hypothalamus in both *in vitro* and *in vivo* experiments, indicating that BDNF is an important downstream mediator of MC4R signaling in regulation of food intake. The effect of BDNF in MC4R signaling is dependent on TrkB activation (Tsao *et al.,* 2008).

The *BDNF* rs6265 G/A polymorphism is a missense change (G196A) in the coding exon of the *BDNF* gene at position 66 which results in a non-conservative amino acid change from valine to methionine and it appears to disrupt cellular processing, protein trafficking, and activity-dependent BDNF secretion (Egan *et al.,* 2003; Chen *et al.,* 2004). This SNP was also associated with the dendritic targeting disruption of *BDNF* mRNA (Chiaruttini *et al.,* 2009). The Met allele (or A allele) has been associated with decreased BDNF in schizophrenia patients (Zakharyan and Boyajyan, 2014), while the Val/Val genotype was associated with the improvement in clinical symptom response to olanzapine (Nikolac Perkovic *et al.,* 2014) and clozapine (Zai *et al.,* 2012; Zhang *et al.,* 2013b). However, other studies have found no association of the polymorphism with treatment response to risperidone (Xu *et al.,* 2010), clozapine (Hong *et al.,* 2003), chlorpromazine (Xu *et al.,* 2008), or typical antipsychotic drugs (Anttila *et al.,* 2005). The genetic polymorphism of *BDNF* rs6265 Met/Met (or AA) genotype has been reported to be associated with antipsychotic drug-induced weight gain, especially in a male subgroup in Chinese chronic schizophrenia (Zhang *et al.,* 2008). Furthermore, a haplotype of *BDNF* rs6265 and *BDNF* rs1519480 (G-A haplotype) was associated with atypical antipsychotic drug-induced weight gain in European ancestry chronic schizophrenia (Zai *et al.,* 2012). However, one study did not find the association between the *BDNF* rs6265 G/A polymorphism and antipsychotic drug-induced weight gain in Chinese chronic schizophrenia treated with atypical antipsychotic drugs including clozapine, olanzapine, or risperidone but the *BDNF* rs11030101 TT genotype and the rs11030101-T-allele-related haplotype (rs11030101, rs6265, and rs12291186) was also associated with weight gain (Tsai *et al.,* 2011).

1.4.7 *FTO*

The fat mass and obesity-associated gene (*FTO*) gene was the first obesity-related gene discovered by large-scale GWAS in 2007 and it was strongly associated with BMI and obesity (Frayling *et al.,* 2007) which was replicated in different populations (Fawcett and Barroso, 2010). Epidemiological and functional studies suggest that *FTO* confers an increased risk for obesity through change in food intake and preference (Loos and Yeo, 2014). *FTO* is highly expressed in the hypothalamus which regulates energy balance (Willer *et al.,* 2009). The expression of *FTO* has been associated with food intake. Studies in mice reported that complete or partial inactivation of the *Fto* gene protected from obesity (Church *et al.,* 2009; Fischer *et al.,* 2009) whereas the overexpression of *Fto* increased food intake and obesity (Church *et al.,* 2010). The expression of *FTO* is nutritionally regulated; deprivation of the essential amino acids caused down-regulation of *FTO* mRNA and protein in mouse and human cell lines suggesting a role of *FTO* in sensing of essential amino acid availability (Cheung *et al.,* 2013). The FTO protein is involved in the hypothalamic leptin signaling pathway (Wang *et al.,* 2011). These findings suggested that the expression of *FTO* and leptin signaling pathway may influence the regulation of food intake.

Another biological function of *FTO* is a nucleic acid demethylase. Several *in vitro* studies have shown that FTO is a single-stranded DNA and RNA demethylase and is involved in nucleic acid repair or modification processes (Jia *et al.,* 2008; Gerken *et al.,* 2007; Han *et al.,* 2010), and this role of FTO may influence the expression of certain genes through epigenetic modification.

The *FTO* rs9939609 polymorphism is located in intron-1 of the *FTO* gene (Cheung and Yeo, 2011), and the association of the minor A allele of the polymorphism with increased BMI has been well documented (Frayling *et al.,* 2007; Qi *et al.,* 2014). The *FTO* rs9939609 polymorphism has been associated gene expression; the A allele of *FTO* rs9939609 was associated with increased levels of the *FTO* transcripts studied in skin fibroblasts and peripheral blood (Berulava and Horsthemke, 2010). The *FTO* rs9939609 minor A allele was also associated with increases in total, fat, and protein dietary intake (Timpson *et al.,* 2008; Speakman *et al.,* 2008), and increased hunger and decreased satiety (Wardle *et al.,* 2008; den Hoed *et al.,* 2009). In addition, the

participants with the A allele of the *FTO* rs9939609 polymorphism were more likely to prefer a meat-based diet compared with the TT participants who preferred a plant-based diet (Yang *et al.,* 2014).

There have been several studies that demonstrated the association of polymorphisms of *FTO* with DNA methylation of *FTO* gene itself. Bell *et al.* studied the genotype-epigenetic interaction and identified haplotype-specific methylation in the *FTO* LD block (46kb) T2DM/obesity-susceptibility locus. They found increased DNA methylation on an *FTO* obesity susceptibility haplotype tagged by the rs8050136 risk allele A. The *FTO* obesity susceptibility haplotype contained rs1421085 (C allele), rs17817449 (G allele), rs8050136 (A allele) which was a tagged risk allele, rs3751812 (T allele), rs9939609 (A allele), rs7202116 (G allele), and rs9930506 (G allele) which were in complete linkage disequilibrium (LD) (Bell *et al.,* 2010). Toperoff *et al.* (2012) reported a significant hypomethylation of a CpG site in the first intron of the *FTO* gene of type 2 diabetes mellitus (T2DM) subjects relative to controls and this effect was independent of the polymorphism; the CpG methylation site is located near rs1121980 which is a T2DM/obesity-associated polymorphic site. These studies demonstrate the association of *FTO* DNA methylation and associated diseases as well as the association between the *FTO* polymorphisms and *FTO* DNA methylation that might provide an epigenetic marker of diseases.

Not only *FTO* polymorphisms had an effect on DNA methylation in *FTO* gene itself, it also had an effect on DNA methylation on other genes; the first evidence was provided by Almén *et al.* (2012) using genome-wide analysis to determine the association of the *FTO* rs9939609 with epigenetic changes in Greek preadolescent girls and they reported a significant differential methylation level in many genes between carriers of the *FTO* rs9939609 TT and AA (risk allele), and also found a significant differential methylation level in many genes between obesity and normal-weight female controls.

Not only DNA methylation, but also RNA methylation is influenced by FTO. An *in vitro* study showed that recombinant human FTO protein exhibited slightly higher efficiency in oxidative demethylation of 3-methyluracil (3-meU) in single-stranded RNA (ssRNA) than 3-methylthymine (3-meT) in single-stranded DNA (ssDNA) which suggesting that the methylated RNAs are the preferred substrates for FTO (Jia *et al.,* 2008). Analysis of

global N(6)-methyladenosine (m(6)A) modification of RNA in the midbrain and striatum of *Fto*-deficit mice found that there was an increase in adenosine methylation in a subset of RNAs important for neuronal signaling, including dopamine transmission (Hess *et al.,* 2013). Study in mice showed the *Fto* gene was a transcriptional coactivator that enhanced the binding of the CCAAT/enhancer binding proteins (C/EBPs) to unmethylated and methylated DNA, this results in promotion of the transcriptional functions of C/EBP family, suggesting a role of *Fto* in epigenetic regulation of adipogenesis (Wu *et al.,* 2010).

Association between the *FTO* rs9939609 polymorphism and antipsychotic drug-induced weight gain has been published. A study in first episode Chinese Han schizophrenia patients (n=250) demonstrated that the body weight and BMI of the TT genotype carriers were significantly lower than those of the A allele patients both at baseline and after risperidone treatment for 6 months (Song *et al.,* 2014). In another study of first episode schizophrenia patients in Spain (n=239) found a significant association of the AA genotype of the *FTO* rs9939609 polymorphism with the higher baseline BMI compared to AT/TT group but there was no significant difference of weight increase between two groups of genotype after 1 year antipsychotic treatment (Perez-Iglesias *et al.,* 2010). Reynolds *et al.* (2013) demonstrated that the A allele of the *FTO* rs9939609 polymorphism has been associated with BMI in chronic schizophrenia patients but not with weight gain in first-episode schizophrenia patients, although the AA genotype had higher baseline weight and baseline BMI than T allele carriers. However, other studies in chronic schizophrenia failed to observe the association between the polymorphism and BMI or antipsychotic drug-induced weight gain (Watanabe *et al.,* 2012; Shing *et al.,* 2014).

1.5 Epigenetic factors associated with antipsychotic drug-induced weight gain

1.5.1 Epigenetic mechanisms

Normally, a cell contains the complete hereditary information in the form of DNA. The complete DNA sequences in a cell are known as the genome. The basic building blocks of DNA are nucleotides that contain bases Adenine (A), Thymine (T), Cytosine (C), and Guanine (G). The DNA chain wraps around histones forming a structure known as nucleosome. Multiple nucleosomes form chromatin. The chemical modification on DNA and histones that does not alter the DNA sequences which control gene expression, is known as epigenetics; in other words, epigenetics is the study of changes in gene function that does not involve changes in DNA sequence. The epigenetic field is growing rapidly; but the definition of epigenetic terms is still evolving. Mann (2014) has summarized current definitions of epigenetics as shown in **Table 1.5**.

Table 1.5: Some current definitions of epigenetics

'The study of changes in gene expression, which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression' (Holliday, 1994).
'The study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence' (Riggs *et al.,* 1996).
'...the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states' (Bird, 2007).
'An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence' (Berger *et al.,* 2009).
'...the inheritance of variation (-genetics) above and beyond (epi-) changes in the DNA sequence' (Bonasio *et al.,* 2010).

Source: From (Mann, 2014)

The main mechanisms of epigenetics include DNA methylation, histone protein modifications, and the non-coding RNA strands. DNA methylation and histone protein modifications can regulate gene expression through inducing the change in chromatin structure while the activity of non-coding RNA strands can regulate gene expression at

the transcriptional and post-transcriptional levels; it plays role in chromatin remodeling, histone modification, DNA methylation targeting, and gene silencing. Non-coding RNA molecules are functional RNA that are transcribed from DNA but are not translated into proteins. They have a wide range of functions, including control of chromosome dynamics, splicing, RNA editing, translational inhibition and mRNA destruction (Mattick and Makunin, 2006).

1.5.1.1 DNA methylation

DNA methylation refers to the covalent addition of a methyl group to position 5 of the cytosine pyrimidine ring (C) of a cytosine-guanine dinucleotide (CpG) on DNA, giving rise to 5-methylcytosine (5mC). In mammalian cells, approximately 3% to 5% of the cytosine residues in genomic DNA are present as methylated cytosine (5mC) (Ehrlich *et al.,* 1982), and 70% to 80% of 5mC residues are found in the CpG islands (Bird, 1986). CpG islands are often located at the promoters or regulatory regions of the house-keeping genes and can be also observed in the tissue-specific genes (Antequera, 2003). Unmethylated CpG islands are associated with the housekeeping genes, whereas the CpG islands of many tissue-specific genes are found to be methylated, except in the tissue where they are expressed. CpG islands are also located in coding region of the genes or the downstream of the transcription start site such as human *APOE* gene (Larsen *et al.,* 1992). Methylation of CpG islands downstream of transcription initiation site does not block the transcriptional initiation or elongation in mammalian cells (Jones, 1999).

In mammals, DNA methylation is involved in many biological processes including the genomic imprinting, X-chromosome inactivation, regulation of gene expression, and silencing of transposable elements (Li and Zhang, 2014). The correct pattern of DNA methylation is necessary for normal mammalian development (Li *et al.,* 1993, 2002). Aberrant DNA methylation due to failure to maintain correct methylation patterns can lead to several diseases including neurodevelopmental defects, neurodegenerative, neurological diseases, autoimmune diseases, and cancers (Lv *et al.,* 2012).

DNA methylation is known to be associated with silencing of genes; however, there are an increasing number of genes found to be activated by DNA methylation such as mouse insulin-like growth factor 2 (*Igf2*) in which DNA methylation blocks the binding

of repressor proteins to a silencer element in the gene (Murrell *et al.,* 2001; Eden *et al.,* 2001).

DNA methylation regulates gene expression through several mechanisms including: (1) methylation of cytosine residues can directly prevent the binding of transcription regulatory factors to their target sites on DNA sequences. In this way, DNA methylation affects gene transcription by changing transcription factor binding affinity to a gene promoter. (2) DNA methylation can affect gene expression through many methyl-CpG-binding proteins (MECPs) which read the DNA methylation pattern. There are 6 MECPs that have been identified in mammals including MECP2, MBD1, MBD2, MBD3, MBD4 and Kaiso (Li, 2002). Methyl-CpG-binding protein contains a methyl-CpG-binding domain (MBD) and a transcription repression domain (TRD). The MECPs can bind to methylated DNA and insulates the binding of the transcription factor to DNA. For example, MECP2 forms the complex with histone deacetylase (HDACs) and a co-repressor protein, Sin3a, to repress transcription in methylation-dependent manner (Nan *et al.,* 1998; Jones *et al.,* 1998). The MECP2-Sin3a-HDAC provides the first molecular evidence linking DNA methylation to histone deacetylation in transcription repression. Another example, MBD2 can form a complex with the multisubunit NuRD complex, which contains an ATP-dependent chromatin-remodelling protein, Mi-2, and HDACs (Zhang *et al.,* 1999; Wade *et al.,* 1999). The MBD2-NuRD complex previously known as MeCP1 can repress methylated promoters and also remodel methylated chromatin (Feng and Zhang, 2001). This evidence provides a mechanistic link between DNA methylation and histone deacetylation in transcriptional repression. (3) DNA methylation can alter chromatin structure in which methylated DNA leads to more compact DNA by the action of chromatin remodeling enzymes, thereby affect accessibility of transcription factors and/or DNA binding proteins to heterochromatin. The model of the mechanism of DNA methylation on gene transcription is illustrated in **Figure 1.6**.

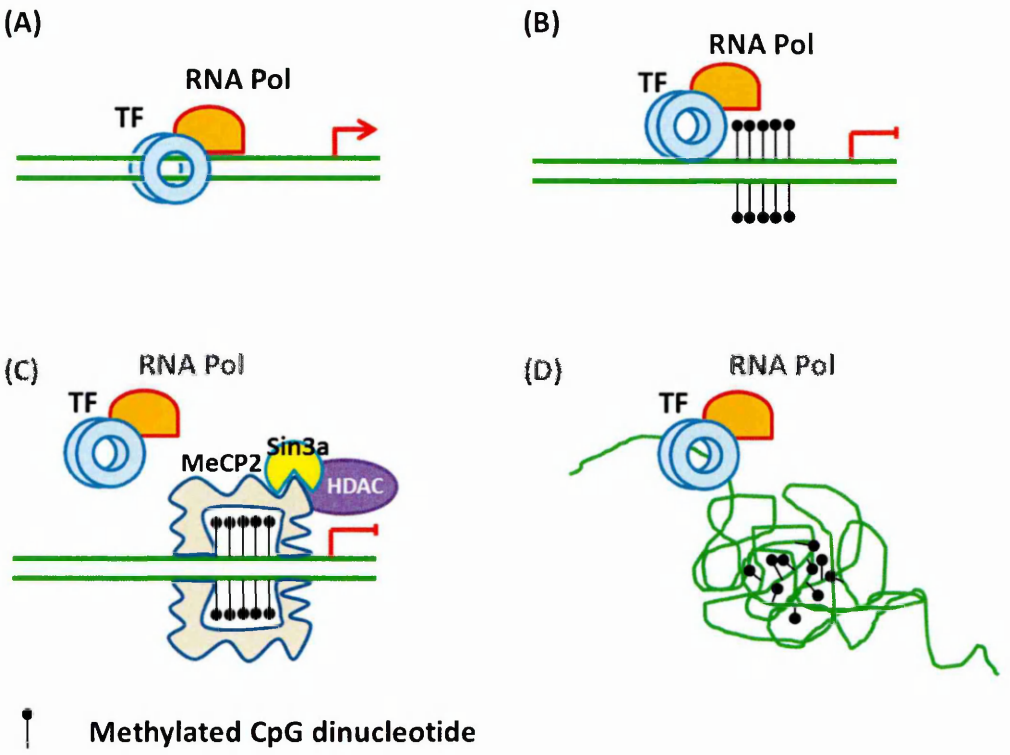


Figure 1.6: Mechanism models of the effect of DNA methylation on gene expression. Transcription can be initiated in unmethylated DNA sequences (A); DNA methylation can affect gene transcription in several ways including; (B) methylated CpG dinucleotide interfere binding of transcription factors that are sensitive to methylated DNA to DNA resulting in initiation of transcription cannot occur; (C) Methyl-CpG-binding proteins can bind to methylated DNA and insulates transcription factor binding to DNA; (D) methylated DNA can be made more compact to heterochromatin by chromatin remodeling enzymes that affect accessibility of the transcription factors. TF, transcription factor; RNA Pol, RNA polymerase; MeCP2, methyl CpG binding protein 2; Sin3a, SIN3 transcription regulator family member A; HDAC, histone deacetylases. *Modified from*: (Zhang and Pradhan, 2014)

DNA methylation involves the enzymatic methylation of CpG sites. DNA methylation takes place after DNA replication and is catalysed by DNA methyltransferase (DNMTs) using S-adenosyl methionine as the methyl donor. A number of DNA methyltransferase enzymes (DNMT1, DNMT2, DNMT3a and DNMT3b) have been identified by biochemical and sequence analysis. The maintenance DNMT1 uses hemi-methylated DNA as a preferential template, so it plays an important role in maintaining the identical methylation pattern after DNA replication that is characteristic for each type of differentiated cell (Turker and Bestor, 1997). DNMT3a and DNMT3b catalyse *de*

novo methylation because they can methylate both hemi-methylated and unmethylated DNA templates with equal efficiency (Xie *et al.,* 1999). Therefore, they are critical for the establishment of DNA methylation during development (Okano *et al.,* 1999). Importantly, the activity of DNMTs and DNA CpG methylation in a variety of tissues, including CNS, are sensitive to the social environment, ischemia, environmental toxins, nicotine, alcohol, psychostimulants, and antipsychotic drugs (reviewed by Akbarian, 2010).

DNA methylation is limited by the availability of the methylation substrate, S-adenosyl methionine, the synthesis of which is under the control of MTHFR as mentioned previously in **section 1.4.1.** MTHFR is thus important in DNA methylation (Lucock, 2000) in which its functional genetic variants can influence the extent of such methylation (de Arruda *et al.,* 2013). The T allele of the common *MTHFR* 677C/T genotype results in reduced enzyme activity, and elevated plasma homocysteine under conditions of impaired folate status (Ueland *et al.,* 2001). This and other polymorphisms of *MTHFR* are reported as genetic risk factors for several disorders and drug-induced side effects including weight gain and metabolic pathology in subjects receiving antipsychotic drugs (van Winkel *et al.,* 2010a; Ellingrod *et al.,* 2008). Conceivably this could indicate that *MTHFR* polymorphisms might interact with SNPs in other genes (e.g. the -759C/T *HTR2C* SNP) in determining the extent of drug-induced weight gain.

1.5.1.2 Histone modifications

Histone modifications refer to the addition and removal of the covalent moieties to the specific amino acid residues of the histone protein. Histones are the main protein component of chromatin. The core histones which are H2A, H2B, H3, and H4 form the nucleosome. Histone modifications are covalent post-translational modifications. These modifications which include acetylation, methylation, and phosphorylation influence the degree to which the DNA associates with the histones. Histone modifications result in chromatin structure remodeling that can affect gene expression regulation. Chromatin condensation to heterochromatin impedes gene expression, while unwinding to euchromatin encourages transcription. Generally, histone acetylation and phosphorylation promote gene transcription whereas histone

methylation has been associated with both repressed and activated gene transcription (reviewed by Jiang *et al.,* 2008; Meaney and Ferguson-Smith, 2010). The effect of each histone modification can be highly residue-specific. The effect of histone methylation on gene expression depends on the exact amino acid residue methylated and also the number of methyl groups being added (reviewed by Rahn *et al.,* 2013).

Each type of histone modification which includes addition and removal of chemical groups is catalysed by specific enzymes. For instance, the addition of acetyl groups to histones in histone acetylation is catalysed by histone acetyltransferases (HATs) whereas the removal of acetyl groups (histone deacetylation) is catalysed by histone deacetylases (HDACs).

1.5.1.3 Non-coding RNAs

Non-coding RNAs (ncRNAs) or non-protein coding RNAs are transcribed, but not translated into proteins. They are transcribed in tissue-specific and cell-specific patterns to regulate cell differentiation and development. Non-coding RNAs act as the regulators of gene expression and epigenetics. They are subdivided into 4 subclasses based on length, characteristics and function including microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and long-non-coding RNAs (lncRNAs) (Kaikkonen *et al.,* 2011). For example, 20-24 nucleotide-long miRNAs have been postulated to control the activity of approximately 50% of all protein-coding genes in a cell at the post-transcriptional level (Krol *et al.,* 2010). A recent report suggests that miRNAs can destabilize the target mRNA to reduce protein expression (Guo *et al.,* 2010).

PiRNAs are small ncRNAs of 24–31 nucleotides that have the primary role in suppression of transposon activity during germ line development (Brennecke *et al.,* 2007; Gunawardane *et al.,* 2007) and in somatic cells (Malone *et al.,* 2009). However, the regulation of the transposon activity in mammals during spermatogenesis also occurs through *de novo* DNA methylation and this process is regulated by piRNAs (Kuramochi-Miyagawa *et al.,* 2008).

SiRNAs (20-24 nucleotides) mediate post-transcriptional silencing similar to miRNA silencing. SiRNAs have also been found to direct gene silencing at sequence-specific

transcriptional level by increasing epigenetic modifications characteristic of heterochromatin (Carthew and Sontheimer, 2009; Grewal, 2010). SiRNAs also play a role in regulating genome functions and it has been suggested that siRNAs may play a similar role as piRNAs in suppressing transposon activity (Watanabe *et al.,* 2006; Yang and Kazazian, 2006).

LncRNAs are the majority of the non-protein-coding transcripts. LncRNAs, which are arbitrarily considered as >200 nucleotides in length are transcribed from various regions of eukaryotic genomes and can be classified according to their proximity to protein coding genes including sense, antisense, bidirectional, intronic, and intergenic (Ponting *et al.,* 2009). These lcnRNAs are important regulators for epigenetic modification, transcription, and translation and they play crucial roles in cell differentiation, development and disease progression processes (Nie *et al.,* 2012). A large number of lncRNAs recruit chromatin modifying enzymes that facilitate chromatin modification and ultimately changing gene expression (Schmitz *et al.,* 2010). The lncRNAs also play roles in X chromosome inactivation. One of two copies of the X chromosome of the female mammalian genome needs to be transcriptionally silent to have proper levels of gene expression. The process of X chromosome inactivation is mediated by the X chromosome inactivation centre which consists of four ncRNAs genes (Xist, Tsix, Jpx, and Ftx). The lncRNA Xist RNA is loaded onto X chromosome by YY1 protein which is a bivalent protein capable of binding both RNA and DNA (Jeon and Lee, 2011). Xist RNA induces chromosome-wide silencing by spreading along the X chromosome and recruiting polycomb repressive complexes (PRC) which mediates DNA methylation, histone hypoacetylation, and MacroH2A deposition throughout the entire targeted X chromosome resulting in transcriptional inactivation of either the paternal or maternal copy of the X chromosome (Pinter *et al.,* 2012).

1.5.2 The role of epigenetic modifications

It is now well known that epigenetics plays an essential role in normal development and disease susceptibility in adults (Dolinoy *et al.,* 2007). Epigenetic alterations contribute to a number of age related disorders including cancer and autoimmune disorders (Lu *et al.,* 2006) as well as many nervous system diseases including

neurodevelopmental disorders, brain cancer, neurodegenerative diseases, and mental illness that includes schizophrenia (Zhang *et al.,* 2013d).

DNA methylation regulates gene expression and also helps stabilize chromatin. DNA hypomethylation can lead to genomic instability by predisposing to strand breakage and derepression of repetitive sequences (Lu *et al.,* 2006). In addition, DNA hypomethylation of CpG in promoter regions can result in gene activation which brings about aberrant gene expression (Attwood *et al.,* 2002).

The effect of DNA hypomethylation on genomic instability is the chromosomal translocations in DNMT (DNMT1 and/or DNMT3b) deficiency studied in DNMT-deficient HCT116 cells (Karpf and Matsui, 2005). In addition, DNA methylation plays an important role in regulating activity of the transposable elements (TEs) which are mobile DNA sequences representing a substantial fraction of most genomes. Insertions of the transposable elements within genome are the source of genetic variation and may be the cause of genetic dysfunction and influence gene expression that ultimately result in cancer and other human diseases. The TE repeats can induce chromosome rearrangement; in addition, the TE insertions can alter gene expression through many modifications for example by creating new polyadenylation sites or new exons (exonization), by exon skipping or splicing, and also by the alteration of regulatory sequences (Chenais, 2015). DNA methylation is known to inhibit transpositional activity of diverse transposons. For example, silent transposable elements are methylated in plants (Martienssen, 1998) and also in animals (Yoder *et al.,* 1997), and they can be reactivated in methylation-defective mutants (Miura *et al.,* 2001; Walsh *et al.,* 1998).

Global DNA hypomethylation is commonly found in a number of cancers, including thyroid, breast, cervical, prostate, stomach, lung, bladder, esophagus, colorectum, and liver (reviewed by Arooj *et al.,* 2013). The alteration in global methylation is considered to be mainly due to hypomethylation of repetitive sequences for example long interspersed nuclear element 1 (*LINE-1*).

In contrast to hypomethylation, DNA hypermethylation, particularly of CpG islands or promoter CpGs can result in inappropriate gene silencing and development of disease states. For example, hypermethylation of CpG islands of the tumor suppressor gene

leads to silencing of the gene and predisposes to carcinogenesis (Attwood *et al.,* 2002). Hypermethylation of specific genes may correlate with decreased gene expression that associated with disease condition. For instance, the fibulin-1 (*FBLN1*), a multi-functional extracellular matrix protein, promoter CpGs hypermethylation is found in accordance with downregulation of FBLN1 protein and mRNA levels in colorectal cancer (Xu *et al.,* 2015), renal cell carcinoma (Xiao *et al.,* 2013), and gastric cancer (Cheng *et al.,* 2008).

1.5.3 Association of DNA methylation and schizophrenia

Schizophrenia is associated with multiple risk factors, including both environmental and genetic influences (**section 1.1.4**). There are several genetic factors that have been reported to be associated with schizophrenia and antipsychotic treatment response and side effects such as *BDNF* and *HTR2A* (**section 1.4**). The study of epigenetic regulators of gene expression including DNA methylation and histone modifications is an attractive field to explore the molecular pathology of schizophrenia.

To date, recent studies provided evidence that pathogenesis of psychiatric disorders may be due to epigenetic aberrations. Researchers focus on the DNA methylation changes in schizophrenia postmortem brain, especially in prefrontal areas of cerebral cortex. Epigenetic alterations in dopaminergic system genes were reported in schizophrenia and bipolar disorder (Abdolmaleky *et al.,* 2008). Hypermethylation-mediated silencing of the reelin gene (*RELN*), which encodes a glycoprotein essential for brain development and neuronal connectivity, was reported in the frontal lobe of post-mortem brain which was correlated with schizophrenia and bipolar disorder (Abdolmaleky *et al.,* 2005; Grayson *et al.,* 2005). Hypomethylation membrane-bound catechol-O-methyltransferase (*MB-COMT*), which is involved in dopamine degradation, was also reported in schizophrenia and bipolar disorder compared to control subjects and this hypomethylation was associated with significant higher *MB-COMT* transcript level (Abdolmaleky *et al.,* 2006).

Related to serotonin receptor genes, it has been found that DNA methylation status was increased in *HTR1A* gene promoter in schizophrenia (Carrard *et al.,* 2011) which may be the cause of the alteration of the receptor expression (Hashimoto *et al.,* 1991; Sumiyoshi *et al.,* 1996). In addition, methylation at a transcription factor-binding site

on the 5-HT1A receptor gene close to the functional polymorphism, -1019C/G (rs6295) correlates with negative symptom treatment response in first episode schizophrenia patients (Tang *et al.,* 2014). In addition, the hypomethylation of 5-HT2A receptor gene at and around 102T/C polymorphic site was reported in schizophrenia and bipolar disorder (Ghadirivasfi *et al.,* 2011; Abdolmaleky *et al.,* 2011), but promoter DNA of the *HTR2A* was hypermethylated at and around the -1438A/G polymorphic site (Abdolmaleky *et al.,* 2011), and these epigenetic changes influence expression of the *HTR2A* gene (Abdolmaleky *et al.,* 2011). DNA hypermethyation of the serotonin transporter (*5-HTT*) promoter and its correlation with the reduction in *5-HTT* expression were reported in drug naïve schizophrenia patients both in DNA from saliva and post-mortem brain samples (Abdolmaleky *et al.,* 2014). These studies support the epigenetic influences in etiology of psychotic disease and might provide new targets in antipsychotic drug development..

1.5.4 Association of epigenetic factors and antipsychotic drug-induced weight gain

The genetic risk factors have been of interest and provide a focus for much research investigating the causes of disease and treatment responses. Recently the epigenetic factors have also been extensively researched. In addition to genetic factors, environmental factors such as nutritional, chemical and physical factors have the potential to alter gene expression through epigenetic modification. In other words, epigenetic mechanisms are influenced by the environment. Several factors have been shown to modulate epigenetic modification, for example, age (Bjornsson *et al.,* 2008), sun exposure (Gronniger *et al.,* 2010), radiation exposure (Chaudhry and Omaruddin, 2012), diet (Heijmans *et al.,* 2008; McKay *et al.,* 2012), alcohol consumption (Philibert *et al.,* 2012; Zhang *et al.,* 2013a), tobacco smoking (Flom *et al.,* 2011; Wangsri *et al.,* 2012), and air pollution (Tarantini *et al.,* 2009; Salam *et al.,* 2012); some of these factors such as age could be a confounding factor in establishing a correlation study relating to DNA methylation. Treatment with antipsychotic drugs can also affect epigenetic modifications which will be described in the next section (**section 1.5.4.1**).

1.5.4.1 *Antipsychotic drugs and epigenetic modifications*

Study of a mouse model with relevance to schizophrenia found that clozapine and sulpiride but not haloperidol or olanzapine treatment induced DNA demethylation as well as histone acetylation at reelin and *GAD67* promoters in the frontal cortex and striatum (Dong *et al.,* 2008; Guidotti *et al.,* 2009). However, olanzapine treatment in rats caused genome-wide DNA methylation change in genes of dopamine neurotransmission in hippocampus, cerebellum and liver (Melka *et al.,* 2014). This research group analysed DNA methylation extents within a total 40 genes in dopamine (DA) pathway and found that 19 genes including genes encoding for DA receptors (D1, D2, and D5), DA transporter, DA synthesis, and DA metabolism (*COMT*) had different methylation between olanzapine and control rats, and most (17/19) genes showed increased methylation (Melka *et al.,* 2013). This study supports the dopamine hypothesis of schizophrenia that olanzapine may reduce DA activity by changing DNA methylation. Melas *et al.* (2012) found decreased global DNA methylation in patients with schizophrenia compared to normal controls, especially in early onset of disease, and they also found that global DNA methylation was increased to nearly the levels of normal controls in patients receiving haloperidol. These data suggest that epigenetic changes influenced by disease and/or antipsychotic treatment may involve, in part, the symptom response and also may account for certain adverse effects including weight gain after antipsychotic drug treatment. Further studies are required to understand the effect of antipsychotic drugs on epigenetic change, gene expression and variable outcome to treatment.

1.5.4.2 *Epigenetic modifications and antipsychotic drug-induced weight gain*

A recent study reported the association of lower DNA methylation of the *HTR2A* gene promoter with decreased body weight, BMI, and fat mass after six months of weight loss treatment in subjects with metabolic syndrome (Perez-Cornago *et al.,* 2014) indicating the influence of epigenetic modification in genes of the serotonin system on body weight change in response to drug treatment. Few studies have investigated the relationship between epigenetic mechanisms and antipsychotic drug-induced weight gain. Two studies have investigated the association between DNA methylation of the *MTHFR* and *COMT* genes and metabolic syndrome in schizophrenia. Burghardt *et al.*

(2012) studied the *MTHFR* 677C/T poylmorphism and *LINE-1* DNA methylation in 133 patients with schizophrenic disorder. They did not find an association between global *LINE-1* methylation and *MTHFR* genotypes, but they found a significant interaction between the *MTHFR* 677C/T variant and gender on global *LINE-1* DNA methylation, in which females with the *MTHFR* 677TT genotype had the lowest global *LINE-1* DNA methylation level compared with other groups.

Another study found a significant association between the *COMT* Val158Met (rs4680) polymorphism, *COMT* promoter methylation, physical activity, and metabolic syndrome in 85 patients with schizophrenia receiving atypical antipsychotic treatment (Lott *et al.,* 2013). They observed that the *COMT* genotype was a significant indicator of methylation status at two CpG sites in the *COMT* promoter region. They found that physical activity had a negative correlation with COMT promoter methylation in Val/Val homozygous patients whereas positive correlation was found in Met/Met carriers. In addition, patients with Met/Met genotype had a positive correlation between *COMT* promoter methylation and metabolic syndrome. These findings indicate that promoter methylation of *COMT* is influenced by its genotype and physical activity. These data indicate the influence of a genetic polymorphism on DNA methylation and this relationship may influence the interindividual variation in symptom response and adverse effects in schizophrenia patients received antipsychotic drugs.

Many of the functional consequences of genetic polymorphisms, particularly those in promoter regions that may influence binding of transcription factors, are likely to affect gene expression. Additionally, some polymorphisms may modify the CpG sequences that are the sites of DNA methylation, and thus may influence gene transcription by affecting this methylation. Particularly notable in pharmacogenetic findings is that the SNPs in *HTR2C* that are associated with drug-induced weight gain are often also at CpG sites of DNA methylation – either directly disrupting CpG sequences (e.g. the -759C/T SNP, -697G/C SNP) or close to other CpG sites. Thus DNA methylation provides a further potential influence on drug induced weight gain – by, for example methylation of the -759C site or nearby CpG sites - in addition to the established pharmacogenetic association of *HTR2C* SNPs.

1.6 Main objectives

Overall aim

The hypothesis is that genetic factors may influence antipsychotic drug-induced weight gain. In addition to the -759C/T polymorphism of the *HTR2C* gene, the genetic polymorphisms of several candidate genes related to antipsychotic drug-induced weight gain including *HTR2A*, *ADRA2A*, *BDNF*, *GNB3*, *MC4R*, *FTO*, and *MTHFR* may have influence on antipsychotic drug-induced weight gain. These genes encode drug target receptors, hormones or neuropeptides involved in food intake regulation, or genes that might be involved in DNA methylation modification. In addition, epigenetic factors may also influence antipsychotic drug-induced weight gain. DNA methylation of CpGs in the promoter sequences of the *HTR2C* may associate with antipsychotic drug-induced weight gain, and it also may be affected by those genetic polymorphisms. Moreover, antipsychotic drugs may affect DNA methylation levels of the *HTR2C* promoter sequences that may cause alteration in mRNA expression. Furthermore, antipsychotic drugs may affect leptin secretion from adipocytes which may be mediated by changing *HTR2C* mRNA expression. The genotype discrimination of genetic polymorphisms, the extent of DNA methylation, the expression of mRNA, and the secretion of leptin were measured by using a variety of molecular, biochemical and cell culture based assays.

The specific goals were:

1. To investigate the influence of SNPs on antipsychotic drug-induced weight gain in patients with schizophrenia.
2. To investigate the association of DNA methylation in the promoter region of the *HTR2C* gene on antipsychotic drug-induced weight gain in patients with schizophrenia.
3. To investigate the genotype effect of SNPs on DNA methylation in the *HTR2C* gene.
4. To investigate the effect of antipsychotic drug treatment on DNA methylation and mRNA expression of the *HTR2C* gene in neuroblastoma cells.
5. To investigate the effect of antipsychotic drug treatment on leptin secretion and *Htr2c* gene expression in mouse adipocyte cells.

Chapter 2: Association of genetic polymorphisms and antipsychotic drug-induced weight gain in patients with schizophrenia

2.1 Introduction

Schizophrenia is a severe, complex and chronic disorder which for many patients is inadequately treated. Antipsychotic drugs can in many individuals relieve the positive psychotic symptoms but have various adverse effects; notably several of the drugs can induce a substantial weight gain in susceptible individuals, particularly second generation antipsychotics such as clozapine, olanzapine and risperidone (reviewed by Panariello *et al.*, 2011). However, first-generation antipsychotic drugs (FGAs) also have been linked to weight gain to a lesser degree (Lett *et al.,* 2012). This weight gain may not only increase treatment non-compliance but also affect morbidity from metabolic consequences including lipid abnormalities, insulin resistance and diabetes mellitus (Henderson *et al.,* 2000). Weight gain also reduces quality of life in patients suffering from schizophrenia (Lett *et al.,* 2012; Allison *et al.,* 2003). Patients receiving antipsychotic treatment can develop metabolic abnormalities with increased risk of cardiovascular disease and mortality (Casey *et al.,* 2004; DE Hert *et al.,* 2009). The mean age of death for schizophrenia patients is 22.5 years younger than general population (57.4 years for schizophrenia and 79.9 years for general population) (Tiihonen *et al.,* 2009). This difference is, in part, attributable to the metabolic side-effects of antipsychotic treatment (Gautam and Meena, 2011).

Susceptibility to antipsychotic-induced weight gain varies substantially between individuals in ways that cannot be fully explained by differences between different drug effects or other environmental factors. Thus genetic influences are strongly implicated, and associations between many genetic polymorphisms and antipsychotic drug-induced weight gain have been reported. The most consistently reported genetic factors involved in antipsychotic induced weight gain include polymorphisms in genes for 5-hydroxytryptamine 2C (5-HT2C), 5-HT2A, adrenergic alpha 2A and melanocortin 4

receptors, as well as leptin and fat mass and obesity associated (*FTO*) genes (Reynolds, 2012).

The inter-individual variability of weight gain in response to antipsychotic drug treatment is partly due to genetic variability. The most consistent genetic variant associated with antipsychotic drug-induce weight gain is the -759C/T polymorphism of *HTR2C* gene, of which the T allele is a protective allele against obesity (Yuan *et al.,* 2000) and antipsychotic drug-induced weight gain in both first episode (Templeman *et al.,* 2005; Reynolds *et al.,* 2002) and chronic schizophrenia patients (Miller *et al.,* 2005; Ellingrod *et al.,* 2005). The 5-HT2C receptor is involved in obesity and food intake regulation. Administration of a 5-HT2C receptor antagonist resulted in increased food intake and weight gain in rats (Bonhaus *et al.,* 1997). 5-HT2C receptor antagonist can attenuate the reduced food intake which is induced by 5-HT2C agonists (Hayashi *et al.,* 2005). It is a target for atypical antipsychotic drugs such as clozapine and olanzapine which have high affinities for the 5-HT2C receptor (Roth *et al.,* 2004; Reynolds and Kirk, 2010). Endogenous 5-HT inhibits neuropeptide Y (NPY) (Heisler *et al.,* 2006) which controls satiety. Clozapine (the 5-HT2C receptor antagonist) disinhibits hypothalamic NPY neurons resulting in elevated NPY (Kirk *et al.,* 2006). 5-HT increases POMC activity via 5-HT2C receptor by modulating POMC neuronal excitability (Roepke *et al.,* 2012; Qiu *et al.,* 2007; Sohn *et al.,* 2011). Therefore, it has been suggested the involvement of 5-HT2C receptor mechanisms in atypical antipsychotic drug-induced weight gain is by modulating POMC and NPY activity (Balt *et al.,* 2011).

The *HTR2C* -759C/T polymorphism is located in the promoter region near the regulatory transcription factor binding sites which may affect gene expression (Xie *et al.,* 1996). The transcription factor binding sites located nearby the -759C/T SNP are TCF-1, LF-A1, and bHLH (Xie *et al.,* 1996). Yuan *et al.* (2000) demonstrated that the *HTR2C* promoter haplotype containing either the -997A, -759T or -697C allele has increased promoter activity in Chinese hamster embryonic carcinoma cells. The same promoter haplotype showed decreased expression in SH-SY5Y neuroblastoma cells (Hill and Reynolds, 2007). The haplotype of 4 polymorphisms containing -759T or -977G (the study did not include the -697G/C site) resulted in increased promoter activity in human cell lines, HEK293t and TE671 (Buckland *et al.,* 2005). Another study did not find any significant influence of the promoter haplotype containing -759T and -697C

and other 5' polymorphisms on promoter activity while haplotype with -759T and -697C showed 21% less activity compared to the major haplotype; -759C and -697G (McCarthy *et al.,* 2005). The different findings may be due to the difference of promoter fragment sequences that included -697 or not and addition of other SNPs. Although the function of individual promoter polymorphisms in regulating promoter activity is unclear, the removal or addition of a particular site may affect the promoter activity since some sites may affect the affinity of the transcription factor binding site. The methods used for transfection including transient or stable transfection may also influence the findings. The transfected plasmid DNA is usually not integrated into the host genome in transient transfection; therefore, the foreign DNA is diluted through mitosis or degraded. In stable transfection, the transfected plasmid DNA is integrated into host genome and is replicated when cell mitosis occurs; therefore, the transfected gene remains in the genome of the cell and daughter cells (reviewed by Kim and Eberwine, 2010). In addition, the different cell lines used in transfection may contribute to the different findings; the different cell lines provide different transcriptional machinery or regulatory elements required for transcription of the gene. Furthermore, the different plasmid constructs also contribute to the different findings. The promoter enhancers such as SV40 or CMV viral enhancer in both experimental and reference plasmids used in the luciferase assay have strong activity compared to the plasmids without the enhancer and this may affect the transcription rate of the tested plasmid. It has been proposed that the reduced promoter activity of the T allele of the -759C/T polymorphism of the *HTR2C* may results in the decreased expression of the 5-HT2C receptors leading to subsequent compensatory changes in other systems in regulating food intake (Hill and Reynolds, 2007). The alteration in 5-HT2C receptor expression influenced by promoter polymorphisms might be involved in antipsychotic drug-induced weight gain.

The T allele of *HTR2C* -759C/T polymorphism was associated with higher plasma leptin levels at baseline in schizophrenia patients compared to C/CC genotype (Templeman *et al.,* 2005). However, the mechanistic link or interaction between leptin and 5-HT2C receptor is still unclear and needs further study to elucidate the interaction of these two pathways. Leptin induction of central 5-HT turnover via a nitric oxide dependent pathway has been reported (Calapai *et al.,* 1999). There is evidence showing that

central leptin-induced anorexia is mediated via the 5-HT2C receptor and the specific 5-HT2C receptor antagonist (SB 242084) attenuated anorexia induced by leptin administration (von Meyenburg *et al.,* 2003a). Peripheral administration of the 5-HT precursor (5-hydroxytryptophan) increases serum leptin in mice (Yamada *et al.,* 1999). Immuno-histochemical evidence suggests an inverse relationship between 5-HT and leptin levels in the hypothalamus and dorsal raphe (Fernandez-Galaz *et al.,* 2010). A study in 5-HTT deficient mice found that increased 5-HT in various brain regions was paralleled by increased leptin levels (Chen *et al.,* 2012). However, the change in leptin could be independent of 5-HT and both leptin and 5-HT may have separate pathways in the control of food intake (Halford and Blundell, 2000). Thus, further experimental verification is required.

In addition to the *HTR2C* -759C/T polymorphism, there are other genetic risk factors that are likely to contribute to determining weight gain associated with antipsychotic drug treatment, including polymorphisms for leptin, melanocortin receptor 4, adrenergic α2A and g-protein beta3 among many others (Reynolds, 2012). In addition, the associations of the other genetic polymorphisms with antipsychotic drug-induced weight gain were previously discussed in chapter 1 and are summarized in **Table 2.1**.

Table 2.1: Summary and main finding of the association between SNPs and antipsychotic drug-induced weight gain

SNPs	Patient	Main findings	Reference
MTHFR 677C/T, *MTHFR* 1298A/C	58 schizophrenia patients receiving atypical antipsychotic drugs for ≥12 months (cross-sectional analysis), , treated at least 12 months with various antipsychotics	the 677T allele but not the 1298A/C of *MTHFR* was associated with a greater risk of developing metabolic syndrome and the TT genotype was associated with risk of insulin resistance with greater central adiposity induced by antipsychotic treatment	Ellingrod *et al.,* 2008
MTHFR 677C/T, *MTHFR* 1298A/C	237 subjects with bipolar or schizophrenia receiving an antipsychotic for at least 6 months (cross-sectional analysis), various antipsychotics (54% were under poly-pharmacy),	Not associated with BMI but the *MTHFR* 677T was related to age, smoking, and metabolic syndrome	Ellingrod *et al.,* 2012

SNPs	Patient	Main findings	Reference
	72% were white		
MTHFR 677C/T, *MTHFR* 1298A/C	518 patients with a schizophrenia spectrum disorder, 97.3% were white), olanzapine, clozapine, quetiapine, risperidone, palipeidone	*MTHFR* A1298C, but not C677T, was associated with the metabolic syndrome, C/C genotypes having a 2.4 times higher risk compared to A/A genotypes	van Winkel *et al.*, 2010a
MTHFR 677C/T, *MTHFR* 1298A/C	104 schizophrenia patients, a 3-month follow-up period after initiation of an SGAs, various antipsychotics, some taken medication for other somatic disorders (e.g. hypertension)	The 1298C variant, but not C677T was associated with increased weight , waist circumference, fasting glucose and impaired glucose tolerance	van Winkel *et al.*, 2010b
ADRA2A -291C/G	62 Korean chronic schizophrenia, (not FEP), long-term olanzapine treatment (≥ 3 months)	The G allele was significantly higher frequency in patients who had severe weight gain (>10% weight increased from baseline)	Park *et al.*, 2006
ADRA2A -291C/G	93 Chinese chronic schizophrenia treated with clozapine (14±6.2 months)	The GG genotype had higher body weight gain than CC genotype	Wang *et al.*, 2005b
ADRA2A -291C/G	129 chronic schizophrenia or schizoaffective disorder (60 European-Americans and 39 African-Americans), 6-14 weeks treated with clozapine/olanzapine	The C allele carriers gained more weight compared to GG genotype in European-Americans but not in African-Americans	Sickert *et al.*, 2009
ADRA2A -291C/G	139 schizophrenia patients with various ethnicity, 6-14 weeks treatment with clozapine (91), olanzapine (22), haloperidol (12), risperidone (14)	No association with weight gain	De Luca *et al.*, 2011
ADRA2A -291C/G	470 schizophrenia patients, cross-sectional study	No association with metabolic syndrome	Risselada *et al.*, 2010
HTR2A rs6313	164 schizophrenia patients in Japan, olanzapine	The 102T allele of *HTR2A* was associated with olanzapine-induced weight	Ujike *et al.*, 2008

SNPs	Patient	Main findings	Reference
	treatment (8-24 weeks)	gain	
HTR2A -1438G/A	84 (FEP) Chinese Han schizophrenia patients, 10 weeks of treatment with risperidone or chlorpromazine	No association of the *HTR2A*-1438G/A with weight gain	Mou *et al.*, 2005
MC4R rs17782313	345 white inpatients schizophrenia, various atypical antipsychotics (clozapine, olanzapine, risperidone, paliperidone, quetiapine, or amisulpride) treatment for 4 weeks	The C-allele had a significantly higher risk of weight gain and BMI increase	Czerwensky *et al.*, 2013a
MC4R rs17782313	224 schizophrenia patients (European-ancestry), 14 weeks treatment	No association with weight gain	Chowdhury *et al.*, 2013
MC4R rs489693	4 cohorts consisted of 139 pediatric patients with first exposure to SGAs treated with SGAs for 12 weeks. The 3 additional cohorts consisted of 73, 40, and 92 subjects treated for 6 and 12 weeks.	The AA genotype was associated with greater weight gain	Malhotra *et al.*, 2012
MC4R rs489693	341 Caucasian inpatients schizophrenia receiving at least one SGA drug (olanzapine, clozapine, risperidone, paliperidone, quetiapine, or amisulpride) for 4 weeks	A-allele showed a 2.2 times higher weight increase	Czerwensky *et al.*, 2013b
GNB3 825C/T	164 schizophrenia patients in Japan, olanzapine treatment (8-24 weeks)	T allele was significantly associated with olanzapine-induced weight gain	Ujike *et al.*, 2008
GNB3 825C/T	134 Chinese schizophrenia patients, long-term treatment with clozapine (13.4 months)	Patients with the TT genotype had significantly greater weight gain	Wang *et al.*, 2005a
GNB3	87 treatment-resistant schizophrenic patients,	Not associated with clozapine-induced body	Tsai *et al.*,

SNPs	Patient	Main findings	Reference
825C/T	clozapine treatment for 4 months	weight change	2004
GNB3 825C/T	79 Korean schizophrenic patient group receiving olanzapine treatment at least 3 months	No association with weight gain	Park *et al.*, 2009
GNB3 825C/T	42 schizophrenia patients treated with olanzapine for 6 weeks	No association with weight gain	Bishop *et al.*, 2006
GNB3 825C/T	a meta-analysis of 18,903 subjects	CC genotype showed a trend association with lower BMI	(Souza *et al.*, 2008)
BDNF rs6265	196 Chinese schizophrenia on long-term antipsychotic medication	Met/Met (or AA) genotype was associated with weight gain, with strong effect in male but not female	Zhang *et al.*, 2008
BDNF rs6265	257 schizophrenia patients of European ancestry	a haplotype of rs6265 and rs1519480 (G-A haplotype) was associated with atypical antipsychotic drug-induced weight gain	Zai *et al.*, 2012
BDNF rs6265	481 schizophrenic patients treated with clozapine (n = 266), olanzapine (n = 79), or risperidone (n = 136) for an average of 49.2 ± 28.2 months	The *BDNF* Val66Met SNP was not associated with body weight gain, but the *BDNF* rs11030101 TT genotype was associated with weight gain	Tsai *et al.*, 2011
FTO rs9939609	250 Chinese Han schizophrenia patients (FEP), risperidone treatment for 6 months	The TT genotype carriers had significantly lower body weight and BMI than the A allele (both at baseline and after treatment)	Song *et al.*, 2014
FTO rs9939609	239 schizophrenia patients in Spain (FEP), 1 year antipsychotic treatment	The AA genotype was significant associated with the higher baseline BMI compared to AT/TT group but the weight increase between two groups of genotype was not significant difference after 1 year antipsychotic	Perez-Iglesias *et al.*, 2010

SNPs	Patient	Main findings	Reference
		treatment	
FTO rs9939609	Chronic and FEP schizophrenia patients	The A allele was associated with BMI in chronic schizophrenia patients but not with weight gain in first-episode schizophrenia patients, although the AA genotype had higher baseline weight and baseline BMI than T allele	Reynolds *et al.*, 2013
FTO rs9939609	351 chronic schizophrenia and 342 age- and sex-matched healthy subjects	No association between the SNP and BMI or weight gain in patients with schizophrenia but the carriers of A allele had significant higher BMI than those of TT genotype in healthy subjects	Watanabe *et al.*, 2012
FTO rs9939609	218 chronic schizophrenia or schizoaffective disorder treated mostly with clozapine or olanzapine for up to 14 weeks	No association between the polymorphism and BMI or antipsychotic drug-induced weight gain	Shing *et al.*, 2014

BMI, body mass index; FEP, First episode psychosis; SGAs, Second generation antipsychotics

Therefore, these SNPs were studied to investigate their influence on weight gain in first episode or BMI in chronic patients with schizophrenia. These SNPs are strong candidates relating to epigenetic modification (*MTHFR* polymorphisms), neurotransmitter receptors for antipsychotic drugs (*ADRA2A* and *HTR2A*), the GWAS SNPs associated with BMI and obesity (*FTO*, *MC4R*, and *BDNF*), and the 5-HT signaling transduction (*GNB3*).

2.1.1 TaqMan®-based SNP Genotyping

The principle of the TaqMan®-based SNP genotyping technology relies on the 5'-3' nuclease activity of Taq polymerase and fluorophore-based detection. In addition to unlabelled specific primers (forward and reverse primers) targeting the region flanking the SNP site, each SNP assay contains two TaqMan® fluorescent probes with the same

sequence except at the SNP site, with one probe complementary to the first allele and the other probe complementary to the second allele. 5' end of each probe is labelled or attached covalently with a different fluorophore (VIC or FAM) as a reporter. 3' ends of both probes are covalently linked to a non-fluorescent quencher preventing liberation of the reporter fluorescence if the probe is not degraded.

In the PCR cycling, during the denaturation step DNA was denatured to separate double-stranded DNA into single-stranded DNA providing DNA templates for the following annealing step in which primers bind specifically to DNA template while probes hybridize specifically to the targeted SNP site. During PCR extension or polymerisation step, the binding probe is displaced and cleaved by the 5' nuclease activity of the Taq polymerase which releases the reporter and quencher dyes and then the fluorescence of corresponding fluorophore is detected. Only the perfectly hybridized probes are destroyed by exonuclease activity of Taq polymerase, since a mismatched probe does not bind to DNA template; therefore, it will not be recognized and cut by the Taq polymerase; therefore, un-hybridized probe not complementary to the SNP site remains intact and the fluorescence of that reporter is suppressed (**Figure 2.1**).

The fluorescent signals for the two reporter dyes are measured at the end of the PCR cycle. These signals are normalized using the signal of a third dye for example ROX dye, of which the fluorescent intensity is proportional to the template DNA concentration and the extent of the PCR reaction. Typically, the reporter dye signals are visualized in a plot (**Figure 2.2**). The ratio of the signals will be indicative of the genotype of the sample (**Table 2.2**).

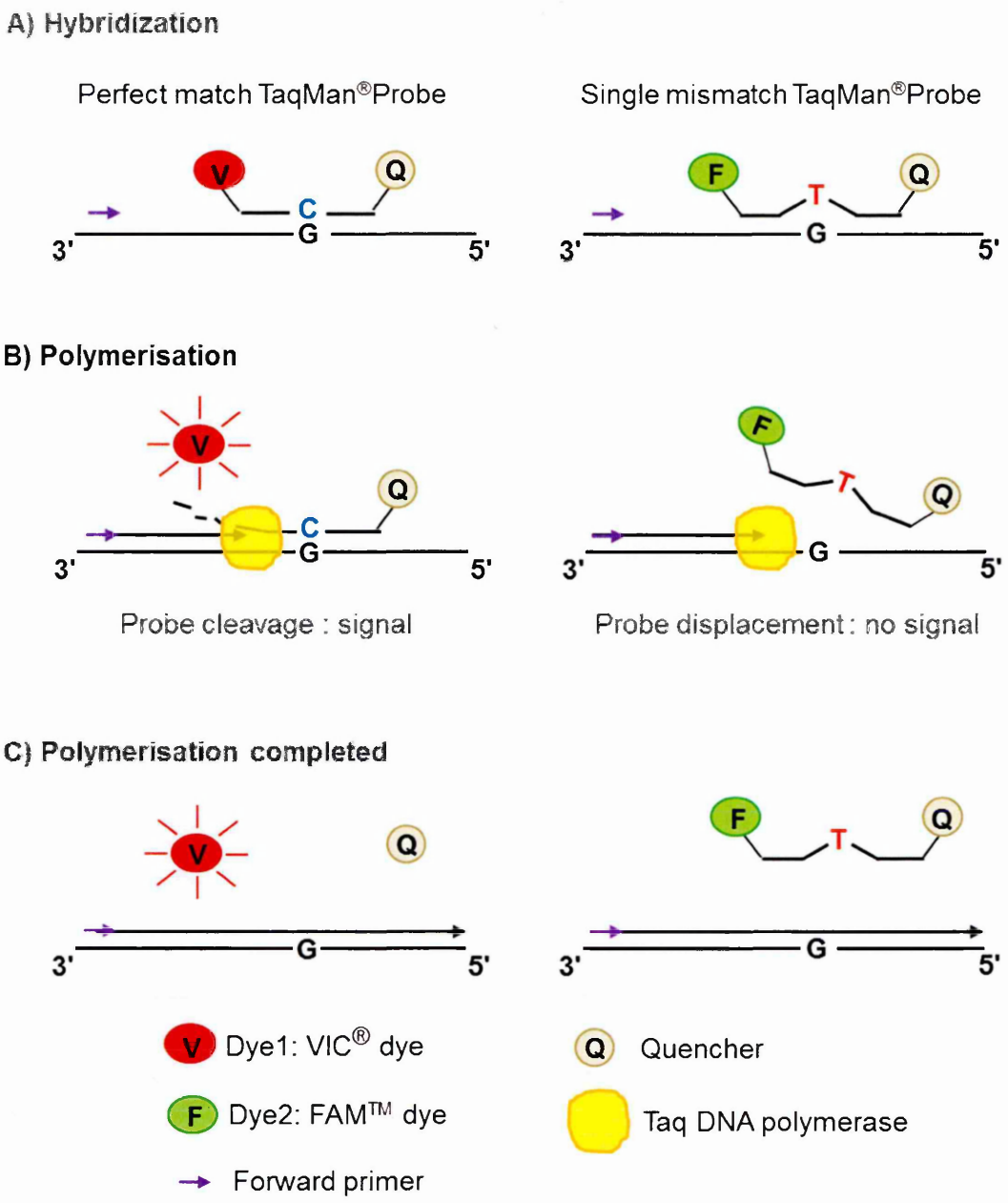


Figure 2.1: Overview of TaqMan® based SNP genotyping principle

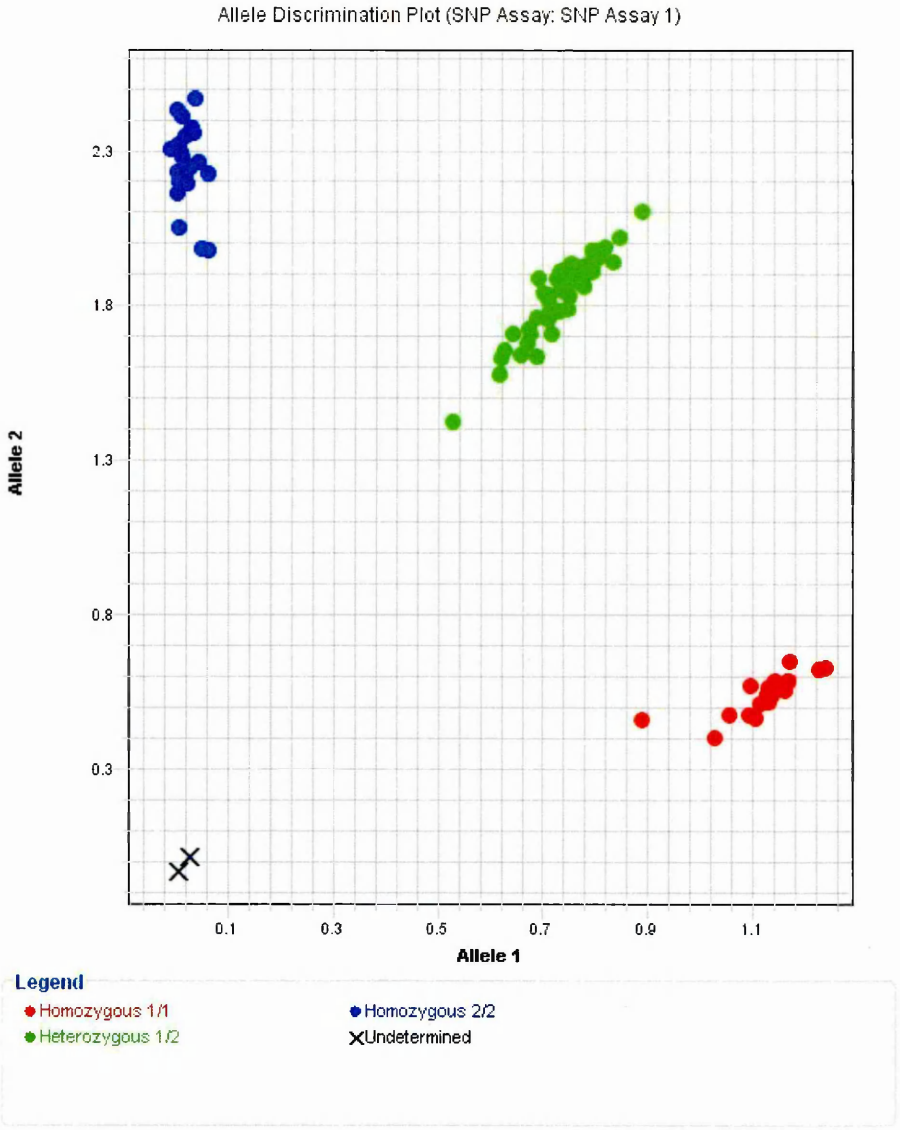


Figure 2.2: Genotyping result discriminating 3 groups of alleles

Figure shows homozygous allele 1 (red), homozygous allele 2 (blue), and heterozygous both alleles (green) with undetermined negative control.

Table 2.2: Categorization of SNP from fluorescent signal ratio after genotyping

A substantial increased in fluorescence signal	SNP allele
VIC® dye fluorescence only	Homozygosity for allele 1
FAM™ dye fluorescence only	Homozygosity for allele 2
Both fluorescence signals	Heterozygosity for allele 1 and allele 2

2.1.2 Aims

The main objective of the work reported in this chapter is to identify single-nucleotide polymorphisms associated with antipsychotic drug–induced weight gain.

1. To investigate the influence of polymorphisms in the *MTHFR, ADRA2A, HTR2A, MC4R, GNB3, BDNF, and FTO* genes on antipsychotic drug-induced weight gain in first episode schizophrenia patients.

2. To investigate the influence of polymorphisms in the *MTHFR* gene on body weight in chronic schizophrenia patients.

3. To investigate the gene-gene and drug-gene interactions on weight gain and body mass index following antipsychotic drug treatment.

4. To investigate the influence of previous findings in the *HTR2C* -759C/T, *FTO* rs9939609, and/or leptin -2548A/G polymorphisms on antipsychotic drug induced weight gain and BMI, as well as the gene-gene and drug-gene interactions.

2.2 Materials and methods

2.2.1 Study population and DNA samples

DNA samples used in this study were from three different populations; two cohorts of first-episode, initially antipsychotic drug-naïve patients with schizophrenia (Spanish and Chinese cohorts) and one cohort of chronic schizophrenia patients (Belfast cohort). All patients received treatment according to normal clinical practice as described below in **section 2.2.1.1-2.2.1.3** and gave written informed consent to the procedure of the study. Ethical approval for the studies was granted by the relevant local ethical committees.

2.2.1.1 Chinese first episode drug naïve schizophrenia patients

Chinese Han (n=182) cohort of first-episode, initially antipsychotic drug-naïve patients who met DSM-IV criteria for schizophrenia were studied. Patients who had evidence of previous antipsychotic drug treatment or other medical or neurological illness, and family history of diabetes or eating disorder were excluded. Height and weight to determine body-mass index (BMI) were measured on initiation of antipsychotic drug treatment and after 8 or 10 weeks and weight gain was determined by change in BMI over the treatment period. Blood samples were collected on the initiation of antipsychotic drug treatment. Initial antipsychotic drug treatment consisted primarily of chlorpromazine (n=60) and risperidone (n=114); eight patients received clozapine, fluphenazine or sulpiride.

2.2.1.2 Spanish first episode drug naïve schizophrenia patients

A Spanish Caucasian (n=72) cohort of first-episode, initially antipsychotic drug-naïve schizophrenia patients diagnosed by DSM-IV criteria were studied. Patients with comorbid DSM-IV diagnosis of substance abuse or dependence, or with any physical illness, were excluded from the study. None of the patients had a prior history of medication with antipsychotic, antidepressant or mood stabilizer drugs. Height and weight to determine BMI were measured on initiation of antipsychotic drug treatment and after 3 months and weight gain was determined by change in BMI over the treatment period. Blood samples were collected on initiation of antipsychotic drug treatment. Initial antipsychotic drug treatment consisted primarily of risperidone

(n=21) or olanzapine (n=22) and two received both, others had quetiapine (n=10), haloperidol (n=8) or ziprasidone (n=6) with three other treatments.

2.2.1.3 Belfast series of chronic schizophrenia patients

In this cross-sectional study, a series of patients from Northern Ireland with a DSM-IV diagnosis of schizophrenia or schizoaffective disorder currently receiving antipsychotic drug therapy (n=72) were studied. All patients were of Irish/British Caucasian descent, except one Malaysian Chinese. Each patient was interviewed about personal disease history and family psychiatric disease, diabetes and smoking history. All patients were tested for random blood glucose level, cholesterol, high-density (HDL) and low-density lipoproteins (LDL), and triglycerides; blood pressure and waist circumference were measured and body mass index (BMI) calculated from weight and height. These data were used to determine metabolic syndrome which was defined using the International Diabetes Federation (IDF) criteria (www.idf.org) by presence of central obesity, i.e. waist circumference ≥94 cm in men and ≥80 cm in women, plus any two further risk factors from the following:

(a) raised triglycerides (≥1.7 mmol/l) or specific treatment for this lipid abnormality;
(b) reduced HDL cholesterol (<1.03 mmol/l in men and <1.29 mmol/l in women) or specific treatment for this lipid abnormality;
(c) raised blood pressure (systolic ≥130 mmHg or diastolic ≥85 mmHg) or treatment of previously diagnosed hypertension;
(d) raised fasting blood glucose (≥5.6 mmol/l) or previously diagnosed type 2 diabetes.

Patients received antipsychotic drugs consist of clozapine (n=12), olanzpine (n=10), risperidone (n=10), haloperidol (n=1), amisulpride (n=3), aripiprazole (n=2), zuclopenthixol (n=2), flupentixol (n=12), quetiapine (n=3), chlorpromazine (n=2), sulpiride (n=2), trifluoperazine (n=3), zotapine (n=5), fluphenazine (n=1), thioridazine (n=1), one received zuclopenthixol and amisulpride, one received zuclopenthixol and chlorpromazine, and one was not receiving antipsychotics.

2.2.2 Genotyping assays

Genomic DNA previously isolated from blood using standard techniques was genotyped for polymorphisms including *MTHFR* 677C/T (rs1801133), *MTHFR* 1298A/C (rs1801131), *ADRA2A* -1291C/G (rs1800544), *MC4R* rs17782313 C/T, *MC4R* rs489693A/C, *GNB3* 825C/T (rs5443), *HTR2A* -1438G/A (rs6311) and *BDNF* 196G/A (rs6265) using Custom TaqMan® SNP Genotyping Assays (Applied Biosystems, USA). The *HTR2C* -759C/T was genotyped previously in all cohorts. The *FTO* rs9939609 genotype was genotyped previously in Spanish and Belfast cohorts by Professor Gavin Reynolds' research group (Reynolds *et al.,* 2013) and was included in analyses in this study of both genetic association and epigenetic association (next chapter) to antipsychotic induced weight gain. The genotyping SNPs and their cohorts are listed in **Table 2.3**.

Table 2.3: Genotyping SNPs and cohorts that have been genotyped.

rs number	Gene	Genetic variants	TaqMan®SNP Genotyping Assay ID	Cohorts	Previous data
rs3813929	*HTR2C*	-759C/T	-	-	Spanish, Chinese, Belfast
rs1801133	*MTHFR*	677C/T Ala222Val	C_1202883_20	Spanish, Chinese, Belfast	-
rs1801131	*MTHFR*	1298 A/C Glu429Ala	C_850486_20	Spanish, Chinese, Belfast	-
rs1800544	*ADRA2A*	1291C/G	C_7611979_10	Chinese	-
rs17782313	*MC4R*	C/T	C_32667060_10	Chinese	-
rs489693	*MC4R*	A/C	C_3058718_10	Chinese	-
rs5443	*GNB3*	825C/T	C_2184734_10	Chinese	-
rs6311	*HTR2A*	-1438G/A	C_8695278_10	Chinese	-
rs6265	*BDNF*	196G/A	C_11592758_10	Chinese	-
rs9939609	*FTO*	A/T	C_30090620_10	Chinese	Spanish, Belfast

Genotyping PCR reactions were set up in a 96-well qPCR plate in a total volume of 10 µl as listed in **Table 2.4**. Control was also set up by adding DNase-free water instead of gDNA. The reactions were set up in duplicate. The plate was covered with adhesive film and centrifuged briefly before running on a StepOne Plus Real-Time PCR System (Applied Biosystems, USA) according to PCR run condition in **Table 2.5**.

Table 2.4: PCR reaction set up for genotyping using TaqMan® SNP Genotyping Assays

PCR reaction component	Volume for 10 µL PCR reaction (µL/well)
TaqMan®GTXpress™ Master Mix (2x)	5.0
Custom TaqMan® SNP Genotyping Assays (40x)	0.25
Genomic DNA template (1-10 ng)	2.0
DNase-free water	2.75

Table 2.5: PCR condition for genotyping using TaqMan® SNP Genotyping Assays

Stage	Step	Temperature	Time
Holding	DNA polymerase activation	95 °C	20 sec
Cycling (40 cycles)	Denature Anneal/Extend	95 °C 60 °C	3 s 30 s

Allelic discrimination was performed using a post-read temperature 25°C.

2.2.3 Statistical analysis

All statistical analysis of results was performed using SPSS version 18.0. Data were expressed as mean ± standard deviation. Stepwise linear regression was used to determine where appropriate, the potential confounding effects of baseline BMI, sex, and age on weight gain. Univariate analysis of variance was used to determine the association between genotypes or genetic risk factor and clinical measures. Also, it was used to determine any gene-gene interaction and drug-gene interaction. The Bonferroni post hoc analysis was performed in olanzapine (Spanish), and risperidone and chlorpromazine (Chinese) subgroups after finding a drug-genotype interaction. The subgroup of antipsychotic drugs was analyzed based on receptor binding

characteristic of antipsychotic drugs. Chi-squared analysis was used to determine the association between categorical measures including genotype, sex distribution, percentage of weight change more or less than 7% which is considered clinically significant and is consistent with the Food and Drug Administration's definition of significant weight gain for studies of psychotropic drugs (Sachs and Guille, 1999), present/absence of central obesity and metabolic syndrome.

Hardy-Weinberg Equilibrium was calculated by web tool (http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html). Linkage disequilibrium (LD) of the *MTHFR* 677C/T and 1298A/C as well as the *MC4R* rs17782313 and rs489693 polymorphisms were analysed by SHEsis (Shi and He, 2005).

In the present study, an association of the -759C/T polymorphism of the *HTR2C* with weight gain which had previously been reported in these cohorts (Reynolds *et al.*, 2002; Templeman *et al.,* 2005; Yevtushenko *et al.,* 2008) was also included in a combined analysis with other SNPs in each cohort. In the Belfast cohort, the *FTO* rs9939609 polymorphism was also included in a combined analysis with other SNPs.

Statistical significance was assumed for p values less than 0.05. The largest sample size of 171 subjects (Chinese cohort) had approximately 90% power to identify a significant genotype difference for a medium effect size of 0.5.

2.3 Results (Part 1): First-episode antipsychotic drug naïve schizophrenia

2.3.1 General characteristics of population studies and genotyping results

General characteristics of population studies of first-episode antipsychotic drug naïve Chinese Han and Spanish patients with schizophrenia are shown in **Table 2.6**. 5-10% of samples from each cohort underwent repeated genotyping and provided reproducible results (data not shown).

2.3.1.1 Chinese Han cohort

The genotype distributions and allele frequency for all genotyped SNPs are listed in **Table 2.7**. Genotype frequencies did not deviate from Hardy-Weinberg equilibrium expectations for all SNPs (p>0.05) (**Table 2.7**). Previous *HTR2C* -759C/T SNP data had genotype distribution C=68, T=13 in male, and CC=73, CT/TT=25 in female, and the allele distribution in each gender was not significantly different (χ^2=2.374, p=0.123).

On regression analyses, baseline BMI but not age had a significant confounding effect on weight gain after 8-10 weeks treatment (F=19.90, P<0.001), whereas age had a significant confounding effect on baseline BMI (F=8.78, p=0.003). Therefore, the subsequent analyses were carried out with adjustment for age or baseline BMI as covariates.

2.3.1.2 Spanish cohort

The genotype distributions and allele frequency for all genotyped SNPs are listed in **Table 2.8**. Genotype frequencies did not deviate from Hardy-Weinberg equilibrium expectations for all genotyped SNPs (p>0.05) (**Table 2.8**). Previous *HTR2C* -759C/T SNP data had genotype distribution C=42, T=11 in male, and CC=12, CT/TT=7 in female, and the genotype distribution in each gender was not significantly different (χ^2=1.93, p=0.165). In addition, previous *FTO* rs9939609 data had genotype distribution AA=14, AT=35, and TT=21 which are in Hardy-Weinberg equilibrium (χ^2=0.0074, p =0.933) and the allele frequency for A allele was 0.45 and T allele was 0.55.

On regression analyses, age but not baseline BMI had a significant confounding effect on weight gain at 3 months after treatment (F=7.026, P=0.010). Therefore, the subsequent analyses were carried out with adjustment for age as a covariate.

Table 2.6: General characteristics of Spanish and Chinese Han cohorts

Chinese Han	**Total**	**Males**	**Females**
Number	182	83	99
Age (years)	26.24 ± 7.35	26.30 ± 7.40	26.19 ± 7.34
Baseline weight (kg)	58.83 ± 10.69	65.12 ± 10.25	53.55 ± 7.84
Baseline BMI (kg/m^2)	21.34 ± 2.87	22.16 ± 2.93	20.65 ± 2.64
Change in BMI (kg/m^2)	1.21 ± 1.21	1.27 ± 1.30	1.15 ± 1.12
Spanish	**Total**	**Males**	**Females**
Number	72	53	19
Age (years)	25.35 ± 6.80	24.19 ± 5.63	28.58 ± 8.70
Baseline weight (kg)	63.36 ± 12.37	67.24 ± 11.38	52.56 ± 7.89
Baseline BMI (kg/m^2)	21.89 ± 3.70	22.46 ± 3.62	20.31 ± 3.53
Change in BMI (kg/m^2)	2.18 ± 1.60	2.28 ± 1.53	1.90 ± 1.80

Data is expressed as mean ± SD.

Table 2.7: Genotype distribution and allele frequency in Chinese Han cohort

rs number	Gene	Genetic variants	Genotype distribution	Allele frequency	Chi-Square, p value
rs1801133	*MTHFR*	677C/T	CC=54, CT=94, TT=28	C=0.57, T=0.43	χ^2=1.4899 p =0.222
rs1801131	*MTHFR*	1298 A/C	AA=114, AC=56, CC=5	A=0.81, C=0.19	χ^2=0.3643 p =0.546
rs1800544	*ADRA2A*	1291C/G	CC=21, CG=81, GG=69	C=0.36, G=0.64	χ^2=0.1383 p =0.710
rs17782313	*MC4R*	C/T	CC=13, CT=51, TT=109	C=0.22, T=0.78	χ^2=3.7890 p =0.052
rs489693	*MC4R*	A/C	AA=7, AC=55, CC=112	A=0.20, C=0.80	χ^2=0.0058 p =0.939
rs5443	*GNB3*	825C/T	CC=40, CT=83, TT=50	C=0.47, T=0.53	χ^2=0.2406 p =0.624
rs6311	*HTR2A*	-1438G/A	GG=38, GA=86, AA=49	G=0.47, A=0.53	χ^2=0.0006 p =0.982
rs6265	*BDNF*	196G/A	GG=39, GA=89, AA=45	G=0.48, A=0.52	χ^2=0.1567 p =0.692
rs9939609	*FTO*	A/T	AA=3, AT=35, TT=141	A=0.11, T=0.89	χ^2=0.2294 p =0.631

Table 2.8: Genotype distribution and allele frequency in Spanish cohort

rs number	Gene	Genetic variants	Genotype distribution	Allele frequency	Ch-Square, p value
rs1801133	*MTHFR*	677C/T	CC=20, CT=36, TT=13	C=0.55, T=0.45	χ^2=0.2047 p =0.652
rs1801131	*MTHFR*	1298 A/C	AA=45, AC=21, CC=3	A=0.80, C=0.20	χ^2=0.0759 p =0.784

2.3.2 Association of *MTHFR* 677C/T and 1298A/C polymorphisms with weight gain in first episode drug naïve schizophrenia patients

2.3.2.1 MTHFR 677C/T

Table 2.9: Effect of *MTHFR* 677C/T polymorphism on changes in body weight in first episode drug naïve schizophrenia patients

	***MTHFR* 677C/T genotype**			
	CC	**CT**	**TT**	**p value**
Chinese Han sample	n=54	n=94	n=28	
Sex M/F (%male)	25/29 (46.3%)	57/65 (46.7%)	13/15 (46.4%)	0.998[a]
Age (years)	25.04 ± 6.84	26.21 ± 7.18	28.45 ± 8.60	0.136
Baseline BMI (kg/m^2)	20.99 ± 2.69	21.43 ± 2.77	21.92 ± 3.53	0.607[c]
Change BMI (kg/m^2)	1.58 ± 1.25	0.92 ± 1.15	1.43 ± 1.10	**0.003**[b]
Spanish sample	n=20	n=36	n=13	
Sex M/F (%male)	14/6 (70.0%)	29/7 (80.6%)	9/4(69.2%)	0.578[a]
Age (years)	27.60 ± 8.34	24.25 ± 5.69	23.92 ± 6.96	0.168
Baseline BMI (kg/m^2)	21.44 ± 3.78	22.06 ± 3.70	22.29 ± 4.01	0.780
Change BMI (kg/m^2)	2.86 ± 1.53	2.09 ± 1.44	1.85 ± 1.81	**0.049**[c]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate. Data is expressed as mean ± SD.

As shown in **Table 2.9**, the baseline BMI, age, and sex distribution of both samples were not significantly different between genotypes of the *MTHFR* 677C/T polymorphism. Dividing genotype into two groups by combining minor risk (T) allele carriers found that the CC genotype had greater changes in BMI than T allele carriers: 1.58±1.25 versus 1.04±1.16 kg/m^2 in Chinese (p=0.012) and 2.86±1.53 versus 2.02±1.54 kg/m^2 in the Spanish sample (p=0.017) (**Figure. 2.3**). Analysis in the subgroup of Chinese Han schizophrenia patients who had received risperidone, the CC carriers had higher BMI change than T allele carriers; 1.69±1.24 (n=33) versus 1.00±1.17 kg/m^2 (n=75), p=0.051.

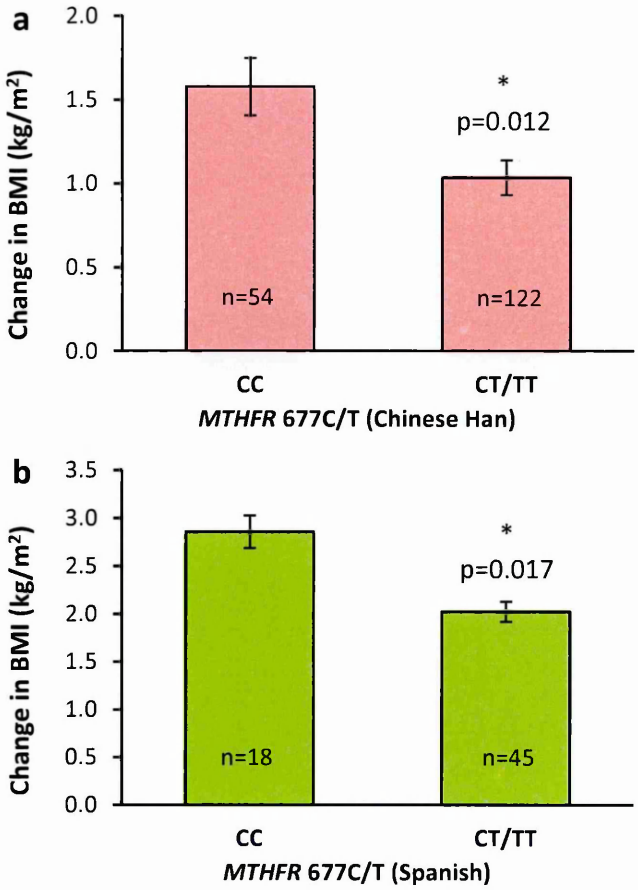


Figure 2.3: The association between *MTHFR* 677C/T genotype and weight gain
The T allele of the *MTHFR* 677C/T polymorphism shows significantly lower in BMI change in both cohorts; (a) Chinese Han and (b) Spanish comparing to CC genotype. Data is expressed as mean ± SEM.

Dividing the Spanish cohort into patients who had received or did not receive olanzapine, and the Chinese cohort into those receiving either risperidone or chlorpromazine, did not identify a significant drug x *MTHFR* 677C/T genotype interaction (p=0.193 and p=0.667 for Spanish and Chinese cohort, respectively). These results indicate that the genotype effect of the *MTHFR* 677C/T on weight gain is independent of drug effect; in other word, the genotype effect on weight gain is not different between drug treatments.

2.3.2.2 MTHFR 1298A/C

The baseline BMI, age, and sex distribution of both study populations were not significantly associated with the *MTHFR* 1298A/C polymorphism (**Table 2.10**). Dividing

the sample into two genotype groups by combining minor risk (C) allele found that the changes in BMI of both study populations were still not significantly different between 1298A/C AA genotype and C allele carriers: 1.27±1.24 versus 1.08±1.17 kg/m^2 in Chinese Han samples (p=0.242) and 2.18±1.66 versus 2.40±1.45 kg/m^2 in Spanish samples (p=0.621) respectively.

Table 2.10: Effect of *MTHFR* 1298A/C polymorphism on changes in body weight in first episode drug naïve schizophrenia patients

	***MTHFR* 1298A/C genotype**			
	AA	**AC**	**CC**	**p value**
Chinese Han sample	n=114	n=56	n=5	
Sex M/F (%male)	53/61 (46.5%)	24/32 (42.9%)	4/1 (80%)	0.279[a]
Age (years)	26.54 ± 7.77	25.61 ± 6.61	27.20 ± 5.54	0.710
Baseline BMI (kg/m^2)	21.47 ± 3.06	21.20 ± 2.59	21.78 ± 1.51	0.895[c]
Change BMI (kg/m^2)	1.27 ± 1.24	1.04 ± 1.18	1.63 ± 0.94	0.228[b]
Spanish sample	n=45	n=21	n=3	
Sex M/F (%male)	32/13 (71.1%)	17/4 (81%)	3/0(100%)	0.412[a]
Age (years)	25.36 ± 7.36	24.76 ± 6.38	25.00 ± 1.73	0.949
Baseline BMI (kg/m^2)	21.92 ± 3.62	21.99 ± 4.16	21.45 ± 3.76	0.973
Change BMI (kg/m^2)	2.18 ± 1.66	2.46 ± 1.52	2.03 ± 0.99	0.807[c]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate. Data is expressed as mean ± SD.

Dividing the Spanish cohort into patients who had received or did not receive olanzapine, and the Chinese cohort into those receiving either risperidone or chlorpromazine, did not identify a significant drug x *MTHFR* 1298A/C genotype interaction (p=0.296 and p=0.363 for Spanish and Chinese cohort, respectively).

The *MTHFR* 677C/T and 1298A/C polymorphisms were in strong linkage disequilibrium in both Chinese and Spanish cohorts: D'=0.866, r^2=0.127 for Chinese and D'1.000, r^2=0.198 for Spanish cohort.

2.3.2.3 Gene-gene interaction

Previous findings in these two cohorts (Reynolds *et al.*, 2002; Templeman *et al.*, 2005) showed the T allele of the *HTR2C* -759C/T polymorphism had a protective effect against antipsychotic-induced weight gain. Association of this polymorphism with changes in BMI were as follows: in the Chinese cohort T allele carriers 0.71±1.11 kg/m^2 (n=38), C/CC genotype 1.33±1.21 kg/m^2 (n=141), p=0.004; in the Spanish cohort T allele carriers 1.24±1.46 kg/m^2 (n=16), C/CC genotype 2.48±1.54 kg/m^2 (n=50) p=0.012. Previous findings in the Spanish cohort showed the *FTO* rs9939609 had no influence on BMI change (Reynolds *et al.*, 2013).

The relationship between the effects of the *HTR2C* -759C/T and the *MTHFR* 677C/T polymorphisms was investigated. Analysing the association of weight gain with both polymorphisms together in each cohort, no significant interaction between the polymorphisms was detected but a significant overall effect was observed (p<0.001 in Chinese sample; p=0.019 in Spanish sample) indicating an independent effect of the two polymorphisms. Thus carriage of two risk factors (*HTR2C* C/CC genotype and *MTHFR* 677 CC genotype) was significantly associated with mean BMI gains in Chinese and Spanish cohorts (**Table 2.11**).

Table 2.11: Combined genetic risk genotype of *HTR2C* -759C/T and *MTHFR* 677C/T

Number of risk genotype	Chinese Han			Spanish		
	BMI change (kg/m^2)	n	p value	BMI change (kg/m^2)	n	p value
0	0.63 ± 1.20	25	**0.001**	1.36 ± 1.57	10	**0.005**
1	1.10 ± 1.11	107		2.14 ± 1.46	39	
2	1.81 ± 1.26	41		3.23 ± 1.46	14	

BMI change values are expressed as mean ± SD. p value was obtained from univariate analysis of variance with baseline BMI and age as covariates for Chinese and Spanish cohorts, respectively.

2.3.3 Association of *ADRA2A* rs1800544 (-1291C/G) polymorphism with weight gain in Chinese Han schizophrenia patients

The baseline BMI, age, and sex distribution of were not significantly associated with the *ADRA2A* -1291C/G polymorphism (**Table 2.12**). Dividing genotypes into two groups by combining risk (G) allele carriers found that the G allele carriers tended to have a greater change in BMI than that of CC genotype but this did not reach statistical significance: 1.25±1.21 versus 0.84±1.09 kg/m^2 (p=0.126). However, there was a non-significant indication of drug x *ADRA2A* -1291C/G genotype interaction in the analysis of patients receiving either risperidone or chlorpromazine (p=0.072). Therefore, post hoc analysis was performed in a subgroup of patients who had received risperidone and this analysis found a significantly greater change in BMI in G allele carriers than CC genotype: 1.30±1.19 kg/m^2 (n=91) versus 0.47±1.14 kg/m^2 (n=12), p=0.027 (**Figure 2.4**).

Table 2.12: Effect of *ADRA2A* -1291C/G polymorphism on changes in body weight in Chinese Han schizophrenia patients

	***ADRA2A* -1291C/G genotype**			
	CC	**CG**	**GG**	**p value**
Chinese Han sample	n=21	n=81	n=69	
Sex M/F (%male)	8/13 (38.10%)	39/42 (48.15%)	33/36 (47.8%)	0.695[a]
Age (years)	25.14 ± 7.02	25.80 ± 6.88	26.88 ± 8.09	0.537
Baseline BMI (kg/m^2)	21.36 ± 3.07	21.13 ± 2.79	21.69 ± 2.94	0.601[c]
Change BMI (kg/m^2)	0.84 ± 1.09	1.24 ± 1.29	1.25 ± 1.13	0.279[b]
Baseline weight (kg)	57.76 ± 12.18	58.28 ± 10.48	60.23 ± 10.62	0.496[d]
Weight change (kg)	2.17 ± 3.05	3.38 ± 3.60	3.43 ± 3.16	0.206[e]
Weight change (%)	4.34 ± 4.91	6.16 ± 6.55	6.11 ± 5.51	0.247[e]
Weight increase>7%	n=8/13 (38.1%)	n=37/81 (45.7%)	n=33/69 (47.8%)	0.735[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate, [d] analyses with age and sex as covariates, [e] analyses with baseline weight as a covariate. Data is expressed as mean ± SD.

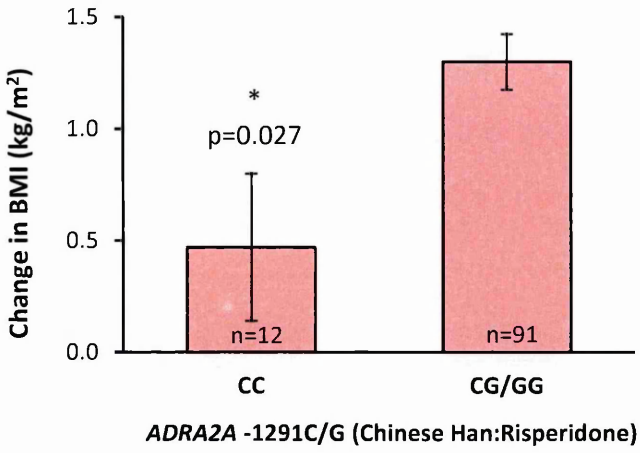


Figure 2.4: The association between *ADRA2A* -1291C/G genotype and weight gain in Risperidone treatment group of Chinese Han cohort

The relationship between the effects of the *ADRA2A* -1291C/G, the *HTR2C* -759C/T, and the *MTHFR* 677C/T polymorphisms was investigated. Analysing the association of weight gain with *ADRA2A* -1291C/G and *HTR2C* -759C/T or *ADRA2A* -1291C/G and *MTHFR* 677C/T polymorphisms or all three polymorphisms together, no significant interaction between the polymorphisms was detected but a significant overall effect was observed ($p \leq 0.001$) both in total cases and in the subgroup receiving risperidone treatment indicating an additive effect of the three polymorphisms.

2.3.4 Association of *HTR2A* rs6311 (-1438G/A) polymorphism with weight gain in Chinese Han schizophrenia patients

The baseline BMI, age, and sex distribution were not significantly associated with the *HTR2A* -1438G/A polymorphism (**Table 2.13**). Dividing genotype into two groups by combining risk (A) allele carriers found that the A allele carriers had a slightly greater change in BMI than that of GG genotype but this did not reach statistical significance: 1.25±1.15 versus 1.03±1.36 kg/m^2 (p=0.326). However, there was a significant drug x *HTR2A* -1438G/A genotype interaction in the analysis of patients receiving either risperidone or chlorpromazine (p=0.017) (**Figure 2.5**). Therefore, post hoc analysis was performed in the subgroup of patients who had received risperidone and this found a significant greater change in BMI in A allele carriers than GG genotype: 1.35±1.18 kg/m^2 (n=79) versus 0.79±1.20 kg/m^2 (n=26), p=0.047 (**Figure 2.6**).

Table 2.13: Effect of *HTR2A* -1438G/A polymorphism on changes in body weight in Chinese Han schizophrenia patients

	***HTR2A* -1438G/A genotype**			
	GG	**GA**	**AA**	**p value**
Chinese Han sample	n=38	n=86	n=49	
Sex M/F (%male)	18/20 (47.37%)	43/43 (50%)	20/29 (40.8%)	0.588[a]
Age (years)	24.97 ± 7.83	26.91 ± 6.82	25.65 ± 7.87	0.349
Baseline BMI (kg/m^2)	21.45 ± 2.77	21.22 ± 2.89	21.49 ± 3.01	0.657[c]
Change BMI (kg/m^2)	1.03 ± 1.36	1.31 ± 1.17	1.15 ± 1.11	0.509[b]
Baseline weight (kg)	60.21 ± 10.69	58.47 ± 10.88	58.62 ± 10.66	0.310[d]
Weight change (kg)	2.86 ± 3.83	3.54 ± 3.29	3.09 ± 3.11	0.609[e]
Weight change (%)	5.21 ± 7.18	6.51 ± 5.68	5.53 ± 5.38	0.532[e]
Weight increase>7%	n=14/38 (36.8%)	n=46/86 (53.5%)	n=19/49 (38.8%)	0.119[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate, [d] analyses with age and sex as covariates, [e] analyses with baseline weight as a covariate. Data is expressed as mean ± SD.

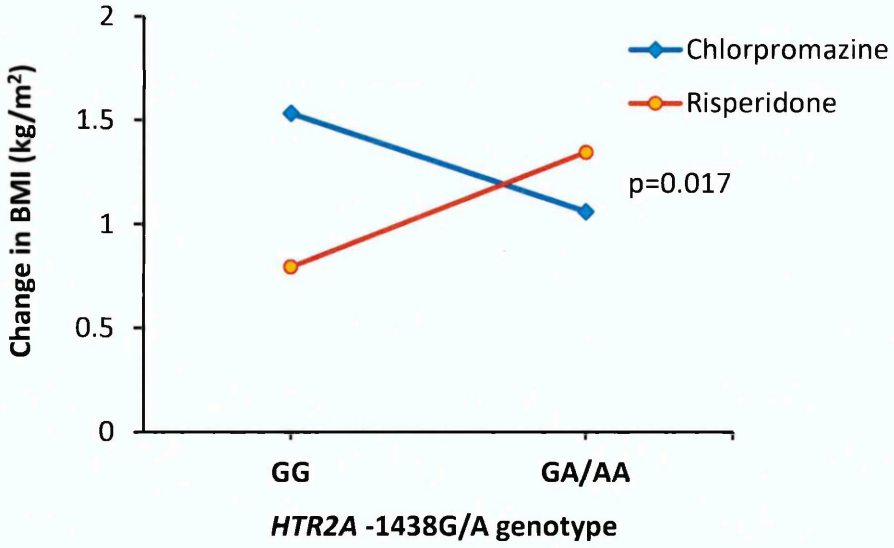


Figure 2.5: The interaction between *HTR2A* -1438G/A genotype and antipsychotic drug treatment on weight gain in Chinese Han cohort in the analysis of subgroup of patients receiving either risperidone or chlorpromazine

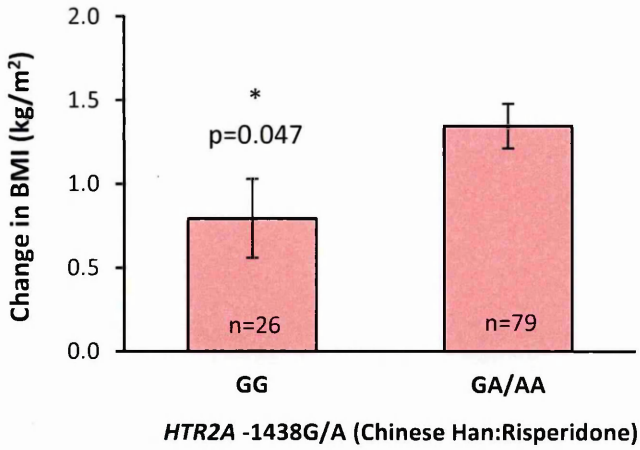


Figure 2.6: The association between *HTR2A* -1438G/A genotype and weight gain in risperidone treatment group of Chinese Han cohort

The relationship between the effects of the *HTR2A* -1438G/A, the *ADRA2A* -1291C/G, the *HTR2C* -759C/T, and the *MTHFR* 677C/T polymorphisms was investigated. Analysing the association of weight gain with *HTR2A* -1438G/A polymorphism and the other three polymorphisms together, no significant interaction between the polymorphisms was detected but a significant overall effect was observed ($p \leq 0.002$) both in the analysis of total cases and in a subgroup of risperidone treatment indicating an additive effect of the four polymorphisms.

2.3.5 Association of rs17782313 and rs489693 polymorphisms near *MC4R* gene with weight gain in Chinese Han schizophrenia patients

The baseline BMI, age, and sex distribution were not significantly associated with polymorphisms near *MC4R*; rs17782313 C/T polymorphism (**Table 2.14**) and rs489693 A/C polymorphism (**Table 2.15**).

Dividing *MC4R* rs17782313 genotype into two groups by combining minor risk (C) allele found that changes in BMI were not significantly different between *MC4R* rs17782313 TT genotype and C allele carriers: 1.31±1.06 (n=109) versus 1.02±1.39 kg/m^2 (n=64), p=0.163. However, TT genotype had a higher frequency of percentage of weight changes over than 7% (n=56/109, 51.38%) compared to C allele carriers (n=23/64, 35.94%), χ^2=3.874, p=0.049. No drug x *MC4R* rs17782313 genotype interaction was

observed when analysing patients receiving either risperidone or chlorpromazine (p=0.822).

The *MC4R* rs489693, dividing by genotype into two groups by combining C allele vs AA genotype based on BMI change found that BMI change in the C allele carriers was significantly greater than that of carriers with AA homozygous genotype: 1.25±1.21 (n=167) versus 0.31±1.19 (n=7) kg/m^2 (*p*=0.040)(**Figure 2.7**). The genotype distribution of AA and AC/CC was not significantly different between groups of patients who had weight changes more than 7% and less than 7%. The percentage of weight change in C allele carriers was higher than that of AA genotype: 6.16±6.00 vs 1.45±5.69% (p=0.052). No drug x *MC4R* rs489693 genotype interaction was observed when analysis patients receiving either risperidone or chlorpromazine (p=0.810).

The relationship between the effects of the *HTR2C* -759C/T, *MTHFR* 677C/T, *MC4R* rs17782313 and *MC4R* rs489693 polymorphisms was investigated. No significant interaction between the *HTR2C* -759C/T or *MTHFR* 677C/T and the *MC4R* rs17782313 as well as *MC4R* rs489693 polymorphisms in their effect on BMI change was detected in this Chinese Han cohort.

The *MC4R* rs17782313 and rs489693 polymorphisms were in weak LD: D'=0.196, r^2=0.007.

Table 2.14: Effect of *MC4R* rs17782313 C/T polymorphism on changes in body weight in Chinese Han schizophrenia patients

	***MC4R* rs17782313 genotype**			
	CC	**CT**	**TT**	**p value**
Chinese Han sample	n=13	n=51	n=109	
Sex M/F (%male)	6/7 (46.15%)	26/25 (50.98%)	49/60 (44.9%)	0.775[a]
Age (years)	24.46 ± 6.63	25.45 ± 5.97	26.65 ± 8.00	0.443
Baseline BMI (kg/m^2)	21.11 ± 2.52	21.66 ± 3.32	21.23 ± 2.72	0.529[c]
Change BMI (kg/m^2)	0.97 ± 1.41	1.03 ± 1.40	1.31 ± 1.06	0.354[b]
Baseline weight (kg)	58.92 ± 9.15	60.69 ± 12.35	58.05 ± 10.07	0.328[d]
Weight change (kg)	2.65 ± 3.75	2.78 ± 3.89	3.56 ± 3.02	0.450[e]
Weight change (%)	4.86 ± 7.26	5.35 ± 7.22	6.36 ± 5.10	0.683[e]
Weight increase>7%	n=4/13 (30.8%)	n=19/51 (37.3%)	n=56/109 (51%)	0.132[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate, [d] analyses with age and sex as covariates, [e] analyses with baseline weight as a covariate. Data is expressed as mean ± SD.

Table 2.15: Effect of *MC4R* rs489693 A/C polymorphism on changes in body weight in Chinese Han schizophrenia patients

	***MC4R* rs489693 genotype**			
	AA	**AC**	**CC**	**p value**
Chinese Han sample	n=7	n=55	n=112	
Sex M/F (%male)	4/3 (57.14%)	26/29 (47.27%)	50/62 (44.6%)	0.791[a]
Age (years)	24.71 ± 6.58	25.66 ± 6.55	26.67 ± 7.79	0.603
Baseline BMI (kg/m^2)	21.58 ± 2.49	21.43 ± 3.15	21.38 ± 2.74	0.916[c]
Change BMI (kg/m^2)	0.31 ± 1.19	1.21 ± 1.35	1.26 ± 1.13	0.119[b]
Baseline weight (kg)	61.29 ± 8.06	59.49 ± 11.54	58.49 ± 11.54	0.775[d]
Weight change (kg)	0.93 ± 3.27	3.26 ± 3.71	3.41 ± 3.19	0.205[e]
Weight change (%)	1.45 ± 5.69	6.14 ± 7.03	6.16 ± 5.46	0.149[e]
Weight increase>7%	n=1/7 (14.3%)	n=25/55 (45.5%)	n=54/112 (48%)	0.216[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate, [d] analyses with age and sex as covariates, [e] analyses with baseline weight as a covariate. Data is expressed as mean ± SD.

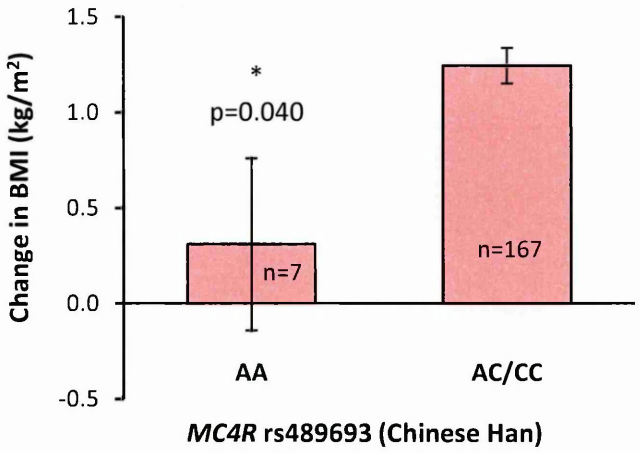


Figure 2.7: The association between *MC4R* rs489693 A/C genotype and weight gain in Chinese Han cohort

2.3.6 Association of *GNB3* rs5443 (825C/T) polymorphism with weight gain in Chinese Han schizophrenia patients

The baseline BMI, age, and sex distribution were not significantly associated with the *GNB3* 825 C/T (**Table 2.16**). Dividing genotype into two groups by combining minor (C) allele carriers found that changes in BMI were not significantly different between *GNB3* rs5443825 C allele carriers and TT genotype: 1.23±1.24 (n=123) versus 1.13±1.10 kg/m^2 (n=50), p=0.503. No significant difference of weight changes and percentage of weight changes were found between TT genotype and C allele carriers. Genotype distribution was not different between groups of patients who had percentages of weight changes over 7% and less than 7%. No drug x *GNB3* 825C/T genotype interaction was observed when analysing patients receiving either risperidone or chlorpromazine (p=0.459).

Table 2.16: Effect of *GNB3* 825 C/T polymorphism on changes in body weight in Chinese Han schizophrenia patients

	GNB3 825C/T genotype			
	CC	**CT**	**TT**	**p value**
Chinese Han sample	n=40	n=83	n=50	
Sex M/F (%male)	20/20 (50%)	37/46 (44.58%)	24/26 (48%)	0.836[a]
Age (years)	26.73 ± 7.01	25.75 ± 7.22	26.28 ± 7.94	0.781
Baseline BMI (kg/m^2)	20.77 ± 2.65	21.71 ± 2.95	21.20 ± 2.93	0.149[c]
Change BMI (kg/m^2)	1.20 ± 1.34	1.25 ± 1.18	1.13 ± 1.10	0.569[b]
Baseline weight (kg)	57.15 ± 10.58	60.17 ± 11.21	58.17 ± 9.97	0.053[d]
Weight change (kg)	3.09 ± 3.77	3.43 ± 3.32	3.11 ± 3.12	0.592[e]
Weight change (%)	6.19 ± 6.90	6.06 ± 6.00	5.57 ± 5.11	0.639[e]
Weight increase>7%	n=21/40(52.5%)	n=37/83 (44.6%)	n=21/50 (42%)	0.588[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate, [d] analyses with age and sex as covariates, [e] analyses with baseline weight as a covariate. Data is expressed as mean ± SD.

2.3.7 Association of *BDNF* rs6265 196G/A (Val66Met) polymorphism with weight gain in Chinese Han schizophrenia patients

The baseline BMI, age, and sex distribution were not significantly associated with the *BDNF* rs6265 G/A (**Table 2.17**). Dividing genotype into two groups by combining minor risk (G) allele carriers found that changes in BMI were not significantly different between *BDNF* rs6265 G allele carriers and AA genotype: 1.14±1.20 (n=128) versus 1.36±1.19 kg/m^2 (n=45), p=0.519. No significant difference of weight changes and percentage of weight changes were found between AA genotype and G allele carriers. Genotype distribution was not different between groups of patients who had percentages of weight changes over 7% and less than 7%. No drug x *BDNF* rs6265 G/A genotype interaction was observed when analysis patients receiving either risperidone or chlorpromazine (p=0.100).

Table 2.17: Effect of *BDNF* rs6265G/A polymorphism on changes in body weight in Chinese Han schizophrenia patients

	***BDNF* rs6265 (Val66Met) genotype**			
	GG	**GA**	**AA**	**p value**
Chinese Han sample	n=39	n=89	n=45	
Sex M/F (%male)	16/23 (41.03%)	41/48 (46.07%)	24/21 (53.3%)	0.519[a]
Age (years)	25.33 ± 7.38	25.98 ± 7.34	27.11 ± 7.44	0.526
Baseline BMI (kg/m^2)	21.68 ± 3.25	21.45 ± 2.92	20.85 ± 2.44	0.234[c]
Change BMI (kg/m^2)	1.16 ± 1.41	1.14 ± 1.10	1.36 ± 1.19	0.788[b]
Baseline weight (kg)	59.54 ± 12.31	59.48 ± 10.45	57.18 ± 9.87	0.054[d]
Weight change (kg)	3.04 ± 3.77	3.13 ± 3.15	3.72 ± 3.41	0.725[e]
Weight change (%)	5.90 ± 7.30	5.54 ± 5.32	6.80 ± 5.91	0.742[e]
Weight increase>7%	n=15/39(38.5%)	n=39/89 (43.8%)	n=25/45 (55.6%)	0.258[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate, [d] analyses with age and sex as covariates, [e] analyses with baseline weight as a covariate. Data is expressed as mean ± SD.

2.3.8 Association of *FTO* rs9939609 A/T polymorphism with weight gain in Chinese Han schizophrenia patients

The baseline BMI, age, and sex distribution were not significantly associated with the *FTO* rs9939609 A/T (**Table 2.18**). Dividing genotype into two groups by combining the risk allele A and AT/TT genotype (Perez-Iglesias *et al.,* 2010) found that changes in BMI were not significantly different between *FTO* rs9939609 AA genotype and T allele carriers: 1.02±1.46 (n=3) versus 1.21±1.20 kg/m^2 (n=176), p=0.937. No significant difference of weight changes and percentage of weight changes were found between AA genotype and T allele carriers. Genotype distribution was not different between groups of patients who had percentages of weight changes over 7% and less than 7%. No drug x *FTO* rs9939609 genotype interaction was observed when analysis patients receiving either risperidone or chlorpromazine (p=0.752).

Table 2.18: Effect of *FTO* rs9939609A/T polymorphism on changes in body weight in Chinese Han schizophrenia patients

	***FTO* rs9939609 genotype**			
	AA	**AT**	**TT**	**p value**
Chinese Han sample	n=3	n=35	n=141	
Sex M/F (%male)	1/2 (33.33%)	14/21 (40%)	67/74 (47.5%)	0.660[a]
Age (years)	32.00 ± 1.73	27.19 ± 7.27	25.92 ± 7.39	0.260
Baseline BMI (kg/m^2)	22.29 ± 2.03	20.98 ± 2.62	21.45 ± 2.93	0.525[c]
Change BMI (kg/m^2)	1.02 ± 1.46	1.29 ± 0.96	1.19 ± 1.26	0.987[b]
Baseline weight (kg)	60.67 ± 12.22	56.80 ± 9.42	59.36 ± 10.92	0.459[d]
Weight change (kg)	2.50 ± 3.91	3.43 ± 2.62	3.23 ± 3.51	0.947[e]
Weight change (%)	4.97 ± 6.65	6.32 ± 4.60	5.89 ± 6.32	0.974[e]
Weight increase>7%	n=2/3(66.7%)	n=18/35 (51.4%)	n=61/141 (43%)	0.517[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with baseline BMI as a covariate, [c] analyses with age as a covariate, [d] analyses with age and sex as covariates, [e] analyses with baseline weight as a covariate. Data is expressed as mean ± SD.

2.3.9 Association of combined five polymorphisms associated with weight gain in Chinese Han schizophrenia patients

Taking the results of part one in the first episode schizophrenia patients together, there were five polymorphisms that showed significant associations with BMI gains from both the analysis of total cases as well as a subgroup of patients who had received risperidone; *HTR2C* -759C/T, *MTHFR* 677C/T, *MC4R* rs489693 A/C, *ADRA2A* -1291C/G, and *HTR2A* -1438 G/A polymorphisms. The *HTR2C* -759C/T, *MTHFR* 677C/T, and *MC4R* rs489693 A/C polymorphisms were associated with weight gain in the analysis of total cases, while the *ADRA2A* -1291C/G and *HTR2A* -1438 G/A polymorphisms were associated with weight gain in the analysis of a subgroup of patients who had received risperidone, although *MTHFR* 677C/T showed a trend of CC genotype with a higher BMI gain than T allele carriers (p=0.051).

Analysis of the gene-gene interactions among these polymorphisms did not show a significant interaction in ethier the analysis of total cases or as a subgroup of patients

who had received risperidone, but always found a significant overall effect indicating an additive effect of these polymorphisms on BMI change.

Analysing the association of weight gain with five polymorphisms together, carriage of 5 risk genotypes (*HTR2C* C/CC, *MTHFR* 677 CC, *MC4R* rs489693 AC/CC, *ADRA2A* CG/GG, *HTR2A* GA/AA genotype) was significantly associated with mean BMI gains of 1.98 kg/m^2 (n=24); smaller BMI gain values for subjects carrying fewer risk genotypes was observed; however, no patients carried either 0 or 1 risk genotype as shown in **Table 2.19**.

Table 2.19: Combined genetic risk genotype of the *HTR2C* -759C/T, *MTHFR* 677C/T, *MC4R* rs489693 A/C, *ADRA2A* -1291C/G, and *HTR2A* -1438 G/A polymorphisms in Chinese Han cohort

Number of risk genotype	**Chinese Han (total cases)**			**Chinese Han (risperidone subgroup)**		
	BMI change (kg/m^2)	n	p value	BMI change (kg/m^2)	n	p value
2	0.05 ± 0.52	10	**<0.001**	-0.05 ± 0.44	5	**<0.001**
3	0.93 ± 1.24	53		0.76 ± 1.19	30	
4	1.28 ± 1.18	78		1.31 ± 1.21	48	
5	1.98 ± 0.93	24		2.21 ± 0.75	14	

BMI change values are expressed as mean ± SD. p value was obtained from univariate analysis of variance with baseline BMI as a covariate.

2.4 Results (Part 2): Chronic schizophrenia patients

2.4.1 General characteristics of population studies and genotyping results

The general characteristics of Belfast chronic schizophrenia patients are listed in **Table 2.20**. The genotype distributions for all genotyped SNPs are listed in **Table 2.21**. A 5% of samples underwent repeated genotyping and obtained reproducible results (data not shown). Genotype frequencies for *MTHFR* 677C/T and 1298A/C did not deviate from Hardy-Weinberg equilibrium expectations (p>0.05) (**Table 2.21**). Previous *HTR2C* -759C/T SNP data had genotype distribution C=33, T=8 in male, and CC=20, CT/TT=11 in female, and the genotype distribution in each gender was not significantly different (χ^2=2.318, p=0.128). In addition, previous *FTO* rs9939609 data had genotype distribution AA=14, AT=27, and TT=31 which was in Hardy-Weinberg equilibrium (χ^2=3.0496, p =0.081) and the allele frequency for A allele was 0.38 and T allele was 0.62.

On regression analyses, age and sex did not have a significant confounding effect on BMI. Sex had significant confounding effects on plasma leptin (df=1, F=61.364, p<0.001 and df=1, F=19.491, p<0.001 respectively) and waist:hip ratio (df=1, F=6.320, p=0.014), and HDL (df=1, F=7.817, p=0.007). Therefore, the subsequent analyses were carried out with adjustment for sex variables as covariates as appropriate.

Table 2.20: General characteristics of chronic schizophrenia patients (Belfast cohort)

DNA series	Belfast
Sex (M,F)	n=72 (41,31)
Age (years)	44.07 ± 11.28
BMI (kg/m^2)	28.46 ± 6.87
Weight (kg)	81.94 ± 19.37

Table 2.21: Genotype distribution and allele frequency in Belfast cohort

rs number	Gene	Genetic variants	Genotype distribution	Allele frequency	Ch-Square, p value
rs1801133	*MTHFR*	677C/T	CC=34, CT=31, TT=7	C=0.69, T=0.31	χ^2=0.0003 p =986
rs1801131	*MTHFR*	1298 A/C	AA=38, AC=30, CC=4	A=0.74, C=0.26	χ^2=0.3771 p =0.539

2.4.2 Association of *MTHFR* 677C/T and 1298A/C polymorphisms with body weight in chronic schizophrenia patients

2.4.2.1 MTHFR 677C/T

Table 2.22: Effect of *MTHFR* 677C/T polymorphism on the measurements of obesity and presence of metabolic syndrome in chronic schizophrenia patients

	***MTHFR* 677C/T genotype**			
	CC	**CT**	**TT**	**p value**
n=72	34	31	7	
Sex M/F (%male)	18/16 (52.9%)	19/12 (61.3%)	4/3 (57.1%)	0.794[a]
Age (years)	43.29 ± 11.72	45.42 ± 9.70	41.86 ± 16.15	0.652
BMI (kg/m^2)	29.35 ± 8.39	27.81 ± 5.18	27.06 ± 5.31	0.580
Weight (kg)	82.83 ± 23.01	82.64 ± 18.78	78.93 ± 15.01	0.897
Waist circumference (cm)	101.42 ± 19.35	99.13 ± 15.22	98.36 ± 14.74	0.836
Waist : hip ratio	0.94 ± 0.12	0.90 ± 0.10	0.92 ± 0.12	0.221[b]
BMI ≥ 30 kg/m^2	n=15/33(45.5%)	n=9/30(30%)	n=2/7 (28.6%)	0.396[a]
Triglycerides (mmol/l)	2.44 ± 1.51	2.19 ± 1.59	1.43 ± 0.56	0.264
HDL (mmol/l)	1.07 ± 0.33	1.19 ± 0.28	1.26 ± 0.33	0.101[b]
Central obesity	n=24/32(68.8%)	n=24/31(77.4%)	n=4/7(57.1%)	0.537[a]
Metabolic syndrome	n=13/33(39.4%)	n=14/31(45.2%)	n=3/7(42.9%)	0.896[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with sex as a covariate. Data is expressed as mean ± SD.

As shown in **Table 2.22**, sex distribution, age, BMI, weight, waist circumference, waist:hip ratio, triglycerides, HDL, and presence of central obesity and metabolic syndrome were not significantly associated with the 677C/T *MTHFR* polymorphism.

Nor were the BMI significantly different between 677C/T CC genotype and T allele carriers: 29.35±8.39 kg/m^2 (n=33) versus 27.66±5.14 kg/m^2(n=37), p=0.310; however, it was noted that T allele carriers had 1.69 kg/m^2 lower BMI compared to CC genotype. Similar to BMI, a lower frequency of patients who had BMI over than 30 kg/m^2 was observed in T allele carriers (n=11/37, 29.73%) compared to CC genotype group (n=15/33, 45.45%) but it did not reach a statistically significant level.

2.4.2.2 MTHFR 1298A/C

Table 2.23: Effect of *MTHFR* 1298A/C polymorphism on the measurements of obesity and presence of metabolic syndrome in chronic schizophrenia patients

	***MTHFR* 1298A/C genotype**			
	AA	**AC**	**CC**	**p value**
n=72	38	30	4	
Sex M/F (%male)	23/15 (60.5%)	17/13 (56.2%)	1/3 (33.3%)	0.394[a]
Age (years)	43.79 ± 10.11	45.00 ± 13.07	39.75 ± 8.06	0.672
BMI (kg/m^2)	28.53 ± 6.95	27.85 ± 6.12	32.35 ± 11.59	0.472
Weight (kg)	83.32 ± 19.94	80.05 ± 19.06	90.75 ± 35.47	0.570
Waist circumference (cm)	100.89 ± 16.75	97.83 ± 15.28	109.25 ± 30.63	0.422
Waist : hip ratio	0.92 ± 0.10	0.90 ± 0.12	0.97 ± 1.16	0.298[b]
BMI ≥ 30 kg/m^2	n=12/36(33.3%)	n=12/30(40%)	n=2/4(50%)	0.736[a]
Triglycerides (mmol/l)	2.27 ± 1.56	2.18 ± 1.40	2.27 ± 2.07	0.972
HDL (mmol/l)	1.17 ± 0.31	1.12 ± 0.31	0.97 ± 0.34	0.212[b]
Central obesity	n=29/37(78.4%)	n=20/29(69%)	n=3/4(75%)	0.685[a]
Metabolic syndrome	n=18/37(48.7%)	n=11/30(36.7%)	n=1/4(25%)	0.474[a]

[a] p values obtained from Chi-squared test, other p values obtained from univariate analysis of variance test, [b] analyses with sex as a covariate. Data is expressed as mean ± SD.

As shown in **Table 2.23**, sex distribution, age, BMI, weight, waist circumference, waist:hip ratio, triglycerides, HDL, and presence of central obesity and metabolic syndrome were not significantly associated with the 1298A/C *MTHFR* polymorphism. Nor were the BMI significantly different between 1298A/C AA genotype and C allele carriers: 28.53±6.95 kg/m^2 (n=36) versus 28.38±6.88 kg/m^2 (n=34), p=0.927.

The *MTHFR* 677C/T and 1298A/C polymorphisms were in strong LD in this chronic (Belfast) cohort: D'=1.000, r^2=0.163.

2.4.3 Previous findings and gene-gene interaction

Previous findings in this chronic schizophrenia patient group showed that neither the *HTR2C* -759C/T nor leptin -2548A/G polymorphisms was significantly associated with measures of obesity including BMI, BMI≥30 kg/m^2, central obesity, and waist circumference; whereas, the leptin -2548A/G, but not *HTR2C* -759C/T polymorphism was significantly associated with metabolic syndrome (Yevtushenko *et al.,* 2008). These effects were not apparent in re-analysis in this study probably due to the smaller sample size. In addition, previous findings also reported a significant association between the *FTO* rs9939609 polymorphism and the measurements of obesity in this cohort including BMI, waist circumference, waist:hip ratio, and central obesity (Reynolds *et al.,*, 2013). Re-analysis in this study still observed the association between the *FTO* rs9939609 polymorphism and BMI in which each A allele was associated with greater BMI: AA=31.95±7.27 (n=13), AT=29.26±7.37 (n=27), TT=26.22±5.51 (n=30), p=0.029.

The relationships between the effects of the *FTO* rs9939609A/T polymorphism and other polymorphisms including *HTR2C* -759C/T, *MTHFR* 677C/T, *MTHFR* 1298A/C, and leptin -2548A/G were investigated and no interactions between genotypes of these polymorphisms were observed. No drug-*FTO* rs9939609 genotype interaction was observed when patients were analysed as groups who had received either clozapine or olanzapine (p=0.614).

2.5 Discussion

The experiments in this chapter aimed to investigate the association of genetic polymorphisms with antipsychotic drug-induced weight gain and BMI in patients with schizophrenia. These genetic polymorphisms are in the genes relating to antipsychotic drug-induced weight gain, food intake regulation, and DNA methylation processes. Various functional polymorphisms from strong candidate genes were genotyped using custom TaqMan SNP genotyping assays in first episode and chronic schizophrenia patients.

2.5.1 *MTHFR* 677C/T associated with antipsychotic drug-induced weight gain in first episode patients with schizophrenia

This study indicated that the *MTHFR* 677C/T polymorphism is associated with antipsychotic induced weight gain in first-episode patients with schizophrenia. Individuals carrying the T allele showed less weight gain compared to the common CC genotype after 8-10 weeks or 3 months treatment with antipsychotic drugs. This finding was observed in two patient cohorts of different ethnicity, this indicates the effect to be a robust and reproducible one. The study had 90% power to identify a medium (0.50) effect size in the main cohort; previous studies of association of the well-replicated -759C/T polymorphism of *HTR2C* with antipsychotic drug-induced weight gain in a subgroup of the Chinese sample and in the Spanish sample have demonstrated substantially larger effect sizes of 0.90 and 0.86 respectively (Reynolds et al., 2002; Templeman *et al.,* 2005). In order for pharmacogenetic risk factors to explain a good proportion of the variance and thereby to have substantial predictive value, strong effects are needed. This study is aided substantially by the cohorts studied here; each only included first-episode patients who had never previously received antipsychotic drug treatment. This eliminates much of the variance associated with prior drug treatment, which can induce significant weight gain within a few weeks of initial treatment (Zhang *et al.,* 2004).

The absence of an effect in the 1298A/C polymorphism, despite it being in strong linkage disequilibrium (high D' values) with the significantly associated 677 genotype,

presumably relates to the large differences in allele frequency between the two polymorphisms, as reflected by low r^2 values.

In two previous cross-sectional studies the 677C/T polymorphism was associated with metabolic syndrome following antipsychotic drug treatment (Ellingrod *et al.,* 2008; 2012), although these authors find the 677T allele to be a risk factor, while this study found a consistent effect of the 677T allele in protecting against antipsychotic drug-induced weight gain. This may well indicate the difference between effects on initial weight gain and its long-term consequences, in which differing pharmacogenetic influences are apparent (Reynolds *et al.,* 2013). In another study the 1298A/C but not 677C/T polymorphism was associated with metabolic syndrome in schizophrenia (van Winkel *et al.,* 2010a). The one previous longitudinal study of changes in weight and metabolic parameters following 3 months treatment with second generation antipsychotics also found an association with the 1298A/C but not 677C/T polymorphism (van Winkel *et al.,* 2010b). This study differed from the present investigation of first episode drug naïve patients in that weight but not BMI were measured, and the 104 patients were older (mean 31.3y) with first admission on average over 6y previously; thus prior treatment may well have confounded subsequent weight gain. However their finding that the 1298A allele is associated with less weight gain is not inconsistent with the result of this study given the close linkage disequilibrium between the two polymorphisms studied. As discussed by van Winkel *et al* (2010b), there are no clinical or ethnic factors identified that may be responsible for the discrepancies between these findings, although it is notable that most studies were not powered to identify significant differences between the effects of the two closely linked polymorphisms. Nevertheless these various reports all indicate that functional genetic variation in *MTHFR* can influence antipsychotic drug-induced weight gain.

It is conceivable that pharmacogenetic associations such as that identified here may vary depending on the treatment regime. Different drugs may have differing mechanisms underlying their effect on body weight – certainly the greater effect of olanzapine over risperidone and several other antipsychotics supports this – and these pharmacological mechanisms may be differentially influenced by genetic

polymorphisms; however, further work needs to address the possible drug specificity of such pharmacogenetic findings.

There was no significant interaction between -759C/T of *HTR2C* and 677C/T of *MTHFR* on antipsychotic induced weight gain indicating that both polymorphisms exert independent influences on this side effect.

The exact mechanism by which *MTHFR* polymorphisms might contribute to determining antipsychotic drug-induced weight gain is unclear. Both variant alleles of 677C/T and 1298A/C *MTHFR* polymorphisms cause decreased enzyme activity (Weisberg *et al.,* 1998), although it is not easy to distinguish effects of two closely-linked polymorphisms *in vivo*. *MTHFR* is an important enzyme in one-carbon metabolism and, via its role in DNA synthesis and methylation (Sugden, 2006), may influence gene expression (Jirtle and Skinner, 2007); such epigenetic effects could be involved in antipsychotic drug-induced weight gain. Diminished levels of genomic DNA methylation (Stern *et al.,* 2000) and gene-specific DNA methylation (Burghardt *et al.,* 2012) have been reported to be associated with the 677TT genotype. It is therefore possible that decreased *MTHFR* enzyme activity in 677TT genotype results in decreased DNA methylation of genes involved in body weight regulation that was investigated in the next chapter.

DNA methylation status is influenced by gene-nutrient interaction. It has been suggested that the *MTHFR* 677TT genotype affects DNA methylation status through an interaction with folate status (Friso and Choi, 2002). These authors found that genomic DNA methylation in peripheral blood mononuclear cells was directly correlated with folate status, inversely correlated with homocysteine levels, and only 677TT subjects with low folate accounted for decreased DNA methylation (Friso *et al.,* 2002). Thus folate status in addition to the 677C/T *MTHFR* polymorphism might modulate DNA methylation of genes relating to the regulation of food intake, energy expenditure, or body weight regulation, and thus could be an unexplored factor contributing to the variance in this and previous studies.

In conclusion, this present study indicates the association of the *MTHFR* 677C/T single polymorphism with weight gain following initial antipsychotic drug treatment in first-episode psychotic patients. Furthermore, the effect of the 677T allele appears to have

a protective effect additional to that of the well-established *HTR2C* -759T allele against antipsychotic induced weight gain. These two polymorphisms, in addition to several other possible genetic factors, might be valuable as pharmacogenetic markers of this important and limiting side effect.

2.5.2 *ADRA2A* rs1800544 (-1291C/G) and *HTR2A* rs6311 (-1438G/A polymorphisms associated with antipsychotic drug-induced weight gain in first episode patients with schizophrenia receiving risperidone

The present finding did not identify an association of the *ADRA2A* -1291C/G and the *HTR2A* -1438G/A polymorphisms with weight gain in first episode Chinese Han schizophrenia patients, but drug-genotype interactions were identified. Post hoc analysis in a subgroup of patients who had received risperidone showed association of the *ADRA2A* -1291C/G and the *HTR2A* -1438G/A polymorphisms with weight gain.

Individuals carrying the G allele of the *ADRA2A* -1291C/G showed more weight gain compared to the common CC genotype after 8-10 weeks treatment with risperidone. This finding is consistent with two Asian population studies; although with differences in drug treatment: olanzapine treatment at least 3 months in Korean chronic schizophrenia patients (Park *et al.,* 2006) and clozapine treatment in chronic Chinese schizophrenia patients (Wang *et al.,* 2005b). Taken together, this suggests that this polymorphism may have an influence in both initial drug naïve and long-term weight gain. However, this finding is not inconsistent with Sickert *et al* who found that the G allele was a protective allele against clozapine-/olanzapine-induced weight gain in European schizophrenia patients (Sickert *et al.,* 2009). This study differed from the present investigation in that ethnicity was European-Americans and African-Americans, and the drug treatment consisted of olanzapine and clozapine for 6-14 weeks. Thus it may well indicate the difference in ethnicity may be responsible for the discrepancies between these findings.

The underlying mechanism of the association between the *ADRA2A* -1291C/G polymorphism with risperidone induced weight gain is unclear. It may relate to the role of the adrenergic system on energy balance regulation via the control of

thermogenesis, lipolysis (Park *et al.,* 2006; Arner, 1992), food intake (Wellman *et al.,* 1993), and glucoregulation (Levin and Planas, 1993). The *ADRA2A* -1291C/G polymorphism has been found to be associated with body fat accumulation (Garenc *et al.,* 2002). The genetic influence of this polymorphism on weight change might be due to an influence on the sympathetic-HPA system (Rosmond *et al.*, 2002a) and the regulation of neurotransmitter release (Langer, 1997). As the *ADRA2A* -1291C/G polymorphism is located in the promoter region of the alpha2a-adrenoceptor gene it may influence transcription regulation and gene expression. The alteration in receptor expression due to a genetic variant may cause changes in hypothalamic regulation of food intake and lipid metabolism that might result in weight gain. Further study regarding the influence of *ADRA2A* -1291C/G on receptor expression is required. Risperidone has high affinity at the alpha-2 adrenergic receptor and a trend of risperidone-*ADRA2A* -1291C/G genotype interaction was observed in this study; thus, via its effect on the receptor, the drug may cause glucose and lipid metabolism changes via sympathetic-HPA system resulting finally in changes in body weight (Wang *et al.,* 2005b; Rosmond *et al.*, 2002a).

Individuals carrying the A allele of the *HTR2A* -1438G/A showed more weight gain compared to the common GG genotype after 8-10 weeks treatment with risperidone. This finding is consistent with previous studies which reported the association of the 102T allele of the *HTR2A* (rs6313), which is in complete LD with -1438A allele of *HTR2A* (rs6311), and antipsychotic drug-induced weight gain across different ethnicities (Ujike *et al.,* 2008; Balt *et al.,* 2011), although there were difference in drugs; olanzapine in Japanese, risperidone in Chinese, and multiple drug treatment in Caucasian patients with schizophrenia. However, one finding by Mou *et al.* showing no association of the *HTR2A* -1438G/A and weight gain is not inconsistent with results in the present study even though the first episode Chinese Han received risperidone or chlorpromazine for 10 weeks (Mou *et al.,* 2005). The lack of significant association in the Mou study might be due to smaller sample size (n=84).

The A allele of the *HTR2A* -1438G/A was associated with weight gain in patients who had received risperidone treatment in this study but this is not in line with findings in general population studies as those found the GG genotype was associated with higher energy intake (Aubert *et al.,* 2000) and greater BMI (Rosmond *et al.*, 2002b). The

different finding may well indicate that the effect of the polymorphism together with the effect of antipsychotic drugs on weight gain in patients with schizophrenia was different from the general population as well as different between different kinds of antipsychotic drugs as shown in the drug-genotype interaction results **section 2.3.4**. On the other hand, there are studies that did not find the association between the *HTR2A* -1438G/A and BMI or obesity (Aubert *et al.,* 2000; Hinney *et al.,* 1997). The different findings might be due to the different methods used for assessing energy intake, the individual factors such as disease symptoms, diet, physical activity, the environmental factors, and the psychosocial and socioeconomic handicaps, as well as ethnic differences. Therefore, the association of *HTR2A* -1438G/A polymorphism and obesity in the general population as well as in antipsychotic drug treatments need to be confirmed.

The mechanisms underlying the association of the *HTR2A* -1438G/A polymorphism and antipsychotic drug-induced weight gain are unknown. The explanation might be that risperidone, which is a potent antagonist of 5-HT2A receptor may increase NPY to stimulate food intake and may also change the cortisol level and ACTH concentrations through effects on 5-HT2A (Currie and Coscina, 1998; Rittenhouse *et al.,* 1994). The *HTR2A* -1438G/A polymorphism is located in the promoter region and is associated with promoter activity; the presence of an A allele has greater promoter activity relative to the G allele (Parsons *et al.,* 2004). In addition, the A allele of the *HTR2A* -1438G/A polymorphism was associated with better clinical response to clozapine (Arranz *et al.,* 1998). Therefore, it is possible that the genetic polymorphism may affect transcription of *HTR2A* gene to bring about a difference in receptor expression and consequently affect treatment response as well as weight gain through 5-HT2A receptor regulating food intake and cortisol secretion. The interaction between the receptor action of risperidone and the *HTR2A* -1438G/A polymorphism may explain the association with risperidone-induced weight gain found in this study.

There was no significant interaction between the associations of -1291C/G of *ADRA2A*, -1438G/A of *HTR2A*, -759C/T of *HTR2C*, and 677C/T of *MTHFR* with antipsychotic drug-induced weight gain indicating that these polymorphisms exert independent influences on this side effect.

In conclusion, this study demonstrates novel associations of the *ADRA2A* -1291C/G and *HTR2A* -1348G/A polymorphisms with weight gain following risperidone treatment in first-episode Chinese Han schizophrenia patients.

2.5.3 Association of rs17782313 and rs489693 polymorphisms near *MC4R* gene and antipsychotic drug-induced weight gain in first episode patients with schizophrenia

The human melanocortin 4 receptor gene (*MC4R*) is a very strong candidate gene for involvement in antipsychotic drug-induced weight gain. The aim of this study was to investigate whether the genetic polymorphisms near the *MC4R* gene, rs17782313 and rs489693, associate with antipsychotic drug-induced weight gain in first-episode Chinese Han schizophrenia patients. The results in this study indicated that rs489693 but not rs17782313 was associated with antipsychotic drug-induced weight gain in these subjects. Individuals carrying the C allele of rs489693 showed more weight gain compared to the AA genotype after 8-10 weeks treatment. This finding is opposite to previous findings that found the significant association of the AA genotype with weight gain in schizophrenia patients who had received second generation antipsychotics (Malhotra *et al.,* 2012; Czerwensky *et al.*, 2013b). The different findings compared with the present study may be due to the difference in ethnicity. Other factors may also influence the result such as prior drug treatments, environmental factors, and clinical factors including age (e.g. Czerwensky *et al.* (2013b) studied patients aged 19 years of age or younger). Czerwensky *et al.* (2013a) also found the association of the C allele of rs17782313 with weight gain after 4-week treatment. A lack of significant association of this SNP with weight gain in the present study may be due to the longer treatment duration (8-10 week-treatment). This result was similar to a study by Chowdhury *et al.,* (2013) that did not find an association of this SNP with weight gain after 14-week treatment. Further studies are needed to confirm the association of this polymorphism and antipsychotic drug-induced weight gain.

The rs489693 and rs17782313 are not in linkage disequilibrium in this study while Malhotra *et al* (2012) find in their sample that they are in strong LD; although with low r^2 values. The minor allele frequencies (A=0.20 for rs489693 and C=0.22 for rs17782313) were lower than those in previous studies; however, they did not deviate

from HapMap data for Chinese Han (A=0.189 for rs489693, C=0.193 for rs17782313) (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap28_B36). This may be another factor responsible for the discrepancies of the findings.

There was no significant interaction between the rs489693, rs17782313, *HTR2C* -759C/T, and *MTHFR* 677C/T polymorphisms on antipsychotic drug-induced weight gain indicating that these polymorphisms exert independent influences on this side effect.

The exact mechanism underlying the association of rs489693 and antipsychotic drug-induced weight gain is unknown. No functional relevance of this polymorphism is known. The rs489693 may be controlled by other remote regulatory sites located at considerable distance from the polymorphism such as the folding of chromosome (Espinoza and Ren, 2011), thus factors affecting chromosome folding may influence the role of this polymorphism on weight gain. It is also possible that rs489693 may be linked to another polymorphism that has actual biological function in the regulation of body weight; for example: a polymorphism in the *MC4R* promoter region that might influence *MC4R* gene expression.

2.5.4 Association of *GNB3* rs5443825 C/T, *BDNF* rs6265 (Val66Met), and *FTO* rs9939609 A/T polymorphisms with weight gain in Chinese Han schizophrenia patients

The G-protein beta3 subunit gene (*GNB3*) 825C/T polymorphism is a strong candidate gene related to antipsychotic drug-induced weight gain (Balt *et al.,* 2011). Therefore, the association of this polymorphism and antipsychotic drug-induced weight gain was investigated. No association between the *GNB3* 825C/T polymorphism and weight gain in first episode Chinese Han schizophrenia was observed in this study. This result has not replicated previous findings (Ujike *et al.,* 2008; Wang *et al.,* 2005a) that found the T allele to be associated with antipsychotic drug-induced weight gain. Those studies differed from the present study in that inpatients with schizophrenia receiving olanzapine for 17.9 weeks or clozapine for 13.4 months were investigated, whereas risperidone was the main drug treatment in first-episode schizophrenia patients in this study; thus prior treatment, current treatment and treatment duration may influence the finding. It is possible that this polymorphism might be associated with weight gain in long-term treatment and response to certain antipsychotic drug treatments.

However, the lack of significant association between the polymorphism and weight gain found in this study supported several previous studies showing no association in Asian and Caucasian studies (Tsai *et al.,* 2004; Park *et al.,* 2009; Bishop *et al.,* 2006). The present study found a trend for association of the CC genotype with lower baseline weight (p=0.053) as well as lower baseline BMI. These trends were similar to previous studies; there was a trend of TT allele with weight gain after olanzapine treatment for 6 weeks in Caucasians (Bishop *et al.,* 2006) and a meta-analysis also reported a trend of CC genotype association with lower BMI and lower antipsychotic drug-induced weight gain (Souza *et al.,* 2008). The lack of significant association found in this study is likely the result of a modest gene effect and the small sample size. Thus with inconsistent findings of the association between the *GNB3* 825C/T polymorphism and antipsychotic drug-induced weight gain, further studies with larger sample sizes are needed.

Recent studies in animals and humans have found that BDNF plays an important role in food intake and body weight regulation (Unger *et al.,* 2007; Toriya *et al.,* 2010; Zhang *et al.,* 2007). The role of the *BDNF* polymorphism in antipsychotic drug-induced weight gain has not been examined in first-episode schizophrenia patients; therefore, the association between the *BDNF* rs6265 G/A polymorphism and antipsychotic drug-induced weight gain in first-episode schizophrenia was determined in this study. No significant association between the polymorphism and weight gain in first episode Chinese Han schizophrenia was observed in the present study, although the AA genotype (Met/Met) showed a higher BMI change and weight change than carriers of the G allele.

This finding was not consistent with previous studies that found a significant association between the Met/Met (AA) genotype of the *BDNF* rs6265 polymorphism and greater body weight gain after chronic antipsychotic drug treatment in Chinese patients with schizophrenia (Zhang *et al.,* 2008). That study differed from the present investigation in that subjects received chronic treatment with multiple antipsychotic drugs for 18y on average; thus *BDNF* rs6265 may be a marker for long-term rather than initial weight gain. However, other studies found haplotypes which included *BDNF* rs6265 G/A polymorphism (rs6265-rs11030101-rs12291186, G-T-A) associated with antipsychotic drug-induced weight gain in Chinese, chronic patients with schizophrenia

(Tsai *et al.,* 2011). The A allele of *BDNF* rs1519480 and the haplotype of *BDNF* rs6265 and *BDNF* rs1519480 (G-A haplotype) were also found to be associated with weight gain in European ancestry chronic patients with schizophrenia (Zai *et al.,* 2012). The *BDNF* rs6265 is in strong linkage disequilibrium with the *BDNF* rs1103010 (Licinio *et al.*, 2009) and rs1519480 (Zai *et al.,* 2012). It is also possible that rs6265 does not have a direct effect or has a modest effect on antipsychotic drug-induced weight gain, but it is the other SNPs in linkage disequilibrium or haplotype patterns in the *BDNF* gene that might have the strong effect on antipsychotic drug-induced weight gain.

The association of the *FTO* rs9939609 polymorphism with food intake has been well documented and it is a gene that is strongly associated with BMI and obesity in different populations (Frayling *et al.,* 2007, Fawcett and Barroso, 2010). In addition, FTO is a demethylase that can modulate epigenetic processes in both DNA and RNA that might affect expression of a certain subgroup of genes and eventually influence food intake and body weight regulation. The present study aimed to determine the association of the *FTO* rs9939609 polymorphism with antipsychotic drug-induced weight gain in Chinese first-episode schizophrenia patients. No significant association between the polymorphism and weight gain or baseline BMI was observed in this study, although those homozygous for the risk allele A had a higher baseline BMI and baseline weight that the AT/TT group.

This result is consistent with a previous study (Reynolds *et al.,* 2013) that found no significant association between the *FTO* rs9939609 polymorphism with antipsychotic drug-induced weight gain in first-episode Caucasian schizophrenia patients although the AA genotype had higher baseline weight and baseline BMI than T allele carriers. Studies in larger samples are more likely to detect any significant association between genetic polymorphism and BMI or body weight at baseline (Perez-Iglesias *et al.,* 2010; Song *et al.,* 2014). However, after antipsychotic treatment, changes in BMI or body weight were inconsistent. Therefore, the absence of significant association between the SNP and weight gain in this study might be due to a small sample size relative to the modest *FTO* genotype effect on antipsychotic drug-induced weight gain. Further studies in independent large sample size are needed to elucidate the role of the *FTO* polymorphism in antipsychotic drug-induced weight gain.

2.5.5 Association of genetic polymorphisms with body weight in chronic schizophrenia patients

The association of the *MTHFR* 677C/T and *MTHFR* 1298A/C polymorphisms with body weight in chronic schizophrenia patients was also determined in this study. No significant association between the *MTHFR* 677C/T or *MTHFR* 1298A/C polymorphism and BMI was detected, although the CC genotype of the *MTHFR* 677C/T polymorphism showed the higher BMI, waist-hip ratio, frequency of subjects who have BMI≥30kg/m^2, and triglyceride level than T allele group. This might be due to the modest effect of the *MTHFR* polymorphisms on body weight together with a very small sample size that reduces the statistical power to detect the modest genotype effect on body weight.

However, the effect of *FTO* rs9939609 A/T polymorphism on BMI was still found in this subgroup that has been reported previously (Reynolds *et al.,* 2013); the AA genotype was associated with higher BMI than the T allele carriers. The present finding was not in line with other studies; the significant association of the A allele with greater BMI was found in healthy subjects but not in Japanese chronic schizophrenia patients (Watanabe *et al.,* 2012). Another study found a trend of AA genotype gained higher weight in European chronic schizophrenia patients receiving antipsychotic drugs for up to 14 weeks (Shing *et al.,* 2014). These studies differed from the present investigation in that the 351 patients were older (mean 52.32y) than this study (mean 44.07y) and there was a ethnicity difference in Watanabe's study, while there was multiple antipsychotic drug treatments for 6 or 14 weeks in 3 subgroups of patients with small sample sizes in Shing's study.

The mechanisms underlying the association of the *FTO* rs9939609 polymorphism and antipsychotic drug-induced weight gain remain poorly understood. The *FTO* gene is highly expressed in the hypothalamus which regulates the energy balance (Willer *et al.,* 2009). The overexpression of *FTO* has been associated with increased food intake and obesity (Church *et al.,* 2009; Church *et al.,* 2010). The A allele of *FTO* rs9939609 was associated with increased levels of the *FTO* transcripts (Berulava and Horsthemke, 2010). The FTO protein is a demethylase involved in demethylation of DNA and RNA (Jia *et al.,* 2008; Gerken *et al.,* 2007; Han *et al.,* 2010) that may influence the expression of the *FTO* gene itself (Bell *et al.,* 2010) or other genes (Almen *et al.,* 2012).

Fto-deficient mice showed an increase in adenosine methylation in a subset of RNAs important for neuronal signaling, including dopamine transmission (Hess *et al.,* 2013). Therefore, it is possible that FTO may also influence other neurotransmitter pathways that could influence food intake regulation. The FTO protein is involved in the hypothalamic leptin signaling pathway (Wang *et al.,* 2011). Study in mice showed the *Fto* gene was a transcriptional coactivator that enhanced the binding of C/EBPs to both unmethylated and methylated DNA that promoted transcriptional functions of the C/EBP family in adipogenesis (Wu *et al.,* 2010). The SNPs located in non-coding regions such as *FTO* rs9930609, which is located in the first intron, may influence promoter activity (gene expression), messenger RNA (mRNA) conformation (stability), and/or proteins (Shastry, 2009).This may cause differences in expression and structure of FTO that may result in differential functional ability such as the binding of FTO protein to the C/EBPs and subsequent binding to unmethylated and methylated DNA which may change the extent of adipogenesis. Collectively, these findings suggest that the *FTO* polymorphism may modulate food intake through epigenetic modification of certain genes as well as certain groups of RNAs relating to neurotransmitter systems involved in energy balance and may result in the alteration of *FTO* and other genes' expression that bring about the modification of energy balance and also adipogenesis. Further studies are required to elucidate the role of *FTO* in antipsychotic drug-induced weight gain or obesity risk.

2.5.6 Limitations in this study

This study has some limitations. First, the sample size is small that leads to a decrease in statistical power to detect small or modest genetic effects. However, every cohort has been observed previously and a significant association of the polymorphism and weight gain or BMI can be detected depending on the effect size. Larger sample size studies are needed to confirm the association between the polymorphism and antipsychotic drug-induced weight gain. Second, the chronic study is a cross-sectional study and could not determine the change in BMI during each antipsychotic drug treatment. Third, environmental factors which could contribute to the weight gain such as diet, physical activity, were not assessed. These factors may influence body weight in this study. Taking these limitations into account, further investigations are needed to understand the genetic risk of antipsychotic drug- induced weight gain.

2.5.7 Conclusions

The findings in this chapter demonstrate that the *MTHFR* 677C/T polymorphism is associated with antipsychotic drug-induced weight gain in the first-episode drug-naïve schizophrenia in both Chinese Han and Spanish patients in which the T allele was a protective allele for antipsychotic drug-induced weight gain.

This *MTHFR* 677C/T showed an additive effect to the well-established *HTR2C* -759C/T polymorphism. Therefore, these two genetic polymorphisms might be valuable as pharmacogenetic markers of weight gain following antipsychotic treatment. The association between the *MC4R* rs489693 and antipsychotic drug-induced weight gain observed in this study remains in need of further study to elucidate and replicate this association.

The analysis of a subgroup of patients who had received risperidone indicated that the polymorphisms of the *ADRA2A* -1291C/G and *HTR2A* -1438G/A were associated with risperidone-induced weight gain. In addition, the CC genotype of the *MTHFR* 677C/T polymorphism showed a trend of higher BMI gain than the T allele carriers in this group.

Combined analysis of five genetic polymorphisms showed that there were no gene-gene interactions between each pair of polymorphisms indicating the additive effect of each significant finding on BMI change.

Therefore, in addition to the genetic polymorphism of the *HTR2C* promoter region, the *MTHFR* 677C/T and the *MC4R* rs489693 polymorphisms may be used as genetic markers for antipsychotic drug-induced weight gain in first episode schizophrenia patients. In addition, the *ADRA2A* -1291C/G and *HTR2A* -1438G/A polymorphisms may be used as genetic markers for risperidone-induced weight gain. The gene-gene and gene-drug interactions provide more understanding of the mechanism underlying antipsychotic drug-induced weight gain. However, further studies with larger sample sizes are required to confirm these findings.

Chapter 3: Association of DNA methylation and antipsychotic drug-induced weight gain in patients with schizophrenia

3.1 Introduction

Not only genetic factors influence antipsychotic drug-induced weight gain as described in the previous chapter, but also epigenetic factors may play important roles in antipsychotic drug-induced weight gain. DNA methylation is one of the most widely studied epigenetic mechanisms in the regulation of gene expression. Changes in DNA methylation both in genome-wide and gene-specific levels have been reported relating to body weight. For example, Crujeiras *et al.* (2013) found that the weight-regainers had higher methylation levels than non-regainers in *POMC* and lower levels on *NPY* CpG sites; the lower methylation levels of *POMC* were associated with weight-loss maintenance, while lower methylation levels of the *NPY* promoter were associated with higher risk of weight regain. Burgio *et al.* (2015) summarise the recent findings of epigenetic biomarkers which include many genes related to obesity and type 2 diabetes mellitus; for example: the methylation status of CpG islands located in clock genes (*CLOCK*, *BMAL1* and *PER2*) was associated with obesity, metabolic syndrome and weight loss; lower methylation of the leptin gene was found in infants born to pre-pregnancy obese mothers; increased DNA methylation of the insulin promoter was found in type 2 diabetes mellitus. Moreover, many factors have influences on DNA methylation such as exercise, diet, medications, as well as medical conditions or diseases.

Several studies have investigated the changes in DNA methylation in patients with schizophrenia. Melas *et al.* (2012) determined genome-wide DNA methylation levels in blood from schizophrenia patients and reported a global DNA hypomethylation independent of drug treatment which was most prominent in early-onset schizophrenia. In addition, haloperidol treatment was associated with greater methylation. Gene-specific DNA methylation associated with schizophrenia has also been examined. Hypermethylation of the promoter regions of soluble catechol-o-

methyltransferase (*S-COMT*) in patients with schizophrenia was reported (Melas *et al.,* 2012). In addition, DNA hypermethylation of *5-HTT* promoter and its correlation with the reduction in *5-HTT* expression were reported in drug naïve schizophrenia patients both in DNA from saliva and post-mortem brain samples (Abdolmaleky *et al.,* 2014). The correlation between GTP cyclohydrolase (*GCH1*) promoter hypermethylation and the reduction in *GCH1* expression was reported in the blood of first-episode schizophrenia patients (Ota *et al.,* 2014). This enzyme catalyzes the conversion of GTP to D-erythro-7,8-dihydroneopterin triphosphate, the first and rate-limiting step in tetrahydrobiopterin (BH4) synthesis. BH4 is an important cofactor for the hydroxylation of the aromatic amino acids by phenylalanine hydroxylase (PAH), tyrosone-3-hydroxylase (TH), and tryptophan 5-hydroxylase (TPH); therefore, *GCH1* plays an important role in the synthesis of dopamine and serotonin (Richardson *et al.,* 2005). In addition, downregulation of *GAD67*, reelin (*RELN*), *BDNF*, and other genes expressed in telencephalic GABAergic and glutamatergic neurons in schizophrenia and bipolar disorder patients was correlated with hypermethylation on their promoter domains (Guidotti *et al.,* 2005; Costa *et al.,* 2007). These data indicate the important role of DNA methylation in regulating gene expression as well as its involvement in the pathophysiology of schizophrenia. Dysregulation of DNA methylation may therefore provide a new target for antipsychotic drug action. The reversal of DNA hypermethylation in specific genes involved in schizophrenia in order to restore their gene expression is a potential pharmacological strategy (Guidotti and Grayson, 2014).

In addition to schizophrenia itself, other environmental factors affecting schizophrenic patients including antipsychotic medication also influence epigenetic modification. Study in a mouse model with relevance to schizophrenia demonstrated that clozapine and sulpiride but not haloperidol or olanzapine treatment induced DNA demethylation as well as histone acetylation at reelin and *GAD67* promoters in the mouse frontal cortex and striatum (Dong *et al.,* 2008; Guidotti *et al.,* 2009). However, olanzapine treatment in rats caused genome-wide DNA methylation change in genes of dopamine neurotransmission in the hippocampus, cerebellum and liver (Melka *et al.,* 2014). The changes in methylation include increases in methylation in 1,140, 1,294 and 1,313 genes and a decrease in 633, 565 and 532 genes in the hippocampus, cerebellum and liver, respectively. Most genes affected are tissue specific. Only 41 affected genes

showed an increase and no genes showed a decrease in methylation in all three tissues. The affected genes are involved in pathways affecting dopamine signalling, molecular transport, neuronal development and functions in the hippocampus; adrenergic receptor signalling and synaptic long-term potentiation in the cerebellum; and tissue morphology, cellular assembly and organization in the liver. These data suggest that epigenetic changes after antipsychotic drug treatment may underlie the improvement of symptoms and may also account for certain adverse effects including weight gain after antipsychotic drug treatment.

Although epigenetic mechanisms are targets for environmental factors, genetic factors also influence epigenetic modifications and this genetic-epigenetic interaction may contribute to the phenotypes. The interaction between the *MTHFR* 677C/T variant, gender, and global DNA methylation, as measured using a *LINE-1* DNA methylation assay has been reported to be associated with metabolic syndrome in schizophrenia patients, in which females with the *MTHFR* 677TT genotype had the lowest global methylation level compared with other groups (Burghardt *et al.,* 2012). Another study reported a significant association between the *COMT* Val158Met (rs4680) polymorphism, *COMT* promoter methylation, physical activity, and metabolic syndrome in patients with schizophrenia (Lott *et al.,* 2013). In addition, they observed that the *COMT* genotype was a significant indicator of methylation status at two CpG sites in the *COMT* promoter region. These data indicate the influence of genetic polymorphisms on DNA methylation and this relationship may influence the inter-individual variation in symptom response and adverse effects in schizophrenia patients receiving antipsychotic drugs.

This chapter describes the examination of the association between antipsychotic drug-induced weight gain and DNA methylation in the *HTR2C* promoter sequences near the *HTR2C* -759C/T, the most consistent polymorphism associated with antipsychotic drug-induced weight gain in first episode and chronic schizophrenia patients. In addition, associations between polymorphisms, especially *MTHFR* and *FTO* polymorphisms that play an important role in DNA methylation, and the extent of DNA methylation, were determined. The results from this chapter may provide the mechanistic link between the genetic risk factor of the *HTR2C, MTHFR, and FTO* polymorphisms and antipsychotic drug-induced weight gain and may also provide an epigenetic marker for

this adverse effect. DNA methylation levels were measured using pyrosequencing of the bisulfite modified genomic DNA by PyroMark Q24 pyrosequencer.

3.1.1 Principles of pyrosequencing method

DNA methylation levels of the target DNA sequences can be determined using bisulfite conversion and pyrosequencing. There are many steps to complete in determining DNA methylation status including genomic DNA extraction, bisulfite treatment, PCR and gel electrophoresis, and pyrosequencing. A work flow is shown in **Figure 3.1**.

Figure 3.1: Work flow for DNA methylation study

3.1.1.1 Bisulfite conversion of genomic DNA

The bisulfite treatment of extracted genomic DNA results in conversion of unmethylated cytosine to uracil while methylated cytosine remains a methylated cytosine. Therefore, bisulfite treatment creates different DNA sequences for methylated and unmethylated DNA as shown in **Table 3.1** for example. It is the most critical step for the correct measurement of DNA methylation because incomplete conversion of unmethylated cytosine residues results in higher methylation levels than the correct level. The complete conversion is achieved by incubating the DNA in high concentration of bisulfite salt at high temperature and low pH that are harsh conditions which often lead to DNA fragmentation and loss of DNA during further purification. As shown in **Figure 3.2**, in the bisulfite treatment reaction, all unmethylated cytosines are sulfonated, deaminated and desulfonated, converting to uracils, while methylated cytosines (5-methylcytosines) remain unaltered.

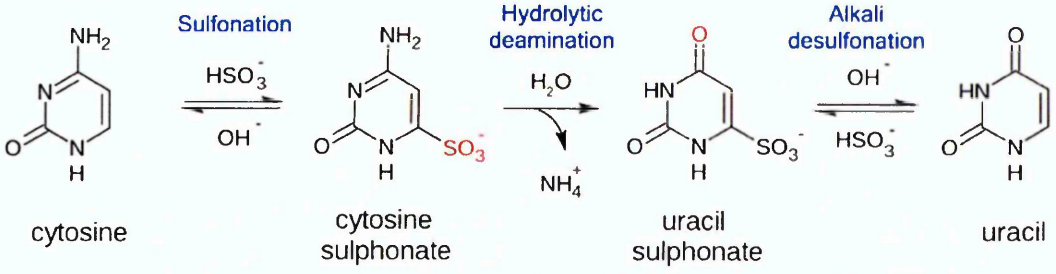


Figure 3.2: Bisulfite reaction converting unmethylated cytosine to uracil base
Modified from Pappas *et al.*, (2013)

Table 3.1: Modification of DNA sequence after bisulfite treatment

	Original sequence	Bisulfite modified sequence
Unmethylated DNA	N-C-G-N-C-N-C-G-N-C-N	N-**U**-G-N-U-N-**U**-G-N-U-N
Methylated DNA	N-C-G-N-C-N-C-G-N-C-N	N-**C**-G-N-U-N-**C**-G-N-U-N

3.1.1.2 Amplification of bisulfite converted DNA

Bisulfite treated DNA is then amplified by PCR. In this step, the uracil bases are converted to thymine while methylated cytosine is presented as cytosine (**Figure 3.3**). In the PCR reaction, it is necessary that one of the PCR primers in the opposite direction of the sequencing primer must be labelled with biotin. The biotinylated primer is important in the preparation of the single-stranded DNA template for pyrosequencing; in the vacuum work station, biotin molecules are immobilized by binding to the streptavidin beads. The principle of PCR is described in the next chapter (**see section 4.1.3**).

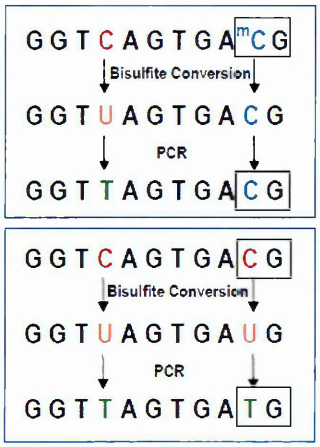


Figure 3.3: Changing in DNA sequences following bisulfite conversion and PCR steps
Bisulfite treatment converts unmethylated cytosine to uracil while methylated cytosine remains cytosine. During PCR, uracil is converted to thymine while methylated cytosine was presented as cytosine.

3.1.1.3 Gel electrophoresis of amplified DNA

The PCR product is run on agarose gel to determine that the product of the expected length has been obtained before processing the subsequent pyrosequencing. The result from gel electrophoresis also indicates the intensity of the PCR product that can be used to approximate the amount of PCR product used in pyrosequencing as well as checking for contamination in the negative PCR reaction.

3.1.1.4 Pyrosequencing

Pyrosequencing is based on sequencing by synthesis which can be used to quantify DNA methylation at specific CpG sites within the target region of interest. In addition, pyrosequencing can be used for DNA sequencing, genotyping and SNP analysis, allele quantification, and whole genome sequencing.

The principle of pyrosequencing is described by the manufacturer's resource as follows;

Step 1: Preparation of DNA template and hybridization with sequencing primer

The amplified PCR products are separated into the single-stranded DNA for pyrosequencing by the PyroMark Q24 vacuum workstation which consists of a series of solutions for denaturation and washing the DNA. After denaturation, a single-stranded biotinylated PCR product is separated and allowed to hybridize with the sequencing primer (**Figure 3.4**). The hybridized primer and template are incubated with the enzymes (DNA polymerase, ATP sulfurylase, luciferase, and apyrase) as well as the substrates (adenosine 5' phosphosulfate (APS), and luciferin).

Step 2: Incorporation of the first dNTP to DNA template

When the first deoxyribonucleotide triphosphate (dNTP) is added to the reaction and if it is complementary to the base in the template strand, the DNA polymerase enzyme catalyzes the incorporation of the dNTP into the new strand DNA next to the sequencing primer. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide (**Figure 3.4**).

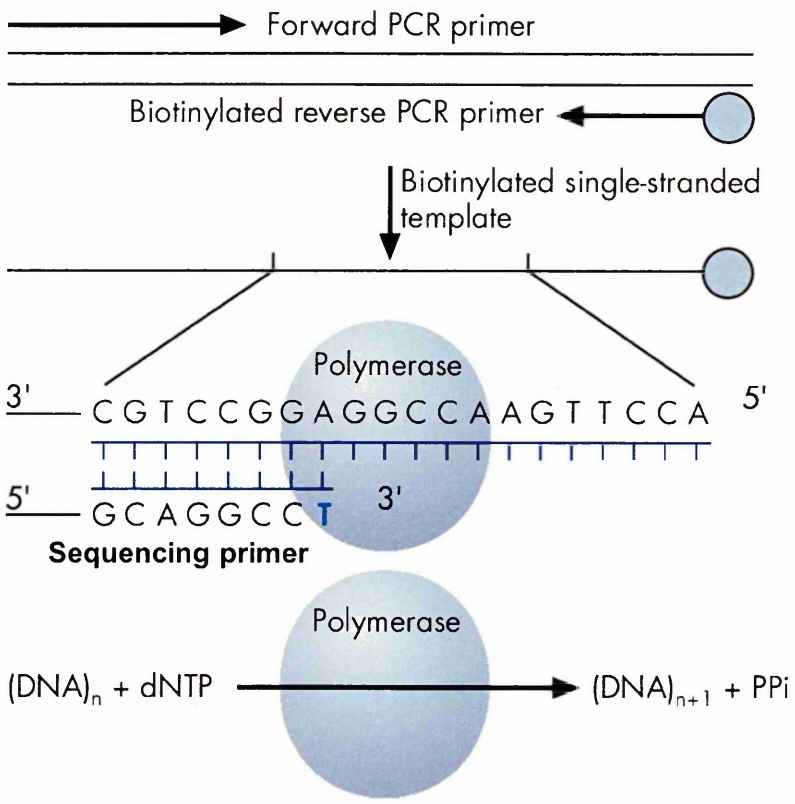


Figure 3.4: Pyrosequencing showing hybridization of sequencing primer with biotinylated single-stranded DNA template (step 1) and incorporation of the complementary nucleotide into sequencing primer (step 2).

Step 3: Enzymatic reactions convert PPi to pyrogram peak

A released pyrophosphate (PPi) is converted to ATP by the enzyme ATP sulfurylase in the presence of adenosine 5' phosphosulfate (APS) substrate. This ATP drives the conversion of luciferin to oxyluciferin mediated by the luciferase enzyme. This conversion reaction generates visible light in amounts that are proportional to the amount of ATP. The light signal is detected by a charge coupled device (CCD) chip and seen as a peak (pyrogram) in the raw data output. The height of each peak (light signal) is proportional to the number of nucleotides that are incorporated (**Figure 3.5**).

Step 4: Degradation of unincorporated nucleotides and ATP

Unincorporated nucleotides and ATP are degraded continuously by apyrase which is a nucleotide-degrading enzyme (**Figure 3.6**). When degradation is complete, another nucleotide is added to start the next cycle of enzymatic reactions.

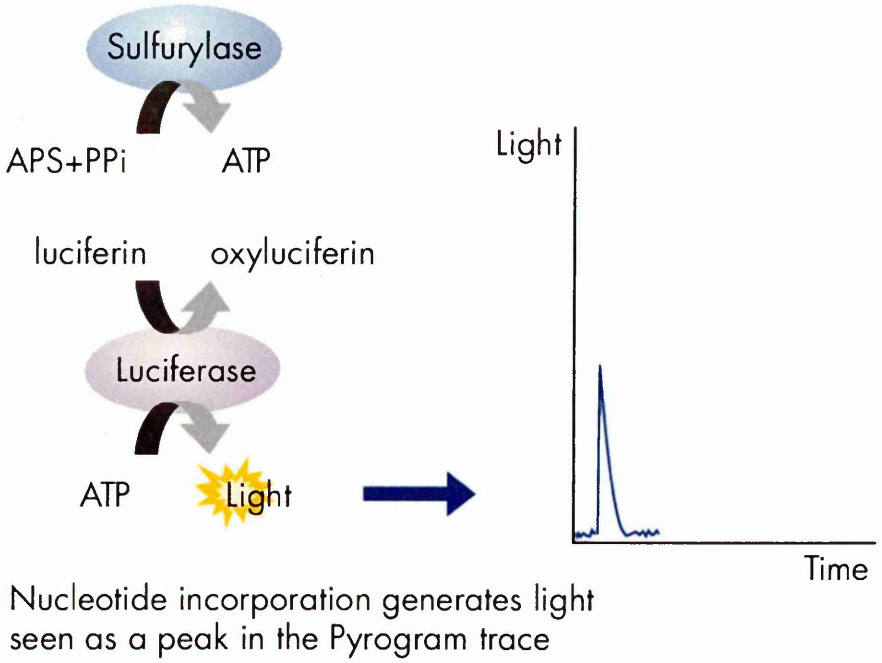


Figure 3.5: Pyrosequencing step 3 showing enzymatic reactions convert PPi to pyrogram peak.

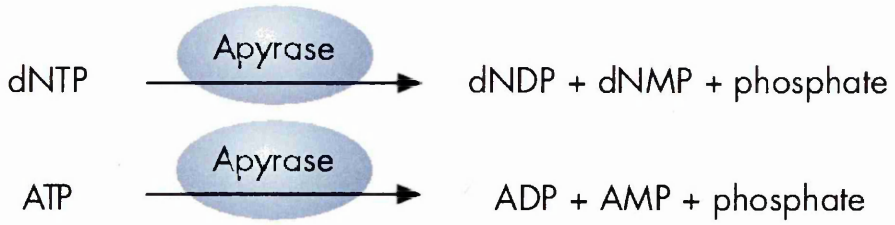


Figure 3.6: Pyrosequencing step 4 showing degradation of unincorporated nucleotides and ATP by apyrase enzyme.

Step 5: Sequential addition of dNTPs

Addition of dNTPs is performed sequentially according to the dispensation sequences. As the process continues, the complementary DNA strand is elongated and the nucleotide sequence is determined from the signal peaks in the pyrogram trace (**Figure**

3.7). In the pyrosequencing processes, the deoxyadenosine alpha-thio triphosphate (dATPαS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP), because it is efficiently used by the DNA polymerase, but not recognized by the luciferase.

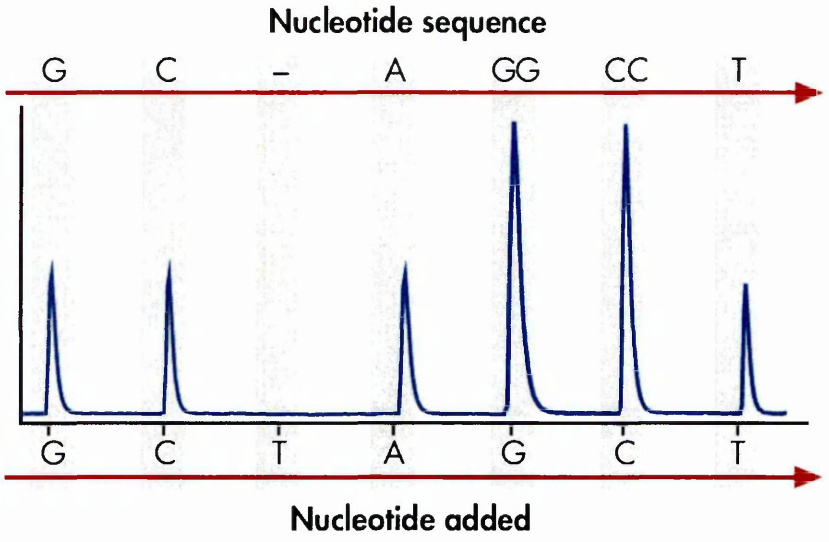


Figure 3.7: Pyrosequencing step 5 showing a sequential addition of dNTPs to generate the nucleotide sequence represented by signal peaks in the pyrogram trace.

Analysing DNA methylation level at each CpG site

The ratio of cytosine and thymine showing at each CpG site is determined, and it reflects the methylation level of that CpG site in genomic DNA. The calculation equation of the methylation level at each CpG site is as following equation.

Methylation level (%) = peak height of C*100/(peak height of C + peak height of T)

3.1.2 Aims

1. To investigate the relationship between DNA methylation of the *HTR2C* promoter sequences near the *HTR2C* -759C/T polymorphism, and the sequences from position +1 to +14 relative to the -759C/T polymorphism, and antipsychotic drug-induced weight gain in first episode schizophrenia patients.

2. To investigate the effect of DNA methylation of the *HTR2C* promoter sequence near the *HTR2C* -759C/T polymorphism, and global DNA methylation, with BMI in chronic schizophrenia patients.
3. To investigate the influence of genetic polymorphisms of the *HTR2C* -759C/T, *MTHFR* 677C/T, *MTHFR* 1289A/C and *FTO* rs9939609 A/T on the extent of DNA methylation in first episode and chronic schizophrenia patients.

3.2 Materials and methods

3.2.1 Study population and DNA samples

The study populations in this chapter were the same as in the previous chapter including the two cohorts of first-episode, initially antipsychotic drug-naïve patients with schizophrenia (Spanish and Chinese cohorts) and one cohort of chronic schizophrenia patients (Belfast cohort). All patients received treatment according to normal clinical practice and gave written informed consent to the procedure of the study; which was approved by local ethical committees. The characteristics of studied populations were described previously in the **section 2.2.1**.

3.2.2 Primer design

There were two regions of interest for DNA methylation of *HTR2C* gene; the first region is located in the promoter region of *HTR2C* gene near the -759C/T and includes the -697G/C polymorphism (from -698 to -640 relative to -759C/T), identified by the PubMed gene bank (GeneBank accession number NG_012082.1). The second region is located at position +1 to +14 relative to the -759C/T polymorphism identified and designed by Qiagen, the Hs_*HTR2C*_01_PM PyroMark® CpG assay (Cat.no. PM00033691). The sequence alignments of both regions are shown in **Figure 3.8**.

Another region of interest was the *LINE-1* human transposon DNA consensus sequence including three CpG sites in positions of 331 to 318 of *LINE-1* (GeneBank accession number X58075.1). *LINE-1* methylation represents the genome-wide or global DNA methylation.

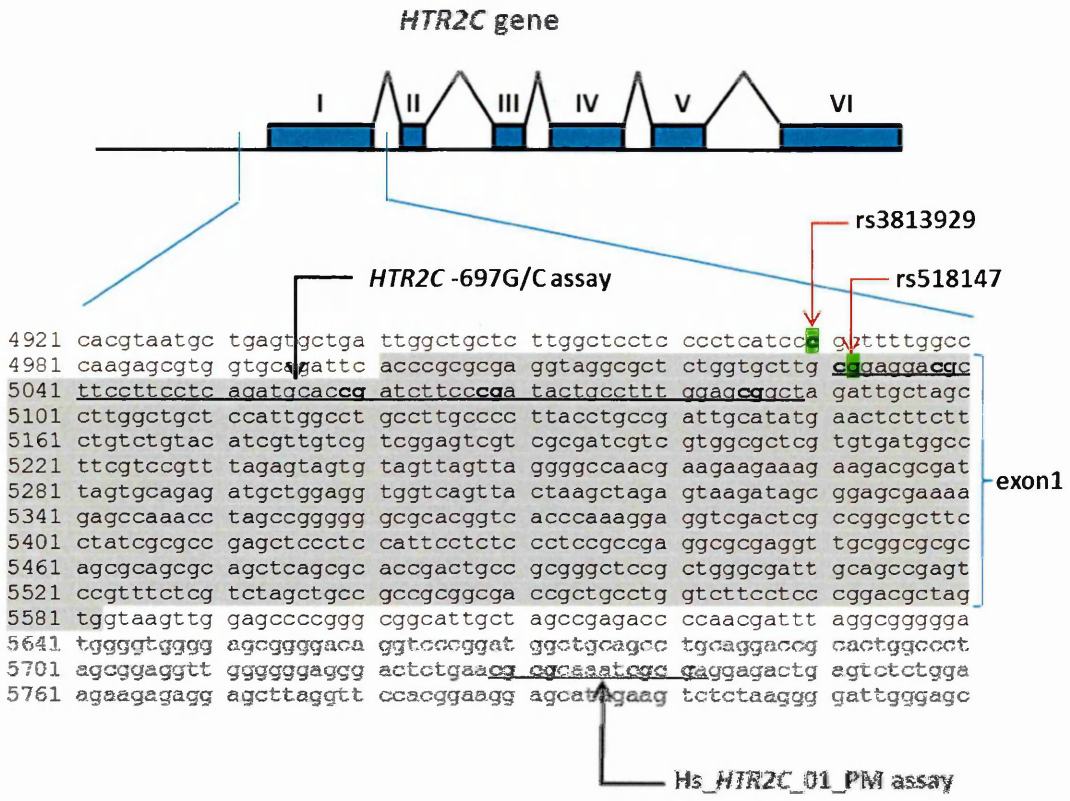


Figure 3.8: Sequence alignments of *HTR2C* gene containing two regions for DNA methylation study

Figure shows *HTR2C* -697G/C and Hs_*HTR2C*_01_PM as underlined sequences with CpG methylation sites shown in bold. *HTR2C* rs3813929 (-759C/T) and rs518147 (-697G/C) were highlighted in green.

The *HTR2C* -697G/C primers were designed using PyroMark Assay Design Software 2.0 (Qiagen) whereas *LINE-1* primers (PyroMark® Q24 CpG *LINE-1*) and Hs_*HTR2C*_01_PM PyroMark® CpG assays were purchased from Qiagen. Primer sequences for PCR and pyrosequencing are listed in **Table 3.2**.

Table 3.2: The sequences of primers used in DNA methylation study

Primer	Sequence	PCR length
HTR2C -697G/C	Fwd: 5'- GTTAGTAGGTTTTAGATGTATTAAGAGAT-3' Rev: 5'-[btn]AACAACCAAAACTAACAATCTAAC-3' Seq: 5'- GAGGTAGGAGTTTTGGTGTTTG-3'	241 bp
Hs_*HTR2C*_01_PM PyroMark CpG assay	Unknown primer sequences but included the sequence to analyse of **C**G**C**GCAAAT**C**G**C**GA	159 bp
LINE-1 PyroMark® CpG assay	Unknown primer sequences but included the sequence to analyse of CT**C**GTGGTG**C**GC**C**GTTT	146 bp

3.2.3 DNA methylation in different cohorts

Table 3.3 shows the DNA methylation assays in each cohort.

Table 3.3: Different DNA methylation assays were investigated in each study population.

	HTR2C-697G/C	Hs_*HTR2C*_01_PM	*LINE-1*
Belfast	✓	✓	✓
Chinese	✓	✓	×
Spanish	✓	✓	×

3.2.4 Bisulfite conversion of genomic DNA

There were two bisulfite conversion kits used in this study, the EpiTech Plus DNA Bisulfite kit and EpiTech Fast DNA Bisulfite kit (Qiagen). The general procedure was the same for bisulfite conversion; genomic DNA was exposed to bisulfite which mediated conversion of unmethylated cytosines to uracils. The converted single-stranded DNA was then bound to the membrane of a MinElute DNA spin column followed by washing and desulfonation. Then washing was performed to remove the desulfonation agent. Finally, converted DNA was eluted.

These two kits from Qiagen used spin column technology and provided reagents in aliquots that more convenient. In addition, the reagents are supplied in a unique formulation, for example, bisulfite mix which provided the optimal pH for complete conversion of cytosine to uracil without the need for tedious pH adjustment. The difference between the two kits was the Bisulfite Mix of the EpiTech Plus Bisulfite Kit was a powder that need to be dissolved in 800 µl RNase-free water before use while the bisulfite solution in the EpiTech Fast Bisulfite Conversion kit was ready-to-use solution. In addition, DNA protect buffer was used to prevent the fragmentation of DNA usually associated with bisulfite treatment at high temperatures and low pH. In addition, the DNA protect buffer also provided effective denaturation to separate double-stranded to single-stranded DNA that is necessary for complete cytosine conversion. Moreover, the DNA protect buffer contains a pH indicator dye which has a green colour which should turn to blue after addition to the bisulfite mixture indicating

sufficient mixing and correct pH for the bisulfite conversion reaction. Another difference from the first kit is a series of incubation steps which were performed in the thermal cycler.

The procedure was carried out according to the manufacturer's protocols as follows. The bisulfite reaction was setup at room temperature in a 0.2 ml PCR tube containing gDNA, bisulfite solution, and DNA protect buffer in a total volume of 140 µl as shown in **Table 3.4**. The combined volume of DNA and RNase-free water was depended on the concentration of DNA, 20 µl for high concentration and up to 40 µl for low concentration DNA samples.

Table 3.4: Bisulfite reaction components

Component	High concentration DNA (1-2 µg)	Low concentration DNA (1-500 ng)
DNA	Variable 1-20 µl	Variable 1-40 µl
RNase-free water	Variable	Variable
Bisulfite solution	85 µl	85 µl
DNA Protect buffer	35 µl	15 µl
Total volume	**140 µl**	**140 µl**

The bisulfite reaction was mixed thoroughly indicated by DNA protect buffer turning from green to blue. Then the reaction mixture was incubated in a thermal cycler with heated lid programmed as listed in **Table 3.5**.

The differences between EpiTech Plus DNA Bisulfite Kit and the EpiTech Fast DNA Bisulfite Conversion kit were the series of incubation steps in the thermal cycler for which the EpiTech Plus required about 5 hours whereas 30-50 minutes were required for the EpiTech Fast kit for DNA denaturation and subsequent sulfonation and cytosine deamination.

Table 3.5: Bisulfite treatment thermal cycler conditions

EpiTech Plus Bisulfite Kit			EpiTech Fast Bisulfite Conversion kit		
Step	Time	Temperature	Step	Time	Temperature
Denaturation	5 min	95°C	Denaturation	5 min	95°C
Incubation	25 min	60°C	Incubation	10-20 min	60°C
Denaturation	5 min	95°C	Denaturation	5 min	95°C
Incubation	85 min	60°C	Incubation	10-20 min	60°C
Denaturation	5 min	95°C	Hold	Indefinite	20°C
Incubation	175 min	60°C			
Hold	Indefinite	20°C			

The next step was cleanup of bisulfite converted DNA. The complete bisulfite reaction from the thermal cycler was briefly centrifuged and transferred to a 1.5 ml tube. Then 310 µl of freshly prepared buffer BL containing 10 µg/ml carrier RNA was added to each tube followed by mixing and brief centrifugation. Absolute ethanol, 250 µl, was added to each tube followed by pulse vortexing for 15 seconds and brief centrifugation. The mixture was transferred into the MinElute DNA spin column and then centrifuged at maximum speed for 1 minute. The flow-through was discarded. Wash buffer (buffer BW) 500 µl was added to each spin column and then centrifuged at maximum speed for 1 minute. The flow-through was discarded. Desulfonation buffer (buffer BD) 500 µl was added and then the lid of the spin column was closed before incubation at room temperature for 15 minutes. Then the spin column was centrifuged at maximum speed for 1 minute. The flow-through was discarded. Wash buffer 500 µl (buffer BW) was added followed by centrifugation and discard the flow-through. The wash step was repeated one additional time. Absolute ethanol, 250 µl, was added to each spin column followed by centrifugation. Another additional centrifugation of the spin column into a new collection tube was performed at maximum speed for 1 minute to remove any residual liquid before the elution step. The spin column was placed into a new 1.5 ml tube and 15-20 µl of elution buffer (buffer EB) was added directly onto the center of the spin column membrane. The lid of the spin column was gently closed. The spin column was incubated at room temperature for 1 minute followed by centrifugation for 1 minute at 15,000xg (12,000 rpm) to elute the DNA. The bisulfite converted DNA was stored at -20°C until use.

3.2.5 Amplification of bisulfite treated DNA by PCR

Bisulfite converted DNA was amplified by PCR to amplify the region of interest using the PyroMark PCR kit (Qiagen). The PCR reaction was set up in a 0.2 ml PCR tube in a total volume of 25 µl containing 10-50 ng of bisulfite converted DNA, PyroMark PCR Master Mix, 2x (HotStartTaq DNA Polymerase, 3 mM $MgCl_2$, dNTPs), and primers according to **Table 3.6**. The final concentration of PCR primers and $MgCl_2$ were 0.2 µM and 1.5 mM, respectively. The negative PCR reaction was also set up in every PCR setup to detect possible contamination. All PCR reaction tubes were briefly centrifuged before placing into the thermal cycler.

Table 3.6: PCR reaction composition for amplification of bisulfite converted DNA

Component	Volume per reaction
PyroMark PCR Master Mix, 2x	12.5 µl
CoralLoad Concentrate, 10x	2.5 µl
PCR Primers (5 µM)	1 µl (0.2 µM final conc.)
RNase-free water	variable
Bisulfite converted DNA template	1-5 µl (10-50 ng)
Total volume	**25 µl**

The thermal cycler instruments used in this study including the Veriti™ Thermal Cycler (Applied Biosystems, USA), the TC-5000 gradient thermal cycler (TECHNE, USA), the MWG-Biotech Primus 96 Plus Thermal Cycler and the Primus 25 Thermal Cycler (Biotech Equipment Sales Inc., CA). The thermal cycler, with heated lid, was programmed according to PCR conditions in **Table 3.7**. The PCR products were stored at 2-8°C overnight or at -20°C for longer storage. 5-20 µl of PCR product was used for subsequent pyrosequencing analysis while 5 µl was run on an agarose gel to check the product and possible contamination.

Table 3.7: Optimized PCR condition using thermal cycler

PCR cycling		Time	Temperature
Initial heat activation		15 min	95°C
45-50 cycles	Denaturation	30 s	94°C
	Annealing	30 s	50°C, 56°C, 60°C *
	Extension	30 s	72°C
Final extension		10 min	72°C
Hold		Indefinite	4°C

*The annealing temperature for *LINE-1, HTR2C* -697G/C and Hs_*HTR2C*_PM were 50°C, 60°C, and 56°C, respectively.

3.2.6 Checking PCR product by agarose gel analysis

The amplified PCR product of bisulfite modified DNA was assessed for the specificity of PCR amplification in which a single product at the expected size was obtained. In addition, the intensity of the PCR product on the gel was used to approximate the amount for use in pyrosequencing. Moreover, any possible contamination during the PCR processes was also assessed.

The 2.0% (w/v) gel was prepared by adding 40 ml of 1xTAE or TBE to 0.8 g of agarose powder. The mixture was then heated to melt and dissolve the agarose thoroughly using a microwave for 1 minute. After the gel was completely melted and cooled down to about 60°C, the gel was mixed with 0.4 µl of 5 mg/ml ethidium bromide solution immediately prior to pouring into a prepared gel casting plate with a plastic comb. The agarose gel was allowed to set at room temperature. Once the agarose gel was ready, it was then placed into an electrophoresis tank filled with 1XTAE buffer.

5 µl of PCR product was loaded directly onto the agarose gel without prior addition of loading buffer because the PCR product contained the CoralLoad® Concentrate which contains a gel loading reagent and 2 gel tracking dyes. 5 µl of 100 bp DNA marker (GeneRuler 100 bp DNA Ladder, Cat.no. SM0241, Thermo Scientific, UK) was also loaded onto the gel.

Electrophoresis was carried out at 100V for approximately 50 minutes or until the dye had reached the bottom of the gel to ensure the appropriate separation of DNA ladder and PCR products. Then the gel was viewed under a UV light or CCD camera fitted to a UV transiluminator. The gel was exposed to UV until a clear image was obtained.

3.2.7 Pyrosequencing

Pyrosequencing was carried out using the PyroMark Q24 systems. There were 4 main steps to complete the analysis of DNA methylation using pyrosequencing including (1) assay and run setup, (2) immobilization of PCR products to streptavidin sepharose HP beads, (3) preparation of samples for pyrosequencing analysis, (4) quantification of CpG methylation as described by manufacturer.

3.2.7.1 Assay and run setup

The assay for CpG methylation analysis was setup in PyroMark Q24 Software (Qiagen) by selecting "New CpG Assay" and entering the nucleotide sequence in "Sequence to Analyze". Dispensation order of nucleotides was created by clicking a "Generate Dispensation Order" button and the internal control for the completion of bisulfite treatment was manually added by adding a C dispensation before or after the dispensation of T that was converted from C. The assay was saved. This assay setup was performed only the first time the assay was run. The gene bank sequence, the sequence to analyze which was the sequence after bisulfite conversion, and the dispensation order (included internal control for bisulfite conversion) of each DNA methylation assay are listed in **Table 3.8**. The dispensation orders were presented as histograms as shown in **Figure 3.9**.

The run file was created by selecting "New Run" and the plate was setup by adding the assay parameters to each wells. The run setup file was saved into a USB stick. A list of required volumes of reagents and the plate setup can be printed out from "Pre Run Information" from the "Tools" menu bar.

More information about how to setup the assays and run the files is provided in the PyroMark® Q24 Software User Guide (Qiagen).

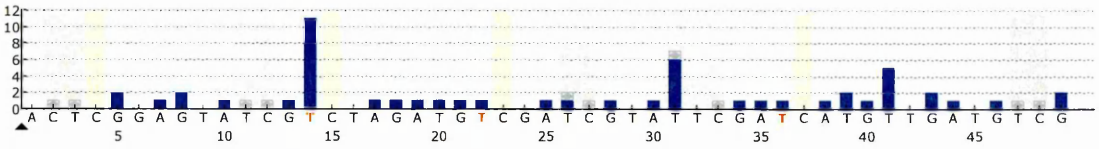
Before starting the next steps, the PyroMark Q24 system and the heat block were turned on to warm up. The heat block was set at 80°C and the plate holder was also placed on the heat block.

Table 3.8: The DNA sequences of each DNA methylation assay

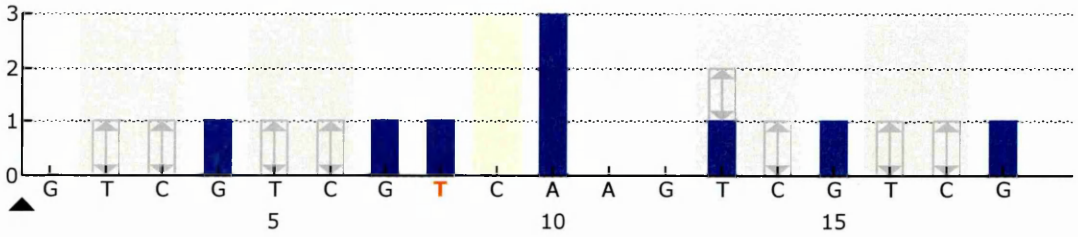
Sequence
***HTR2C* -697G/C assay** (Number of CpG site : 5) Gene bank Sequence: CGGAGGA**C**GCTTCCTTCCTCAGATGCAC**C**GATCTTCC**C**GATACTGCCTTTGGAG**C**GGCT Sequence to Analyze: **Y**GGAGGA**Y**GTTTTTTTTTTTAGATGTAT**Y**GATTTTTT**Y**GATATTGTTTTTGGAG**Y**GGTT Dispensation order: ACTCGGAGTATCGTCTAGATGTCGATCGTATTCGATCATGTTGATGTCG
Hs_*HTR2C*_01_PM PyroMark CpG assay (Number of CpG site : 4) Gene bank Sequence: **C**G**C**GCAAAT**C**G**C**GA Sequence to Analyze: **Y**G**Y**GTAAAT**Y**G**Y**GA Dispensation order: GTCGTCGTCAATCGTCG
Hs_*LINE-1* PyroMark® CpG assay (Number of CpG site : 3) Gene bank Sequence: CT**C**GTGGTG**C**GC**C**GTTT Sequence to Analyze: TT**Y**GTGGTG**Y**GT**Y**GTTT Dispensation order: GCTCGTGTAGTCAGTCG

The methylation cytosine sites are in bold (C or Y) or highlighted in **blue**; the internal controls for completion of bisulfite treatment (C) were highlighted in **yellow**; Y was described for pyrimidine (C or T); the **underline** in *HTR2C* -697G/C assay was the position of -697G/C polymorphism.

(A) *HTR2C* -697G/C CpG assay



(B) Hs_*HTR2C*_01_PM CpG assay



(C) *LINE-1* CpG assay

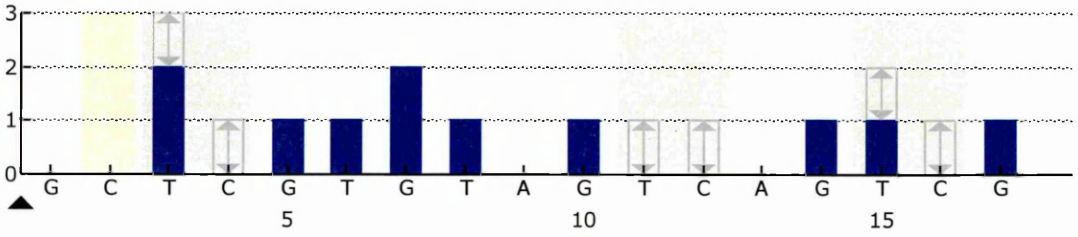


Figure 3.9: Histograms for dispensation order of CpG assays for *HTR2C* -697G/C (A), Hs_*HTR2C*_01_PM (B), and *LINE-1*(C). The controls (C or cytosine) for completion of bisulfite treatment are highlighted in yellow.

3.2.7.2 Immobilization of PCR products to streptavidin sepharose HP beads

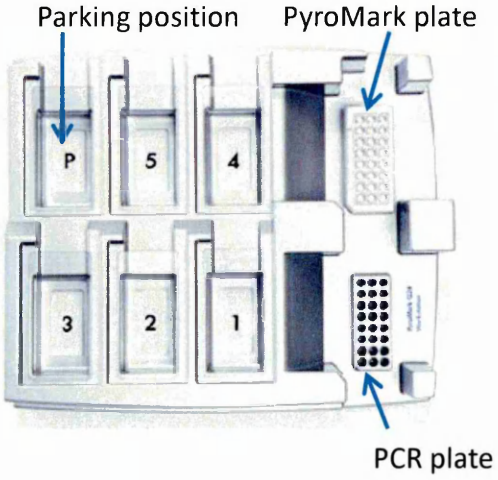
In this step, DNA template was immobilized to streptavidin sepharose HP beads for subsequent analysis using the PyroMark Q24. The reactions were set up in 24-well PCR plates in the total volume of 80 µl according to **Table 3.9**. The PCR plate was sealed using adhesive film to ensure that no leakage is possible between the wells. The PCR plate was agitated constantly at 1,400 rpm for 10 minutes at room temperature (15-25°C) using a mixer. As sepharose beads sediment quickly, the capturing of beads in the next step to prepare samples for pyrosequencing was performed immediately once the agitation was completed. During agitation, the biotin-labelled DNA was immobilized to streptavidin-coated beads.

Table 3.9: Components of master mix and PCR product for DNA immobilization

Component	Volume per sample
Pyromark binding buffer, 2x	40 µl
Streptavidin sepharose HP beads	2 µl
RNase-free water	18 µl or less
PCR product	20 µl or less
Total volume	**80 µl**

3.2.7.3 Preparation of samples for pyrosequencing analysis

This step was carried out using the PyroMark Q24 Vacuum Workstation (Qiagen) to prepare single-stranded DNA template and anneal the sequencing primer to the template before pyrosequencing analysis. The Vacuum work station was prepared before starting by filing five separate troughs according to **Figure 3.10**. The filter probe was washed once time by flushing with high-purity water (Milli-Q 18.2MΩxcm) in trough 5. Trough 5 was refilled with 70 ml high-purity water.



PyroMark Q24 Vacuum Workstation

Trough	Solution	Volume
1	Ethanol (70%)	50 ml
2	Denaturation	40 ml
3	Wash Buffer (1x)	50 ml
4	High-purity water	50 ml
5	High-purity water	70 ml

Figure 3.10: PyroMark Q24 Vacuum Workstation preparation

The sequencing primer was diluted to the concentration of 0.3 μM in annealing buffer (Qiagen) and 25 μl of 0.3 μM sequencing primer was added to the PyroMark Q24 plate.

Once the PCR plate agitation was completed, the beads containing immobilized DNA template were captured immediately within 1 minute since the plate was agitated according to manufacturer's protocol. The PCR plate was placed in the workstation. The pump was switched on and the vacuum switched on to apply vacuum to the tool. The filter probes were carefully lowered into the PCR plate for 15 seconds until all beads were captured. The tool was carefully picked up and transferred to trough 1 containing 70% ethanol to flush the filter probes for 5 seconds. The PCR reaction components except for the immobilized DNA template were washed out in this step. Then the tool was moved to trough 2 containing denaturation solution for 5 seconds. The double-stranded DNA was separated into single-stranded DNA; only the biotinylated strands were captured by beads. Unbiotinylated strands and denaturation solution were completely washed when the filter probes was flushed in trough 3 containing wash buffer for 10 seconds. The tool was raised to beyond 90° vertical for 5 seconds to drain liquid from the filter probes before holding the tool over the PyroMark plate. The vacuum switch was turned off before lowering the filter probes into the PyroMark plate containing sequencing primer. The beads were released by gently shaking the tool in the wells for 15 seconds. The tool was then transferred to trough 4 containing the high-purity water and agitated for 10 seconds before moving to trough 5 containing the high-purity water and applying vacuum to flush the filter probes for 5 seconds. The tool was raised to beyond 90° vertical for 5 seconds to drain the liquid from the filter probes before the vacuum switch was off and then the tool was placed in the parking position. The vacuum pump was turned off.

The PyroMark plate containing the pyrosequencing templates and sequencing primer was heated at 80°C for 2 minutes using the PyroMark Q24 plate holder. The samples were cooled down to room temperature for at least 5 minutes before proceeding in the PyroMark Q24 pyrosequencer. During the time the samples were cooled down, the sequencing primers annealed to the templates.

3.2.7.4 *Quantification of CpG methylation*

The PyroMark Gold Q24 reagents including dNTPs, enzyme, and substrate mixtures, which were purchased from Qiagen were carefully loaded without air bubbles into the PyroMark Q24 cartridge according to the volume reported in the pre run information. All reagents and the cartridge were allowed to reach room temperature before placing into the PyroMark Q24 followed by placing the PyroMark plate on the heating block. The USB stick with set up run file was inserted into pyrosequencer, and then pyrosequencing was started. When pyrosequencing was finished, the processed run file was automatically saved to the USB stick. The plate was discarded and the cartridge was cleaned according to the PyroMark Q24 user manual guide. The PyroMark Q24 was shutdown and switched off.

The processed run file was analyzed in the CpG mode in PyroMark Q24 Software. The quantification of CpG methylation and quality assessment were displayed above each CpG site in the program trace. For reliable results the manufacturer had recommended that the single peak heights should be above 30 relative light units (RLU) which was set as the 'required peak height for passed quality' in assay setup.

3.2.8 Statistical Analyses

All statistical analyses were carried out using SPSS for Window.

The methylation levels of all CpG sites were tested for outliers and normal distribution before testing association. Stepwise linear regression was used to analyse the confounding effect of age or gender on methylation levels. The partial correlation were used in analysing the correlation between methylation at each CpG site and interval or ratio scale variables such as baseline weight, baseline BMI or BMI, weight change, BMI change. The association between genetic polymorphisms and DNA methylation was analyzed using a general linear model (univariate analysis of variance) with covariates as appropriate. Mann-Whitney U test and Kruskal-Wallis one-way ANOVA test were used if the data were not normally distributed. Chi-square was used to analyze the difference between categorical variables. Significant differences between means were determined at a level of $p \leq 0.05$.

3.3 Results

3.3.1 Validation of methodology: DNA methylation study

The validation of the method for determining DNA methylation using bisulfite treatment of genomic DNA and pyrosequencing was determined as described below.

1. DNA samples were tested for the precision of the bisulfite treatment throughout pyrosequencing. Initially, DNA samples were subjected to 3 bisulfite treatments, then each bisulfite modified DNA was amplified by PCR and subsequently pyrosequenced together. **Figure 3.11A** shows the DNA methylation levels of three different bisulfite treatments (per sample) for the determination of *LINE-1* methylation. The coefficients of variation were less than 5% (1.15-3.10%). The result indicates high precision of multiple bisulfite reactions.

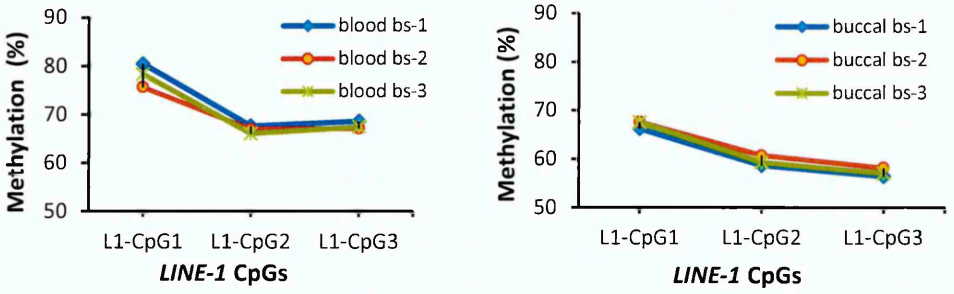
2. 3 modified DNA samples were tested for the precision of the PCR step. Each bisulfite modified DNA was amplified by 3 independent PCRs, and then underwent pyrosequencing together. **Figure 3.11B** shows the DNA methylation levels of three different PCRs (per bisulfite modified DNA sample) in determination of Hs_*HTR2C*_01_PM methylation. The variation coefficients were less than 10% (0.83-7.33%). The result indicates the high precision of multiple PCR reactions.

3. 3 modified DNA samples were tested for the precision of the pyrosequencing step. Each PCR product underwent pyrosequencing separately (3 times). **Figure 3.11C** shows the DNA methylation levels of three different pyrosequencing which were performed using the same PCR product for determination of the *HTR2C* -697A/C methylation. The coefficients of variation were less than 10% (0.5-9.5%). The result indicates the high precision of multiple pyrosequencing.

The results of DNA methylation levels of each sample were calculated for coefficient of variation (CV) using the following formula:

Coefficient of variance (%) = Standard deviation *100 / Mean

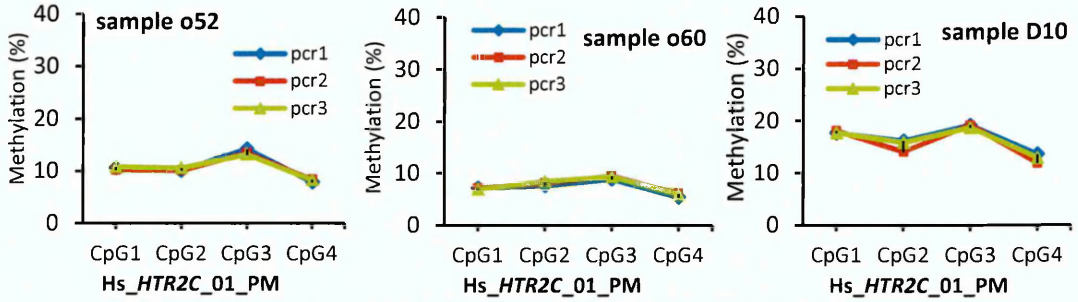
(A) Precision of bisulfite treatment using DNA from peripheral blood and buccal cells



LINE-1	CpG1	CpG2	CpG3
%CV	3.10	1.19	1.15

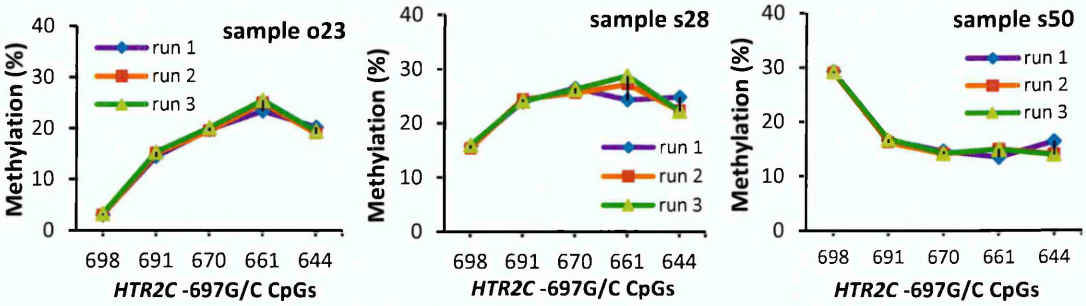
LINE-1	CpG1	CpG2	CpG3
%CV	1.15	1.79	1.58

(B) Precision of PCR step



Hs_*HTR2C*_01_PM	CpG1	CpG2	CpG3	CpG4
%CV sample o52	2.87	2.80	3.79	3.35
%CV sample o60	1.93	5.08	3.36	6.61
%CV sample D10	1.26	7.33	1.37	6.71

(C) Precision of pyrosequencing step



HTR2C -697A/C promoter region	CpG1 -698	CpG2 -691	CpG3 -670	CpG4 -661	CpG5 -644
%CV sample o23	4.4	3.0	1.6	4.4	3.1
%CV sample s28	2.1	1.0	1.8	8.4	6.3
%CV sample s50	0.5	1.9	1.8	5.9	9.5

Figure 3.11: DNA methylation levels and coefficient of variation showing the precision of each step of DNA methylation measurement

(A) precision of bisulfite treatment step, (B) precision of PCR step, and (C) precision of pyrosequencing step.

3.3.2 DNA methylation of the *HTR2C* promoter sequences and global methylation in chronic schizophrenia patients (Belfast cohort)

DNA methylation levels of two regions of the *HTR2C* gene and one region of *LINE-1* were investigated in the chronic Belfast cohort. The mean age of male patients was 42.6y (n=41) and 46.1y for female (n=31). Gender was significantly related to methylation in both *HTR2C* regions in which females had higher methylation levels than males at all CpG sites ($p<0.05$), and the CpG1 of *LINE-1* ($p=0.016$). Age was significantly associated with some CpGs including the CpG -691, -670, -661, and -644 of the *HTR2C*-697G/C region, and the CpG1 and CpG2 of the Hs-*HTR2C*_01_PM region ($p<0.05$) in which methylation levels wereincreased with age. Age did not associate with methylation of *LINE-1*. The mean age of patients receiving olanzapine or clozapine (37.1y) was significantly lower than that of the remaining patients receiving all other drugs (47.2y, $p<0.001$). Therefore, gender and age were included as covariates in subsequent analyses as appropriate. The methylation of *LINE-1* at all CpG sites was normally distributed; thus parametric tests were used in analyses, whereas the non-parametric tests were used in analysing the association of the *HTR2C* -697G/C and Hs_*HTR2C*_01_PM regions where results were not normally distributed.

3.3.2.1 Global DNA methylation

Methylation of *LINE-1* was not associated with age at any of the 3 CpG sites (**Figure 3.12**) in either male or female subgroups ($p>0.05$). However, it was associated with gender; the methylation levels of the *LINE-1* in males was significantly higher than that of females at CpG1 of the *LINE-1* region (male 78.98±1.44%, n=36 vs female 78.04±1.67%, n=30, $p=0.016$) while the other two CpG sites showed the same pattern (**Figure 3.13A**). In addition, tobacco smoking was also associated with methylation of *LINE-1*; smokers had higher methylation of the *LINE-1* than non-smoking patients and the difference reached statistical significance at CpG3 ($p=0.019$) as shown in **Figure 3.13B**.

Patients receiving olanzapine or clozapine had slightly lower global DNA methylation levels than those patients receiving all other drugs (**Figure 3.13C**) but it did not reach statistical significance. No significant association of obesity (BMI>30 kg/m^2), central

obesity, or metabolic syndrome and methylation levels was observed at any CpG sites of this region (**Figure 3.13 D-F**).

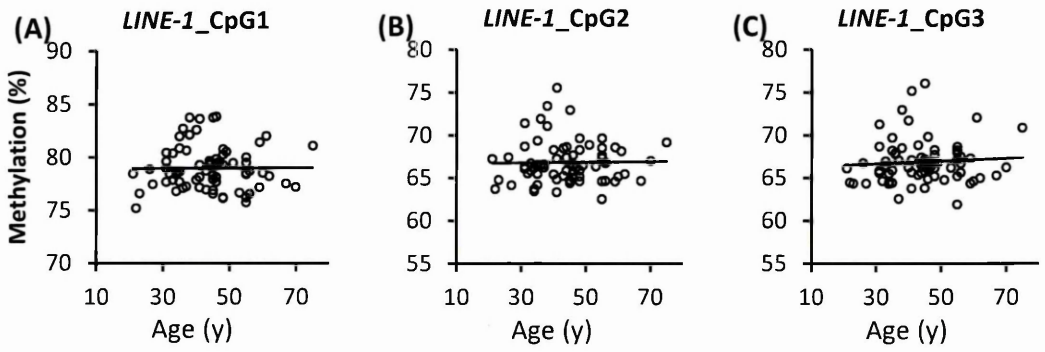


Figure 3.12: Association of age with global *LINE-1* DNA methylation in chronic (Belfast) cohort

The figure shows no correlation of age and global methylation of the *LINE-1* at CpG1 (A), CpG2 (B), and CpG3 (C).

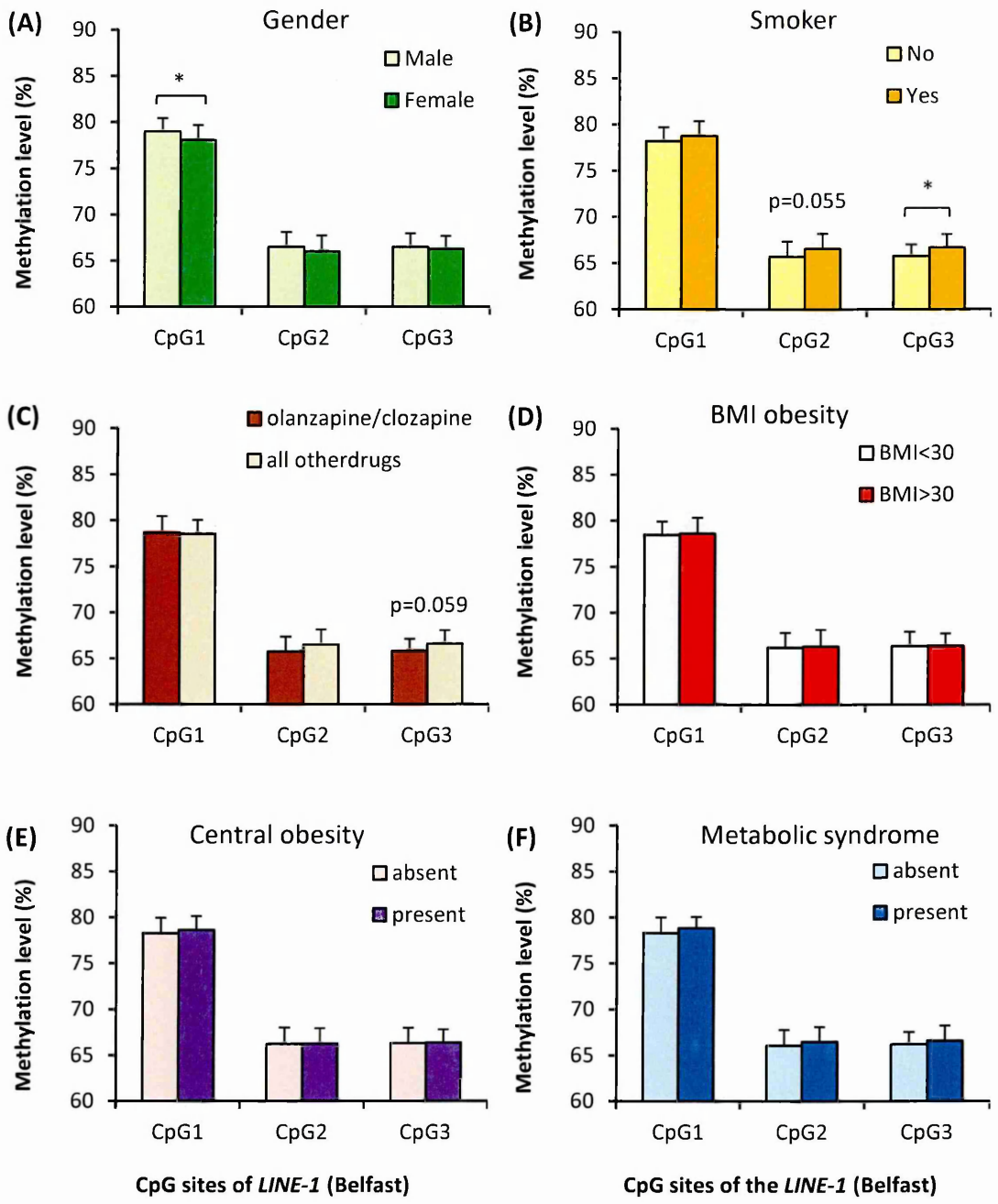


Figure 3.13: DNA methylation of the *LINE-1* in chronic (Belfast) cohort

The global methylation compared between male and female (A), smoker and non-smoker (B), olanzapine or clozapine treatment and all other drugs (C), BMI obesity <30 and >30 kg/m^2 (D), presence of central obesity (E), and metabolic syndrome (F). Data are expressed as mean±SD. * $p<0.05$.

The influence of the polymorphisms and the extent of DNA methylation of the *LINE-1* were investigated and results show that patients who carried the AA genotype of the *FTO* rs9939609, associated with higher BMI in this cohort, were more likely to have higher *LINE-1* methylation than the T allele carriers although this did not reach significance (p=0.055 at CpG3) (**Figure 3-14A**).

The *HTR2C* -759C/T and the *MTHFR* 1298A/C polymorphisms did not influence the methylation levels of *LINE-1* (**Figure 3-14 B and D**). The *MTHFR* 677C/T polymorphism, T allele showed a significant influence on *LINE-1* methylation at CpG3 (p=0.026, correcting for smoking) compared to CC genotype (**Figure 3-14C**).

In addition, the interaction between the *MTHFR* 677C/T polymorphism and gender, controlling for smoking (p=0.042, overall effect 0.006, r^2=0.165) (**Figure 3-15A**).

Females with the T allele had higher methylation than those with CC genotype at CpG3 (p=0.003, overall effect 0.010, r^2=0.255). There was a significant interaction between the *MTHFR* 677C/T polymorphism and smoking on *LINE-1* methylation at CpG3 (p=0.009, overall effect p=0.001, r^2=0.213)(**Figure 3-15B**). Smoking increased *LINE-1* methylation at CpG3 in patients who carried the *MTHFR* 677T allele (67.33±1.37%, n=22) compared to CC genotype carriers (65.88±1.19%, n=18), p=0.001, r^2=0.227.

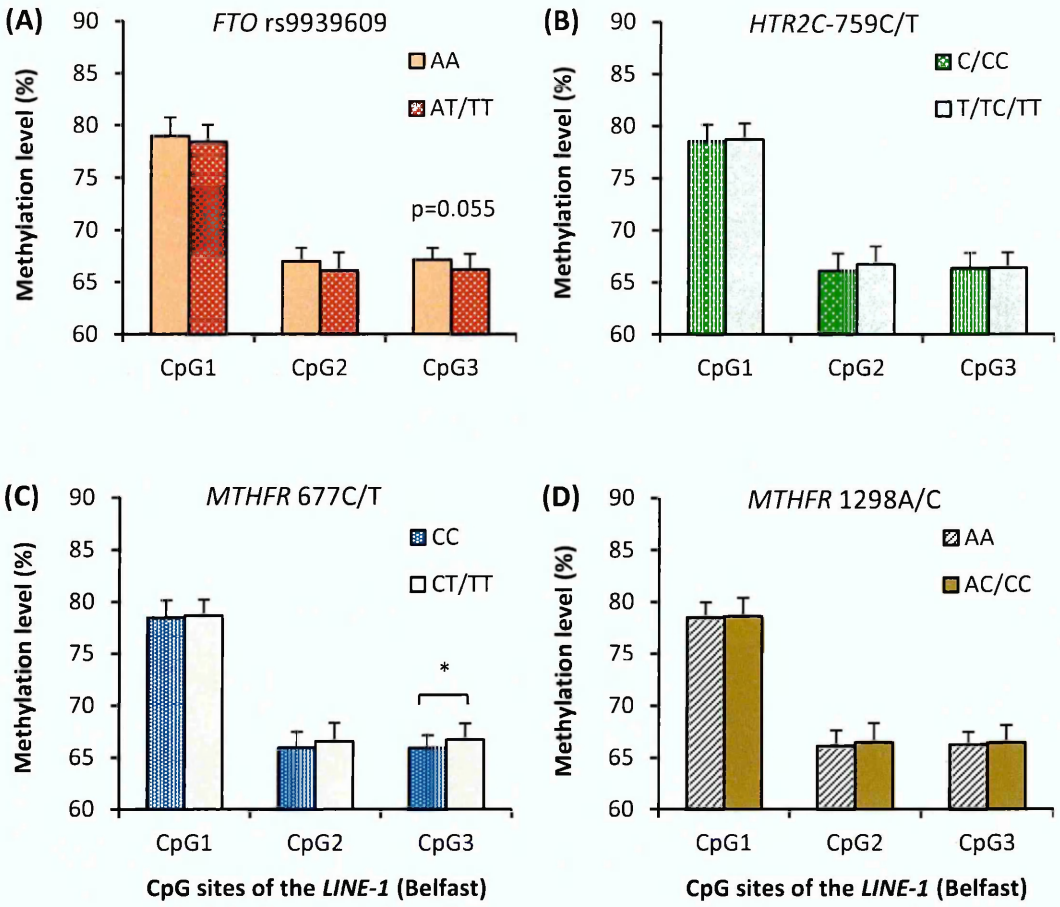


Figure 3.14: Influence of genetic polymorphisms on DNA methylation of the *LINE-1* in chronic (Belfast) cohort.

The global methylation compared between genotype subgroups of the *FTO* rs9939609 (A), *HTR2C* -759C/T (B), *MTHFR* 677C/T (C), *MTHFR* 1298A/C (D) polymorphism. Data are expressed as mean±SD. * p<0.05.

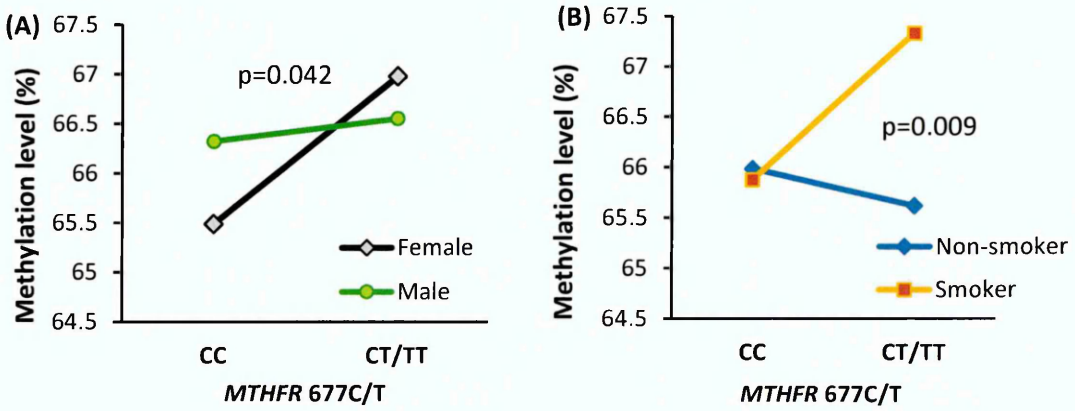


Figure 3.15: Interaction of the *MTHFR* -677C/T genotype with gender (A), and the SNP with tobacco smoking (B) on DNA methylation of *LINE-1* at CpG3 in chronic (Belfast) cohort.

3.3.2.2 DNA methylation of the HTR2C -697G/C region

There was a gender effect on methylation of the *HTR2C* -697G/C region, females had significantly higher methylation than males at all CpG sites ($p<0.001$) as shown in **Figure 3.16A**. This is because of the *HTR2C* gene is located on chromosome X, thus females have more methylation in order to inactivate one of sex chromosome. A similar pattern of lower methylation of the *HTR2C* -697G/C region of smokers was opposite with that of the *LINE-1* (**Figure 3.16B**); however, the olanzapine or clozapine treatment showed similar patterns in *LINE-1* methylation; treatment with these drugs had slightly lower methylation of the *HTR2C*-697G/G region (**Figure 3.16C**). No significant association of the BMI obesity (>30 kg/m^2), central obesity, and metabolic syndrome and methylation levels was observed at any CpG sites of this region (**Figure 3.16 D-F**), although there was a similar pattern of higher methylation in patients who had BMI obesity (>30 kg/m^2) or central obesity.

The influence of polymorphisms on methylation of the *HTR2C* -697G/C regions showed only the *HTR2C* -759C/T polymorphism had a significant association with methylation levels. All CpG sites except CpG -698 showed a similar pattern that carriers with C/CC genotype had lower methylation levels than T allele carriers and the statistical significance were reached at CpG-670 ($p=0.020$) and CpG-661 ($p=0.030$) (**Figure 3.17B**). The GG genotype of the leptin -2548G/A polymorphism showed significantly higher methylation at CpG-698 ($p=0.023$)(**Figure 3.17E**). The *FTO* rs9939609, the *MTHFR* 677C/T, and the *MTHFR* 1298A/C polymorphisms did not influence the methylation of this region of the *HTR2C* (**Figure 3.17 A,C,D**).

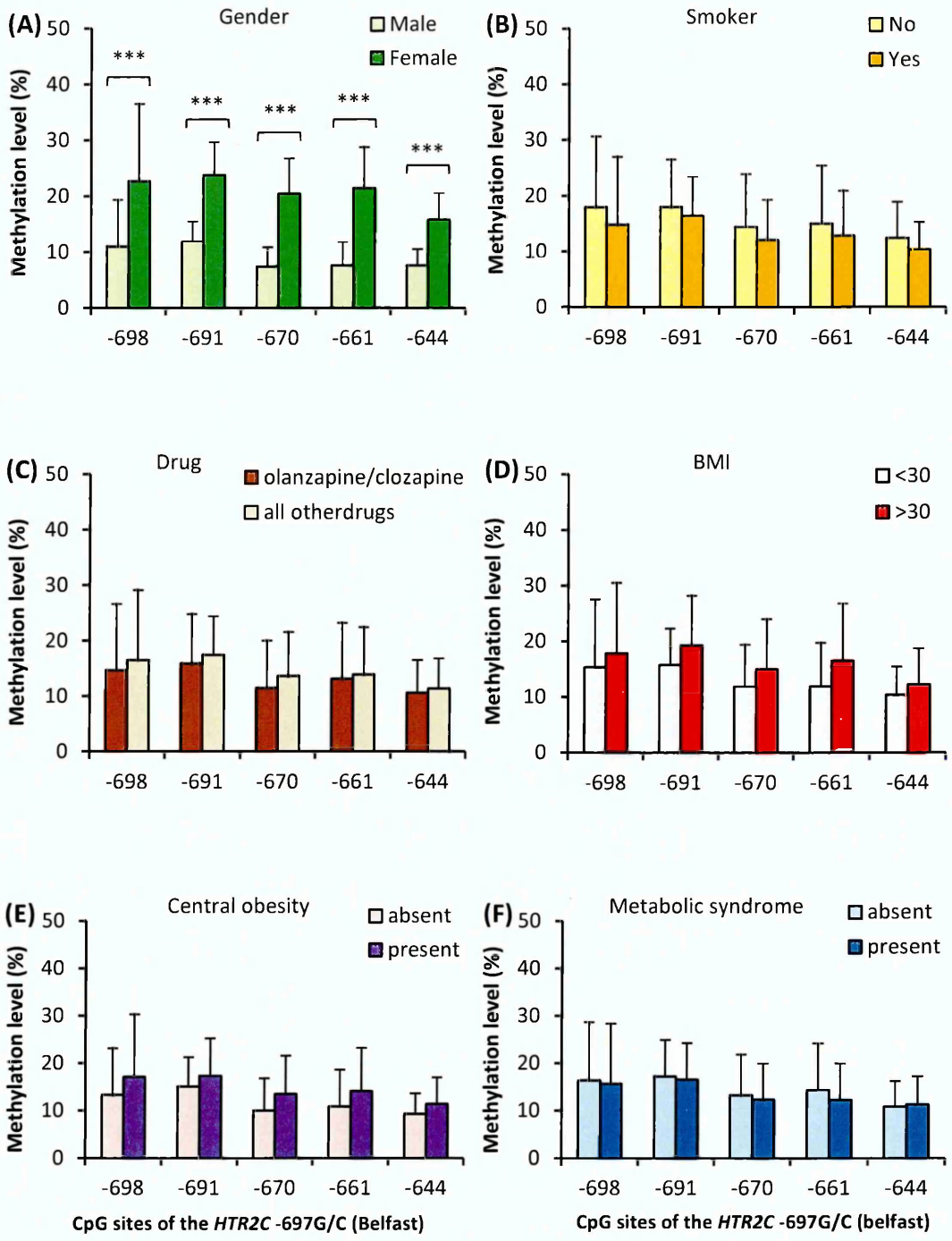


Figure 3.16: DNA methylation of the *HTR2C* -697G/C region in chronic (Belfast) cohort

The methylation of the *HTR2C* -697G/C region compared between male and female (A), smoker and non-smoker (B), olanzapine or clozapine treatment and all other drugs (C), BMI obesity <30 and >30 kg/m^2 (D), presence of central obesity (E), and metabolic syndrome (F). Data are expressed as mean±SD. *** p≤0.001. Gender was included as a covariate in the analyses B-F in the parametric test (GLM; UNIANOVA).

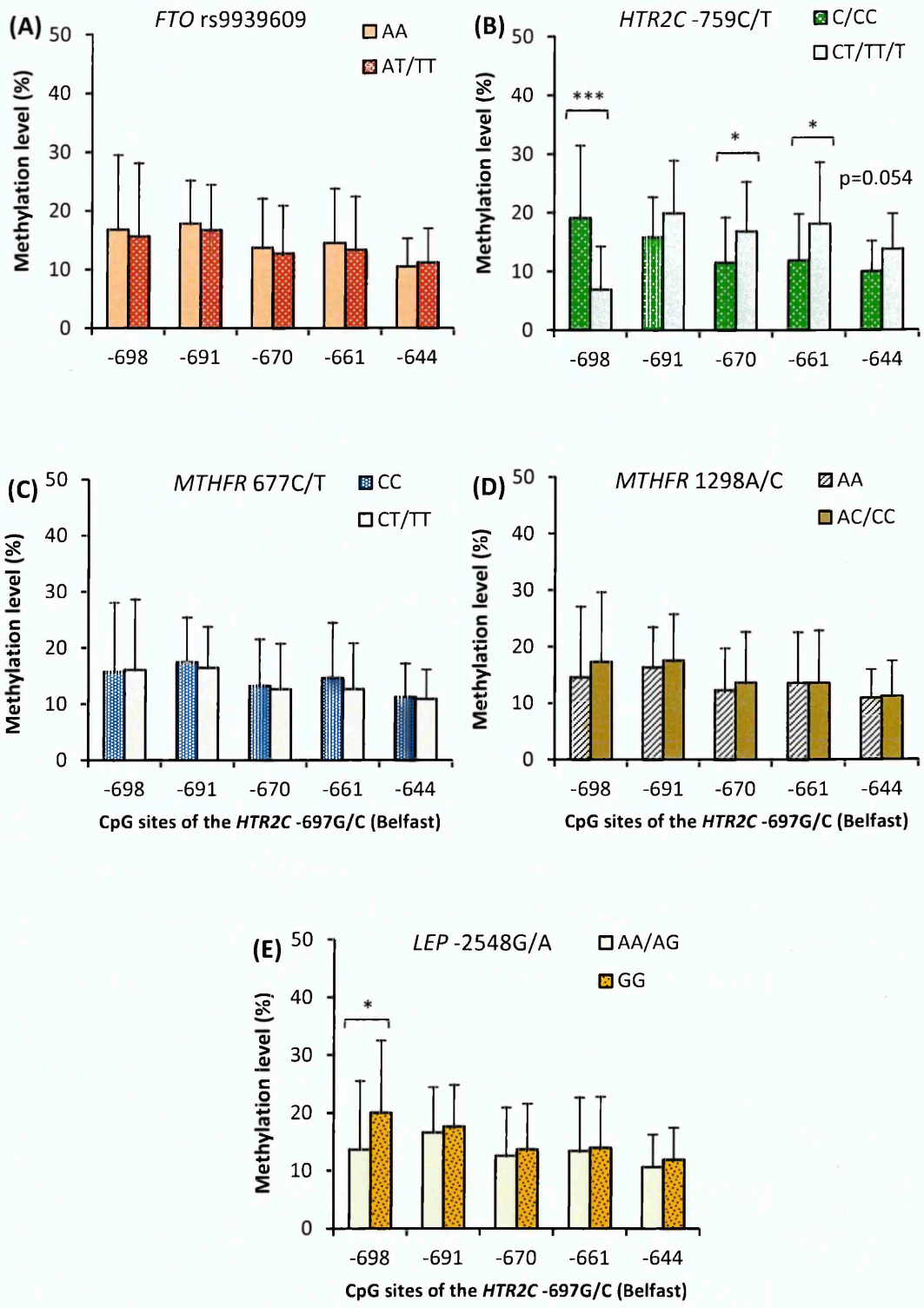


Figure 3.17: Influence of genetic polymorphisms on DNA methylation of the *HTR2C* -697G/C region in chronic (Belfast) cohort

The methylation of the *HTR2C* -697G/C region compared between genotype subgroups of the *FTO* rs9939609 (A), *HTR2C* -759C/T (B), *MTHFR* 677C/T (C), *MTHFR* 1298A/C (D), and leptin -2548G/A (E) polymorphism. Data are expressed as mean±SD. * p<0.05, ***p<0.001.

3.3.2.3 DNA methylation of the Hs_HTR2C_01_PM region

In the Hs_*HTR2C*_01_PM region, females had significantly higher methylation than males at all CpG sites ($p<0.001$) (**Figure 3.18A**). No significant association of DNA methylation with smoking, olanzapine or clozapine treatment, BMI obesity, and metabolic syndrome was observed (**Figure 3.18 B-D, and F**), although the smoker group and olanzapine or clozapine groups were more likely to have lower methylation, and the lower methylation levels were found in BMI obesity <30 kg/m^2, absence of metabolic syndrome and absence of central obesity. The absence of central obesity patient group had significantly lower methylation at CpG1 ($p=0.023$) (**Figure 3.18E**).

There were no significant associations between the *FTO* rs9939609, *HTR2C* -759C/T, *MTHFR* 677C/T, and *MTHFR* 1298A/C polymorphisms and methylation of the Hs_*HTR2C*_01_PM regions. However, there were similar patterns of the results of the *FTO* rs9939609 and the *HTR2C* -759C/T polymorphisms on methylation levels as observed in the *HTR2C*-697G/C region in which the AA genotype of the *FTO* rs9939609 and the T allele of the *HTR2C* -759C/T exhibited slightly higher methylation levels (**Figure 3.19**); for example: the *FTO* rs9939609 AA genotype showed slightly higher levels of methylation than the T allele group at CpG3 (6.92±3.32% vs 5.28±3.13%, $p=0.092$), and the C/CC genotype of the *HTR2C* -759C/T had slightly lower levels of the methylation than T/CT/TT genotype at CpG3 (5.28±3.00% vs 6.57±3.67%, $p=0.150$). Interestingly, the GG genotype of the leptin -2548G/A polymorphism showed significantly higher methylation at CpG1 ($p=0.025$) and CpG4 ($p=0.009$) (**Figure 3.19E**).

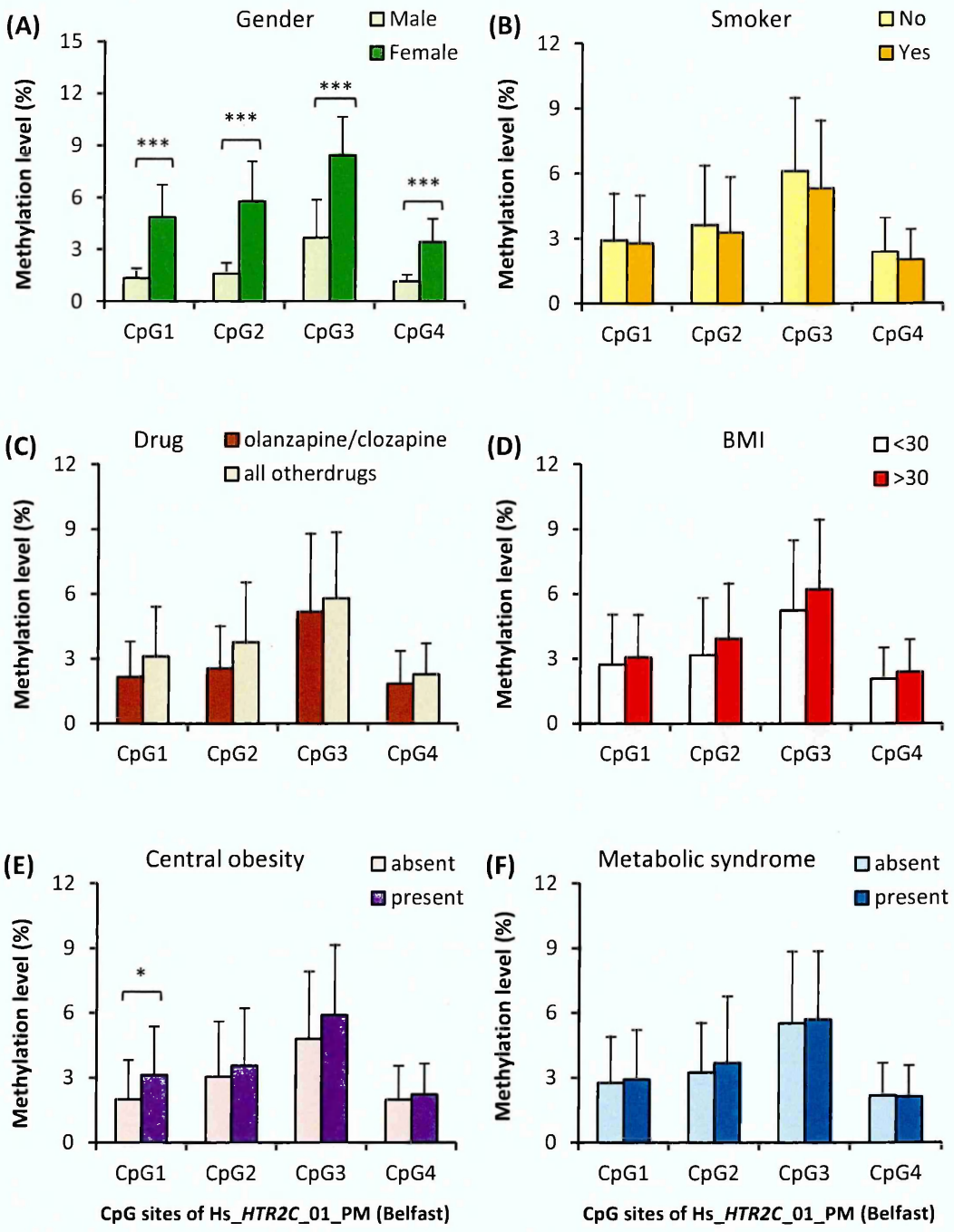


Figure 3.18: DNA methylation of the *Hs_HTR2C_01_PM* in chronic (Belfast) cohort

The methylation of the Hs_*HTR2C*_01_PM region compared between male and female (A), smoker and non-smoker (B), olanzapine or clozapine treatment and all other drugs (C), BMI obesity <30 and >30 kg/m^2 (D), presence of central obesity (E), and metabolic syndrome (F). Data are expressed as mean±SD. * $p<0.05$, *** $p\leq0.001$. Gender was included as a covariate in the analyses B-F in the parametric test (GLM; UNIANOVA).

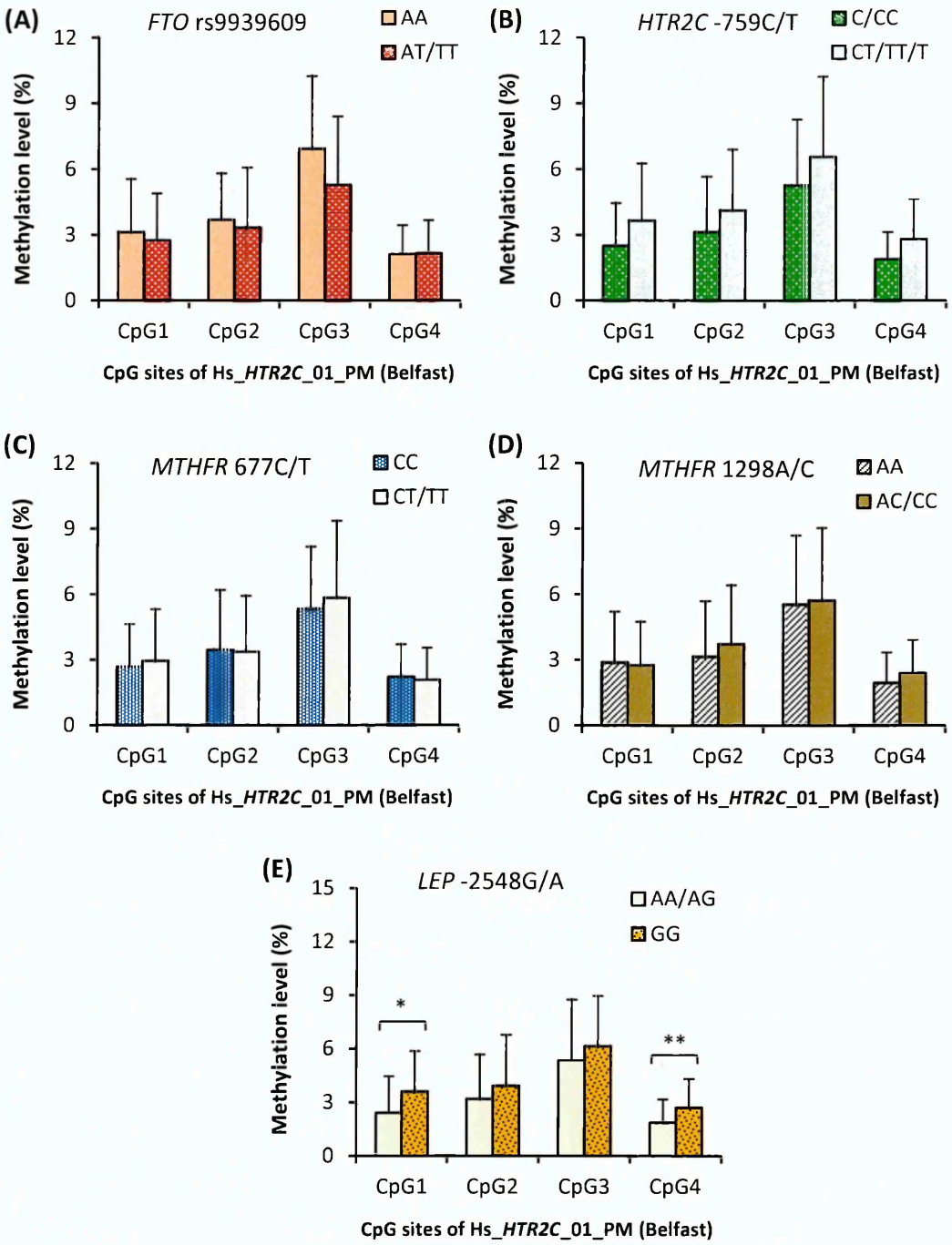


Figure 3.19: Influence of genetic polymorphisms on DNA methylation of the Hs_*HTR2C*_01_PM region in chronic (Belfast) cohort

The methylation of the Hs_*HTR2C*_01_PM region compared between genotype subgroups of the *FTO* rs9939609 (A), *HTR2C* -759C/T (B), *MTHFR* 677C/T (C), *MTHFR* 1298A/C (D), and leptin -2548G/A (E) polymorphism. Data are expressed as mean±SD. * $p<0.05$, ** $p\leq0.01$.

3.3.3 Association of DNA methylation of the *HTR2C* promoter sequences with antipsychotic drug-induced weight gain in first episode Chinese Han and Spanish schizophrenia patients

DNA methylation levels of two regions of the *HTR2C* gene were investigated in both Chinese Han and Spanish cohorts. There were significant differences in DNA methylation levels of *HTR2C* between males and females in that females always had higher methylation levels than males at all CpG sites of both regions.

3.3.3.1 Chinese Han cohort

3.3.3.1.1 Methylation and weight gain

The percentages of methylation levels at each CpG site were tested for normal distribution against testing subgroups; for example: the analysis comparing two subgroups of patients having weight increase >7% and <7% found that methylation levels at all CpG sites of Hs_*HTR2C*_01_PM region and CpG-698, -691, and -644 of the *HTR2C* -697G/C region were not normally distributed and therefore nonparametric statistical analyses were used; whereas the CpG-670 and -661 were normally distributed and parametric statistical analyses were performed. The outliers were also determined at each CpG site against differential testing subgroups (for example, male vs female, weight increase >7% vs <7%, risperidone vs other drug treatment, *HTR2C* -759 T allele vs C/CC genotype, *MTHFR* 677 T allele vs CC genotype, etc.), then the outliers of these tests were sumarized and removed before subsequent statistical analyses. The outlier removal citeria in SPSS is based on Tukey's hinges (Tukey, 1977) which calculate the upper and lower fences using equations as described below.

$$\text{Interquartile range (IQR)} = Q3 - Q1 \qquad \text{(equation 1)}$$

Q1 is the first quartile (the 25^{th} percentile) and Q3 is third quatile (the 75^{th} percentile)

Then the IQR has been used to calculate fences 1.5 hinge-spreads below and above the hinges using equations below.

$$\text{The lower fence } (Fence_{Lower}) = Q1 - (1.5)(IQR) \qquad \text{(equation 2)}$$

$$\text{The upper fence } (Fence_{Upper}) = Q3 + (1.5)(IQR) \qquad \text{(equation 3)}$$

The extreme outliers located ouside the lower or upper fences were exclued. These outliers can be identified from stem-and-leaf plots and boxplots (box-and-whiskers plot) which show the frequncy and the raw data values at lower and upper fence.

Stepwise linear regression indicated a significant effect of gender, but not age on methylation at all CpG sites in both regions of the *HTR2C*; therefore, this was included in subsequent analysis as a covariate. Methylation levels at all CpG sites, except at CpG -691, were normally distributed. No significant correlation of methylation levels with baseline weight, change in weight, baseline BMI, or change in BMI was observed in this cohort (p>0.05, data not shown).

Interestingly, the association between methylation levels at five CpG sites in the *HTR2C* -697G/C regions and weight change groups (>7% vs <7%) was observed in patients who had a weight increase of >7%, these patients had lower mean methylation at all CpG sites compared to patients who had a weight increase of <7%, where only methylation at -644 site showed a significant difference (p=0.046) (**Table 3.10**, the raw data without outlier cut off is shown in Appendix 1, Table 1). Gender distribution between two weight change groups were not significantly different (χ^2=2.160, p=0.142). In this *HTR2C* -697G/C region, there was a genotype effect on methylation at the first CpG (-698) site, because the nucleotide sequences at positions -698,-697(SNP site G/C), and -696 was CGG; thus if the SNP was G, the methylation site was at position -698, but if the SNP was C, the methylation site was at position -697.

Table 3.10: Methylation levels at 5 CpGs in the *HTR2C* -697G/C promoter sequences comparing between two subgroups of weight increase in Chinese Han patients

***HTR2C* -697G/C promoter region**	**CpG1 -698**	**CpG2 -691**	**CpG3 -670**	**CpG4 -661**	**CpG5 -644**
Weight increase>7% (n)	19.52±4.98 (70)	20.39±5.74 (82)	19.12±6.07 (82)	21.30±6.36 (81)	17.84±3.69 (81)
Weight increase<7% (n)	19.82±4.87 (76)	21.19±5.70 (100)	20.62±6.13 (100)	23.28±6.44 (100)	19.20±3.81 (93)
p value	0.654	0.424	0.355	0.165	**0.046**

Data is expressed as mean±SD.

The association between methylation levels at four CpG sites in the Hs_*HTR2C*_01_PM region and weight change groups (>7% vs <7%) were similar to the *HTR2C* -697G/C region; patients who had a weight increase of >7% had lower mean methylation at all

CpG sites compared to patients who had a weight increase of <7%, when only methylation at the CpG3 site showed a significant difference (p=0.042) (**Table 3.11**, the raw data without outlier cut off is shown in Appendix 1, Table 2). Gender distribution between two weight change groups were not significantly different (χ^2=1.774, p=0.183).

Table 3.11: Methylation levels at 4 CpGs in the Hs_*HTR2C*_01_PM sequences comparing between two subgroups of weight increase in Chinese Han patients

Hs_*HTR2C*_01_PM	CpG1	CpG2	CpG3	CpG4
Weight increase>7% (n)	6.39±2.81 (75)	6.85±2.93 (78)	8.94±3.58 (75)	5.27±1.58 (67)
Weight increase<7% (n)	7.08±2.75 (91)	7.59±2.73 (92)	10.29±3.56 (93)	5.60±1.63 (86)
p value	0.296	0.240	**0.042**	0.458

Data is expressed as mean±SD.

These results suggest that the methylation levels in both regions of the *HTR2C* may be able to be used in prediction of weight increase in first episode schizophrenia patients following antipsychotic drug treatment.

3.3.3.1.2 Polymorphisms and methylation

The influence of the polymorphisms and the extent of DNA methylation of the *HTR2C* were investigated and results show that patients who carried the C/CC genotype of the *HTR2C* -759C/T polymorphism (which had missing genotype data n=3) had lower methylation levels compared to T allele carriers at all CpG sites except the -698 site. Methylation at -670 and -644 sites showed significant differences between the two genotype groups (p=0.010 and p=0.014) as shown in **Table 3.12**, the raw data without outlier cut off is shown in Appendix 1, Table 3. Gender distribution between two genotype groups was not significantly different (χ^2=2.374, p=0.123 for CpG -670 and χ^2=2.761, p=0.097 for CpG -644). This result suggests that at some CpG sites DNA methylation of the *HTR2C* is influenced by the *HTR2C* -759C/T polymorphism and the difference in DNA methylation may be a link between the *HTR2C* -759C/T polymorphism and antipsychotic drug-induced weight gain.

Table 3.12: Methylation levels at 5 CpGs in the *HTR2C* -697G/C promoter sequences comparing between two subgroups of the *HTR2C* -759C/T genotype in Chinese Han patients

***HTR2C* -759C/T**	**CpG1 -698**	**CpG2 -691**	**CpG3 -670**	**CpG4 -661**	**CpG5 -644**
C/CC (n)	20.15±5.06 (114)	20.41±5.45 (141)	19.28±5.57 (141)	21.97±6.32 (140)	18.16±3.78 (136)
T/CT/TT (n)	17.26±2.49 (29)	21.99±6.26 (38)	22.55±7.34 (38)	23.99±6.67 (38)	20.20±3.56 (35)
p value	**0.004**	0.214	**0.010**	0.265	**0.014**

Data is expressed as mean±SD.

The results in the Hs_*HTR2C*_01_PM region showed a similar direction in that the C/CC genotype group had lower methylation than that of the T allele group; however, the difference in methylation between the genotype groups did not reach statistical significance (p>0.05) (**Table 3.13**, the raw data without outlier cut off is shown in Appendix 1, Table 4).

Table 3.13: Methylation levels at 4 CpGs in the Hs_*HTR2C*_01_PM sequences comparing between two subgroups of the *HTR2C* -759C/T genotype in Chinese Han patients

***HTR2C* -759C/T**	**CpG1**	**CpG2**	**CpG3**	**CpG4**
C/CC (n)	6.65±2.70 (131)	7.19±2.89 (134)	9.46±3.64 (131)	5.34±1.56 (118)
T/CT/TT (n)	7.40±2.92 (34)	7.66±2.48 (35)	10.84±3.16 (35)	5.85±1.77 (34)
p value	0.352	0.846	0.148	0.236

Data is expressed as mean±SD.

The *MTHFR* 677C/T and *MTHFR* 1298A/C polymorphisms did not influence the methylation of the *HTR2C* at any CpG sites in either region (p>0.05, data not shown). The *FTO* rs9939609 A/T had an effect on methylation at CpG -698 in that there was a trend of the T allele to be associated with lower methylation at CpG -698 than the AA genotype (19.92±4.81 (n=140) vs 14.39±5.61 (n=3), p=0.052). Gender distribution between two subgroups of genotype was not significantly different (χ^2=0.355, p=0.551).

In addition, the *BDNF* rs6265G/A polymorphism also showed a significant difference in DNA methylation at CpG-698; the AA genotype carriers had higher methylation levels

than the G allele carriers (21.22±5.23 (n=38) vs 19.32±4.70 (n=99), p=0.043. Gender distribution between two subgroups of genotype was not significantly different (χ^2=0.650, p=0.420).

The genotype of the *HTR2A* -1438G/A had a significant influence on DNA methylation of the Hs_*HTR2C*_01_PM region at CpG3; the A allele carriers had lower methylation levels than the GG genotype group (9.44±3.70 (n=124) vs 10.74±3.57 (n=36), p=0.030. Gender distribution between two subgroups of genotype was not significantly different (χ^2=0.029, p=0.865).

3.3.3.2 Spanish cohort

3.3.3.2.1 Methylation and weight gain

Methylation data at each CpG site were tested for normal ditribution and outliers. The methylation levels at CpG-698 were not normally distributed, thus non-parametric tests were used in analyses. Gender, but not age, showed a strong effect on methylation extent at all CpG sites in both regions of the *HTR2C* (p<0.05, data not shown).

The methylation levels were not significantly correlated with baseline weight, baseline BMI, BMI change, weight change, or percentage of weight change at all CpG sites of both regions of the *HTR2C* sequences (p>0.05, data not shown).

The association of methylation levels with weight increase of >7% compared to <7% after antipsychotic drug treatment for 3 months (missing data n=6) showed a similar direction to the Chinese Han cohort in that patients who had a weight increase of >7% had lower mean methylation than patients who had a weight increase of <7% at all CpG sites in both regions of the *HTR2C*, the *HTR2C* -697G/C promoter region (**Table 3.14**, the raw data without outlier cut off is shown in Appendix 2, Table 5) and the Hs_*HTR2C*_01_PM region (**Table 3.15**, the raw data without outlier cut off is shown in Appendix 2, Table 6). However, the differences of methylation levels between the two subgroups of weight increase did not reach statistical significance.

Table 3.14: Methylation levels at 5 CpGs in the *HTR2C* -697G/C promoter sequences comparing between two subgroups of weight increase in Spanish patients

***HTR2C* -697G/C promoter region**	**CpG1 -698**	**CpG2 -691**	**CpG3 -670**	**CpG4 -661**	**CpG5 -644**
Weight increase>7% (n)	18.79±16.90 (43)	15.00±9.31 (43)	16.62±10.55 (42)	16.44±11.37 (41)	15.67±9.15 (43)
Weight increase<7% (n)	20.17±17.54 (23)	17.61±11.38 (23)	16.41±10.28 (22)	20.19±12.77 (21)	18.09±10.04 (23)
p value	0.666	0.620	0.496	0.842	0.538

Data is expressed as mean±SD.

Table 3.15: Methylation levels at 4 CpGs in the Hs_*HTR2C*_01_PM sequences comparing between two subgroups of weight increase in Spanish patients

Hs_*HTR2C*_01_PM	**CpG1**	**CpG2**	**CpG3**	**CpG4**
Weight increase>7% (n)	5.05±2.39 (42)	5.28±1.24 (28)	7.27±3.47 (42)	4.19±1.97 (43)
Weight increase<7% (n)	5.95±2.56 (20)	5.52±1.60 (15)	9.11±4.02 (23)	4.77±2.24 (23)
p value	0.337	0.624	0.330	0.949

Data is expressed as mean±SD.

3.3.3.2.2 Polymorphisms and methylation

The influence of the polymorphisms on the extent of DNA methylation of the *HTR2C* were investigated and results show that the *HTR2C* -759C/T polymorphism did not influence the methylation levels at any CpG sites in either regions of the *HTR2C*, except the methylation at CpG-698; the C/CC genotype carriers had higher methylation levels than that of the T allele carriers (C/CC 21.19±17.35 (n=54) vs T/TC/TT 10.94±12.83 (n=18), p=0.037). There is a genotype effect on the methylation at this position as described before (**section 3.3.3.1.1**).

Patients who carried the T allele of the *FTO* rs9939609 polymorphism (which had genotype missing data n=2) had lower methylation levels compared to the AA genotype group at all CpG sites of the *HTR2C* -697G/C region (**Table 3.16**, the raw data without outlier cut off is shown in Appendix 2, Table 7). Gender distribution between two subgroups of the *FTO* genotype was tested and it was not significantly different at any CpGs (p>0.05, data not shown), except the CpG-670. However, there was only one female patient who carried the AA genotype of the *FTO* rs9939609 at all CpG sites and no female patient who carried the AA genotype at CpG-670; therefore, analysis in male

patients was carried out. The results were in a similar direction to the analysis in the total patient group as shown in **Figure 3.20**. There was a trend of the AA genotype of *FTO* rs9939609 associated with higher DNA methylation levels of the Hs_*HTR2C*_01_PM region at CpG4 in male patients (p=0.060). No significant effect of the *HTR2C* -759C/T and the *FTO* rs9939609 A/T genotype interaction on DNA methylation of the *HTR2C* -697G/C region was found indicating an additive effect of these two polymorphisms.

Table 3.16: Methylation levels at 5 CpGs in the *HTR2C* -697G/C promoter sequences comparing between two subgroups of the *FTO* rs9939609 genotype in Spanish patients.

FTO rs9939609	CpG1 -698	CpG2 -691	CpG3 -670	CpG4 -661	CpG5 -644
AA (n)	30.86±16.35 (14)	21.07±8.67 (14)	19.08±7.64 (13)	20.23±10.76 (13)	19.00±9.43 (14)
AT/TT (n)	16.16±15.78 (56)	14.52±10.11 (56)	15.69±11.29 (55)	17.00±12.08 (53)	15.73±9.67 (56)
p value	**0.004**	**0.001**	**0.009**	**0.020**	0.072

Data is expressed as mean±SD.

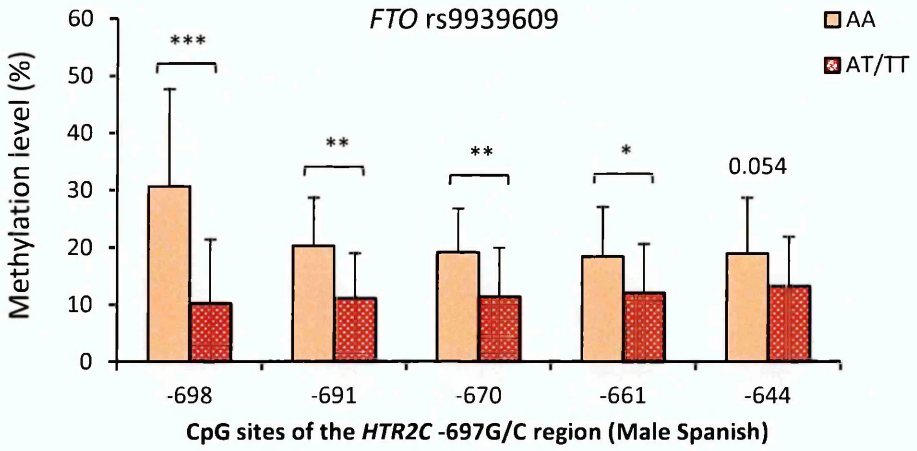


Figure 3.20: DNA methylation levels of the *HTR2C*-697G/C promoter region comparing between two subgroups of the *FTO* rs9939609 genotype.

Data are expressed as mean±SD. * indicates statistical significant difference at p<0.05, ** p<0.01, *** p<0.001.

The *MTHFR* 677C/T and *MTHFR* 1298A/C polymorphisms did not show a significant influence on the methylation of the *HTR2C* at any CpG sites in both regions (p>0.05). However, the CC genotype of the *MTHFR* 677C/T had slightly higher methylation levels than the T allele at all CpG site of the *HTR2C* -697G/C region; whereas, the AA genotype of the *MTHFR* 1298A/C had slightly lower methylation levels than the T allele carriers (**Figure 3.21**).

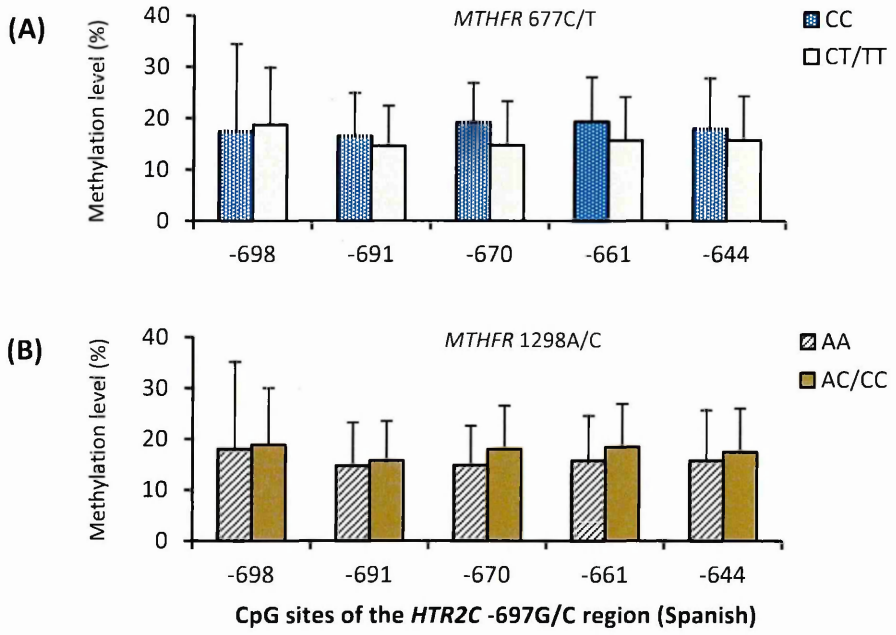


Figure 3.21: DNA methylation levels of the *HTR2C*-697G/C promoter region comparing between two subgroups of genotype of the *MTHFR* 677C/T (A), and the *MTHFR* 1298A/C polymorphism (B).

Data are expressed as mean±SD.

3.4 Discussion

The experiments in this chapter aimed to investigate the association of DNA methylation with antipsychotic drug-induced weight gain and BMI, and the influence of genetic polymorphisms in schizophrenia in both first episode and chronic patients. In chronic schizophrenia patients, DNA methylation of global *LINE-1* repetitive elements and of the specific target gene, the *HTR2C* promoter at and nearby the -697G/C polymorphism and the region nearby TSS, Hs_*HTR2C*_01_PM, were determined using bisulfite pyrosequencing. DNA methylations of the *HTR2C* of both regions were also investigated in first episode schizophrenia patients.

3.4.1 DNA methylation of the *HTR2C* and global DNA methylation in chronic (Belfast) schizophrenia patients - key findings

- *LINE-1* methylation: Gender effect (F<M), no age effect, smoker effect (higher methylation), olanzapine/clozapine (trended lower methylation), no association with BMI, central obesity and metabolic syndrome, *MTHFR* 677C/T (T allele higher methylation) and *FTO* rs9939609 (AA genotype trends higher methylation).
- *HTR2C* (-697G/C sequence) methylation: Gender effect (F>M), methylation increased with age, no association with smoking, drug treatment, BMI, central obesity and metabolic syndrome, *HTR2C* -759C/T SNP (T allele higher methylation), no association with *FTO* and *MTHFR* SNPs.
- *HTR2C* (Hs_*HTR2C*_01_PM) methylation: Gender effect (F>M), methylation increased with age, central obesity (higher methylation), no association with smoking, drugs treatment, BMI, and metabolic syndrome, *FTO* AA trends higher methylation, no *MTHFR*, -759C/T *HTR2C* SNPs effect.

3.4.1.1 Global LINE-1 DNA methylation in chronic (Belfast) patients

The results showed females had lower levels of the global *LINE-1* methylation than males 0.55% on average. This finding is consistent with previous studies in children (Huen *et al.,* 2014; Perng *et al.,* 2013), adults (Wilhelm *et al.,* 2010; El-Maarri *et al.,* 2011; Zhang *et al.,* 2011) and also in cord blood (Burris *et al.,* 2012; Huen *et al.,* 2014). This gender-specific effect on *LINE-1* methylation is likely due to the association of

LINE-1 with X-chromosome inactivation. One recent study measured DNA methylation of 39 *LINE-1*loci on the X-chromosome and 5 loci in autosomes and found the differential methylation in male and female was primarily located in the X chromosome and also found the inactive X chromosome is hypomethylated (Singer *et al.,* 2012).

The lack of the significant correlation between age and *LINE-1* DNA methylation observed in this study confirmed several previous findings that have reported a weak inverse relationship or no association of age with *LINE-1* methylation (Bollati *et al.,* 2009; Jintaridth and Mutirangura, 2010; El-Maarri *et al.,* 2011). The weak or no association may be due to the small sample size and other factors such as life style, smoking, diet, exercise, disease conditions, and antipsychotic drug treatment that may contribute to epigenetic changes.

Smoking was associated with higher levels of *LINE-1* methylation (0.76% on average) in the present study (see a diagram in **Figure 3.22A**) which was in line with some other studies that found smoking is associated with global DNA hypermethylation (Piyathilake *et al.,* 2001; Lin *et al.,* 2007); in contrast to other studies that found hypomethylation (Smith *et al.,* 2007; Hsiung *et al.,* 2007). However, these studies investigated cancer cells or cancer patients that may limit the relevance of these works to the present study. Nicotine is one of the epigenetic modifiers that cause DNA hypermethylation and histone acetylation (Abdolmaleky, *et al.,* 2013). Previous work found that nicotine induced DNA methylation of the fragile histidine triad (*FHIT*) gene and was associated with increased *DNMT3a* expression in human esophageal squamous epithelial cells (Soma *et al.,* 2006). However, nicotine administration in mice caused a decrease in *DNMT1* expression and *GAD67* promoter methylation in GABAergic interneurons which was also associated with an increase in *GAD67* expression in the frontal cortex (Satta *et al.,* 2008). A previous study using blood samples has reported a decrease in global DNA methylation in schizophrenia patients (n=28) while it was increased in healthy controls (n=26) (Bromberg *et al.,* 2008). The differences between this study and the present study are different method to characterize methylation which was based on the restriction by methylation-sensitive or insensitive enzymes; and also the patients were younger (39 years). The present study found a significant association of smoking with *LINE-1* methylation at one of

three CpG sites indicating a weak effect of smoking on global DNA methylation. The influence of smoking on epigenetic modifications may be tissue specific or gene specific and it needs further studies in large sample sizes to elucidate this effect.

The T allele of the *MTHFR* 677C/T polymorphism was associated with higher levels of the methylation of *LINE-1* (CpG3), specifically in female chronic schizophrenia patients (see a diagram in **Figure 3.22A**). The one previous study found the lowest level of *LINE-1* methylation in female patients with the TT genotype. That study differed from the present investigation in the chronic patients in that the 133 patients had a different genotype frequency of the *MTHFR* 677C/T in that CC/CT/TT frequency was 61/30/9% (47/43/10% in the present study). However, further studies in larger sample sizes are required to elucidate the association of the *MTHFR* polymorphism and global DNA methylation.

In addition, this study found an interaction between smoking and the *MTHFR* 677C/T polymorphism on the methylation of *LINE-1* CpG3; smoking *MTHFR* 677T allele carriers had highest *LINE-1* methylation levels. Previous studies in the general population have reported the association of the *MTHFR* 677C/T polymorphism and smoking behavior; the TT genotype had a higher frequency in smokers (Johnson *et al.,* 2001; Linnebank *et al.,* 2012). The interaction of this polymorphism and smoking on plasma homocysteine levels was also observed; plasma homocysteine levels were higher in smokers as well as in the T allele carriers, and the smoking *MTHFR* 677TT individual had the highest plasma homocysteine levels, non-smoking 677CC individuals the lowest (Linnebank *et al.,* 2012). Although the *MTHFR* variant had an association with the decrease of MTHFR enzyme activity and increased plasma homocysteine levels which disrupt DNA methylation (Friso and Choi, 2002), there is a recent study that reported the association of an increment of homocysteine and higher *LINE-1* methylation (Perng *et al.,* 2014). The increased global DNA methylation in smoking *MTHFR* 677T allele in this study may be due to this study investigating chronic schizophrenia patients who were receiving antipsychotic drugs. Responsiveness to the drug treatment, physical activity, and genetic vulnerability may influence DNA methylation. However, this study provides evidence that the *MTHFR* 677C/T polymorphism-smoking interaction plays a role in epigenetic modification that might be implicated in human health and diseases. Further studies are needed to confirm this interaction.

This study did not observe a significant association of the methylation of *LINE-1* with BMI, body weight, waist circumference, waist-hip ratio, triglyceride, LDL, HDL, cholesterol, and plasma leptin. Lack of significant association of *LINE-1* methylation and BMI is in line with a recent study in postmenopausal overweight woman which found no significant difference in *LINE-1* methylation levels in any intervention group (independent and combined effects of a reduced-calorie weight-loss diet, and exercise program for 1 year) versus control and also found no association of *LINE-1* methylation and weight loss at 12 months (Duggan *et al.,* 2014). Other studies reported the association of weight gain and an increased *LINE-1* methylation (Martin-Nunez *et al.,* 2014; Perng *et al.,* 2014). A study in school-age children reported the association of adiposity development and lower *LINE-1* methylation in boys (Perng *et al.,* 2013). Another study in visceral adipose tissue of the severely obese found lower *LINE-1* methylation was negatively associated with fasting glucose, diastolic pressure, and metabolic status (Turcot *et al.,* 2012). Therefore, the association of *LINE-1* methylation and body weight is inconsistent which may be due to the methylation of *LINE-1* may be influenced by gender, age, diet, tissue-specific, and other factors. Further studies are required to confirm this association.

3.4.1.2 DNA methylation of the HTR2C promoter regions in chronic (Belfast) patients

Similar to findings in first episode patients, gender had significant effect on the levels of DNA methylation of the *HTR2C* in both regions in chronic patients in which females had higher methylation levels at all CpG sites than males, this is probably due to the X-chromosome inactivation.

Age was positively associated with DNA methylation of the *HTR2C* in both regions in the chronic (Belfast) study suggesting the influence of age on gene-specific DNA methylation. Increased methylation of the *HTR2C* promoter with age may result in decreased 5-HT2C receptor expression and modulate the control of food intake. The influence of aging on DNA methylation has been reported (Bell *et al.,* 2012). A recent study has reported that CpG islands tended to increase methylation while non-CpG islands lose methylation with increasing age suggesting that the age-related changes in DNA methylation are not homogeneous across a human genome (Christensen *et al.,*

2009). An example of age-related hypermethylation is the increased DNA methylation of *Arc* (activity-regulated cytoskeletal-associated protein) promoter region in the hippocampus which was accompanied by decreased *Arc* transcripts in aged rats compared with adult rats (Penner *et al.,* 2011). There has been a study in postmortem human brain samples that reported age effects on the *HTR2C* expression and/or histone H3 acetylation at lysine 9/14 in schizophrenia patients (Tang *et al.*, 2011). There have been several studies reporting that treatment with histone deacetylase (HDAC) inhibitor as well as DNMT inhibitors can improve the expression of several genes for schizophrenia in mouse brain.

Although the *FTO* rs9939609 polymorphism showed significantly associated with BMI in this chronic Belfast cohort, this polymorphism did not show a significant effect on DNA methylation of the *HTR2C* promoter region. However, the AA genotype which was significantly associated with higher BMI had slightly higher methylation levels of the *HTR2C* in both regions. The AA genotype of the *FTO* polymorphism associated with higher *HTR2C* methylation may decrease 5-HT2C receptor expression to reduce 5-HT neurotransmitter function resulting in increased food intake and obesity. Lack of statistical significance may be due to small sample size, thus further studies in larger sample size are needed to confirm this finding.

The T allele of the *HTR2C* -759C/T polymorphism was significantly associated with higher DNA methylation of the *HTR2C* promoter region (-697G/C region) in which the CpG sites are located near the polymorphism (**Figure 3.22**). This finding indicates the polymorphism may have a strong influence on nearby cytosine methylation rather than the long distance region (Hs_*HTR2C*_01_PM region) or other polymorphisms from other genes such as the *MTHFR* and *FTO* polymorphisms.

However, interestingly, the leptin -2548G/A polymorphism showed significant associations with the methylation of the *HTR2C* in both regions (CpG1 and CpG4 of the Hs_*HTR2C*_01_PM, and CpG-698 of the *HTR2C* -697G/C promoter regions). Finding that carriers with the GG genotype had significantly higher levels of methylation than the A allele carriers (**Figure 3.22**) indicating the influence of the leptin polymorphism on DNA methylation of the *HTR2C* that might further affect 5-HT2C receptor expression. In this cohort of chronic patients, the GG genotype of leptin -2548G/A

polymorphism had slightly higher plasma leptin compared to AG/AA genotype (not significant). However, the role of 5HT-leptin interaction in controlling food intake is still unclear. The 5-HT2C receptors have been reported to be involved in leptin-induced anorexia (von Meyenburg *et al.*, 2003b; Yamada *et al.,* 2003). Other studies reported that leptin did not have a direct influence on 5-HT neurons and did not modulate appetite via 5-HT neuron function (Lam *et al.,* 2011). It also has been suggested that leptin and 5-HT have separate pathways in the control of food intake and suggested that the effects of leptin are long lasting (tonic) whereas 5-HT is involved in short acting (episodic) satiety signals (Weigle *et al.,* 1995).

The present study did not find a significant association of the leptin -2458G/A polymorphism and plasma leptin as well as BMI in chronic schizophrenia patients. However, the GG genotype had slightly higher plasma leptin and this may affect 5-HT2C receptor expression by increased methylation of the *HTR2C* promoter region. No link between leptin polymorphism and methylation of the *HTR2C* was investigated. A study in 39 non-obese female subjects has reported that the AA genotype of the leptin -2548G/A polymorphism was associated with increased plasma leptin and increased adipose tissue leptin mRNA and leptin secretion than G allele subjects (Hoffstedt *et al.,* 2002). However, meta-analysis did not find any difference of the leptin mRNA expression between the leptin -2548G/A genotypes among different ethnicities (He *et al.,* 2013). Templeman *et al.* (2005) has reported a trend of A allele of the -2548G/A polymorphism and higher plasma leptin in first episode schizophrenia patients. The variability of leptin among leptin genotypes in these studies and the present study may be due to differences in subjects that gender, age, obese or non-obese, ethnicity, first-episode or chronic schizophrenia patients, and the antipsychotic drug treatment may affect plasma leptin. In addition, the small sample size may confound the findings. Therefore, further studies are needed to elucidate the association of the leptin polymorphism or plasma leptin and DNA methylation of the *HTR2C* or 5-HT2C receptor expression.

The same direction with global *LINE-1* methylation, antipsychotic drugs (olanzapine or clozapine) treatment decreased methylation of the *HTR2C* promoter sequences was also observed (**Figure 3.22B, and C**) indicating that not only the global DNA methylation but also the specific *HTR2C* promoter was modified by antipsychotic

drugs. Whereas smoking was more likely to increase global DNA methylation, it decreased methylation levels of the *HTR2C* promoter sequences suggesting the gene-specific influence of smoking on DNA methylation.

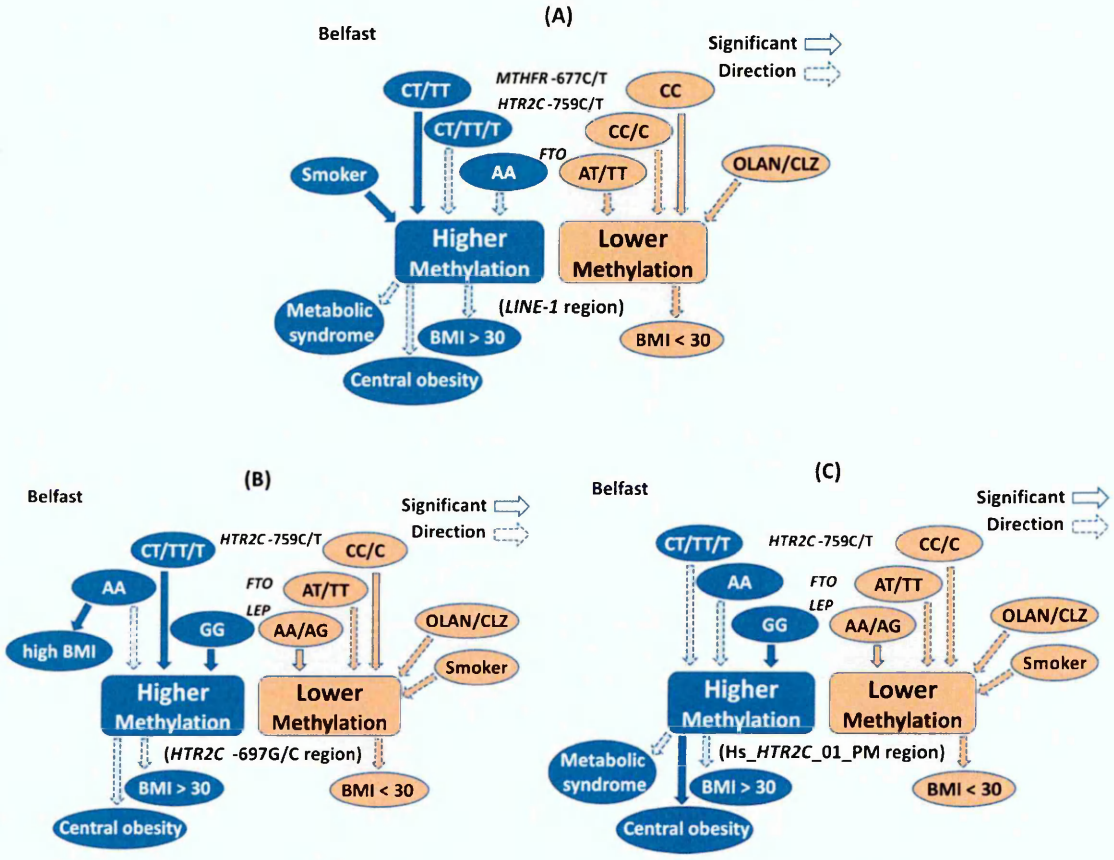


Figure 3.22: Diagrams summarize the associations of DNA methylation of the *LINE-1* methylation (A), the *HTR2C* -697G/C (B), and the Hs_*HTR2C*_01_PM regions (C), with polymorphisms, smoking, antipsychotic drugs, and obesity variables in chronic cohort.

3.4.2 DNA methylation of the *HTR2C* in first episode drug naïve schizophrenia patients - key findings

Chinese Han cohort:

- *HTR2C* (-697G/C sequence) methylation: Gender effect (F>M), no age effect, no association with baseline BMI and change in BMI, *HTR2C* -759C/T SNP (T allele higher methylation), *HTR2C* SNP effect on CpG-698 site, *FTO* rs9939609 T allele (trends lower methylation at -698 site), no *MTHFR* SNPs effect
- *HTR2C* (Hs_*HTR2C*_01_PM) methylation: Gender effect (F>M), no age effect, no association with baseline BMI and change in BMI, no effects of *HTR2C* -759C/T, *FTO* and *MTHFR* SNPs

Spanish cohort:

- *HTR2C* (-697G/C sequence) methylation: Gender effect (F>M), no age effect, no association with baseline BMI and change in BMI, *HTR2C* SNP effect on CpG-698 site, *FTO* rs9939609 (T allele lower methylation, in male), no *MTHFR* SNPs effect
- *HTR2C* (Hs_*HTR2C*_01_PM) methylation: Gender effect (F>M), no age effect, no association with baseline BMI and change in BMI, *FTO* rs9939609 (T allele trends lower methylation, in male), no effects of *HTR2C* -759C/T and *MTHFR* SNPs

The results of this section revealed that the methylation levels of the *HTR2C* -697G/C region and the Hs_*HTR2C*_01_PM region in peripheral blood at baseline were negatively associated with body weight change groups (>7% vs <7%) following antipsychotic drug treatment in first episode schizophrenia patients. In addition, the lower methylation levels of both regions were found in the CC/C genotype of the *HTR2C* -759C/T polymorphism compared to T allele carriers. The results were predominantly seen in the Chinese Han cohort; however, a similar directionwas found in the Spanish cohort indicating replication of the findings (**Figure 3.23**). The statistical significant differences that were found at some CpG sites in Chinese cohort and the lack of significant difference in Spanish cohort may be due to the small sample size or the weak association of methylation levels on weight gain as well as genotype effect

on methylation levels. Further studies in larger sample size are required to confirm these findings.

This is the first study investigating the association of the DNA methylation of the *HTR2C* promoter sequences and antipsychotic drug-induced weight gain. The association between the extent of DNA methylation of the *HTR2C* promoter regions and the weight increase after receiving antipsychotics in both Chinese Han and Spanish cohorts as summarized diagram in **Figure 3.23**, suggests that the epigenetic changes in the *HTR2C* may be used to predict the extent of weight gain following antipsychotic drug treatment in first episode, drug naïve schizophrenia patients.

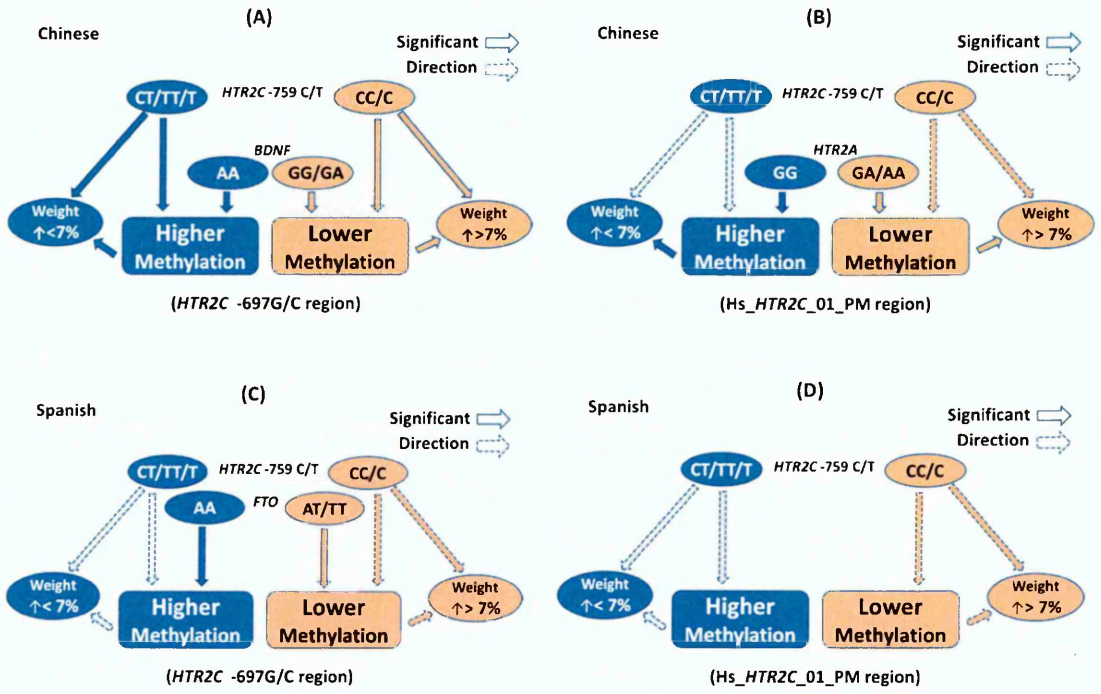


Figure 3.23: Diagrams summarize the association of the DNA methylation of the *HTR2C* -697G/C and Hs_*HTR2C*_01_PM regions and polymorphisms in Chinese (A, B) and Spanish (C, D) cohorts.

The effect of gender on the methylation of the *HTR2C* in this study may be due to the contribution from the X-chromosome inactivation because it is located on the X chromosome and one of the X chromosomes in females is subject to inactivation resulting in CpG sites exhibiting highly significant difference between gender with hemi-methylated patterns in female (Bell *et al.*, 2011). The selection of the X chromosome to be inactivated is random in somatic cells (Goto and Monk, 1998). The

X-chromosome inactivation is accompanied by changing in DNA methylation particularly in CpG islands which the majority of CpG islands show increased methylation on the inactive X chromosome which silences gene expression; however, about 7% of CpG islands show decreased methylation levels (Sharp *et al.,* 2011) and about 5% of X-linked genes have been reported to have increased expression in females compared to male (Johnston *et al.,* 2008). There are some X-linked genes escaping X-chromosome inactivation to some degree and they are expressed from both X chromosomes (Carrel and Willard, 2005). A report shows many CpG sites located at upstream of the *HTR2C* transcription start site (TSS) had similar patterns of DNA methylation in both male and female thus it was suggested that the *HTR2C* gene escapes from X-chromosome inactivation in human (Hernando-Herraez *et al.,* 2013); however, the authors did not provide specific CpG sites or regions. In addition, a female-specific increased expression of the *HTR2C* gene was not observed in the published RNAseq data (Brawand *et al.,* 2011). It has been also suggested that many genes escaping X-chromosome inactivation show no clear gender differences in expression levels (Johnston *et al.,* 2008). Therefore, it is clear that the tested CpG sites of the *HTR2C* in this study are subject to X-chromosome inactivation. The contribution of X-chromosome inactivation of the *HTR2C* in various degrees in individuals may be the causes of the high variability of measured DNA methylation in this study.

Comparing between two regions, the DNA methylation levels of the *HTR2C* -697G/C region were always higher than those of the Hs_*HTR2C*_01_PM region which are located around the TSS of the gene. This result supported previous findings that there are differences in DNA methylation distribution across the genome in which CpG sites located around TSS are not methylated while CpG sites that are located in the gene body, intergenic, and distant to TSS, around 1kb-1.5kb, are highly methylated (Bell *et al.,* 2011; Hernando-Herraez *et al.,* 2013). Methylation of CpG sites of the TSS is associated with long-term silencing such as X-chromosome inactivation, imprinting, and with some tissue specific genes (Jones, 2012). Thus, lower methylation of CpGs at TSS of the gene allows transcription initiation.

The influence of the *HTR2C* -759C/T polymorphism on DNA methylation of the *HTR2C* promoter region provides an additional further mechanism underlying the previous finding by Hill and Reynolds which reported the reduction of promoter activity in the

presence of the *HTR2C* -759T or -697C allele (Hill and Reynolds, 2007)(**Figure 3.24**). The T allele of the *HTR2C* -759C/T polymorphism that was the less active allele was more likely to be methylated than the active C allele. This finding indicates the influence of the *HTR2C* -759C/T polymorphism on the nearby DNA methylation of the promoter region of the *HTR2C* gene that may be a link between the polymorphism and promoter activity, and also antipsychotic drug-induced weight gain. Further study requires investigating the influence of the *HTR2C* -759C/T polymorphism and/or the methylation of this promoter region on 5-HT2C receptor expression.

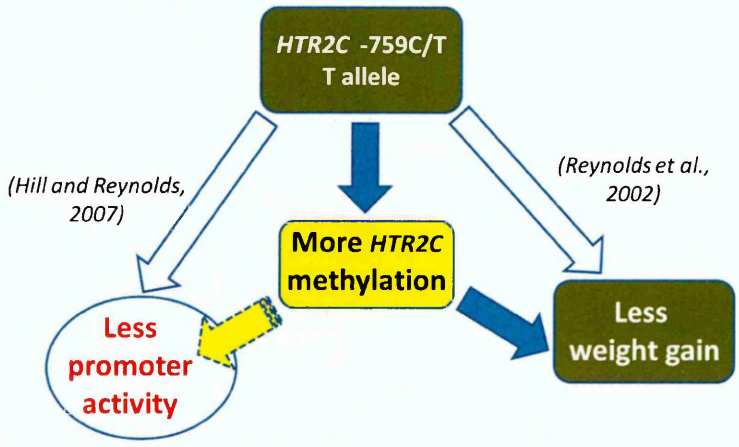


Figure 3.24: Diagram summarizes the association of the DNA methylation of the *HTR2C* -697G/C region and *HTR2C* -759C/T polymorphism.

The *BDNF* rs6265 G/A polymorphism showed significant association with DNA methylation levels at only the CpG-698 SNP site, but a similar pattern at other CpG sites of the *HTR2C* -697G/C promoter region was also observed in the Chinese Han cohort; patients with the AA genotype had higher methylation levels than the G allele carriers. The link between the polymorphism of the *BDNF* and this specific CpG methylation of the *HTR2C* promoter is unknown. The variant methionine (A allele) was associated with decreased BDNF synthesis and secretion (Chen *et al.,* 2004). BDNF influences the serotonergic system and is associated with schizophrenia and eating disorders (Gratacos *et al.,* 2007; Lyons *et al.,* 1999). Brain infusion of BDNF in rats resulted in appetite suppression, weight loss and increased 5-HT (Pelleymounter *et al.,* 1995). The AA genotype which had slightly higher change in BMI (see chapter 2) showed higher DNA methylation of the *HTR2C* promoter compared to G allele. This

result may provide a link between the reduction of BDNF of the AA genotype and the seroternergic dysfunction; high methylation may decrease 5-HT2C receptor expression and result in weight gain (**Figure 3.25**). However, further studies are required to address the role of BDNF on the expression and DNA methylation of the *HTR2C* gene.

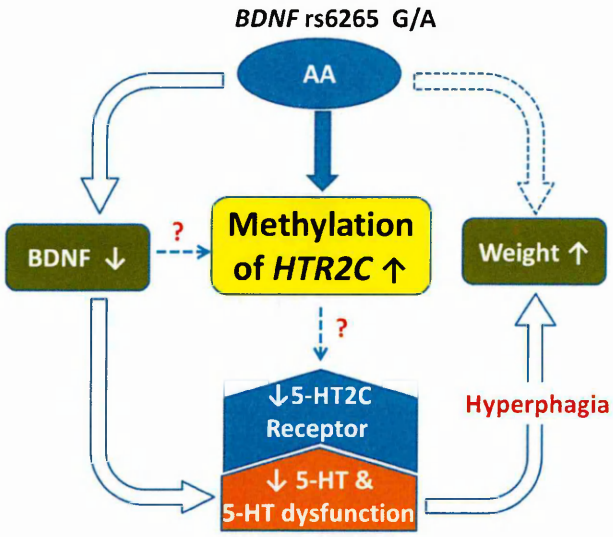


Figure 3.25: Diagram summarizes the association of the DNA methylation of the *HTR2C* -697G/C region and *BDNF* rs6265 G/A polymorphism.

The *HTR2A* polymorphism at CpG3 of the Hs_*HTR2C*_01_PM region was significantly associated with weight gain in the Chinese Han cohort and similar patterns were also found at other CpGs in this region. The link between the *HTR2A* and the methylation of the *HTR2C* is unknown.

In the Spanish cohort, the influence of the *HTR2C* -759C/T polymorphism and DNA methylation of the *HTR2C* show the same direction but did not reach statistical significant levels. However, patients with the AA genotype of the *FTO* rs9939609 A/T showed significantly higher levels of *HTR2C* DNA methylation than the T allele carriers, particularly in males. The A allele of this polymorphism has been reported to be associated with increased BMI (Frayling *et al.,* 2007) and also increased *FTO* transcripts (Berulava and Horsthemke, 2010), although no significant association between the SNP and change in BMI was observed in first episode Chinese and Spanish cohorts. However, in the Spanish cohort, the BMI at baseline of patients who carried the AA genotype of the *FTO* rs9939609 had higher than those of T allele carriers but did not

reach statistically significant differences (23.50±3.41 kg/m^2, n=14 vs 21.58±3.71 kg/m^2, n=56, p= 0.172 correction for gender). The influence of the *FTO* polymorphism on the *HTR2C* promoter methylation may explain the obesity associated with *FTO* rs9939609 (**Figure 3.26**) that the AA genotype of the *FTO* polymorphism caused increase methylation of the *HTR2C* promoter region and may result in decreased 5-HT receptor expression and serotonergic neurotransmitter system and that affects food intake and satiety. However, the exact mechanism is still unknown, as the FTO play a role in DNA/RNA demethylation. FTO may act on other components such as RNA or other genes.

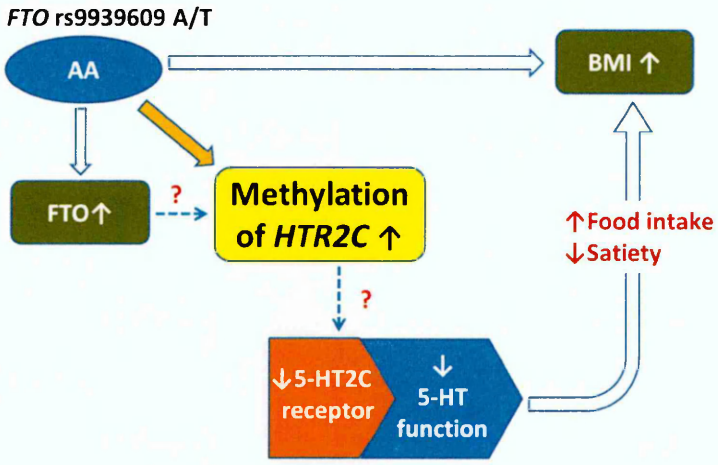


Figure 3.26: Diagram summarizes the association of the DNA methylation of the *HTR2C* -697G/C region and *FTO* rs9939609 A/T polymorphism.

3.4.3 Comparison between chronic and first episode schizophrenia patients

Some findings were observed in both chronic and first episode patients including; 1) there was strong gender effect on DNA methylation of the *HTR2C* sequences between males and females; 2) DNA methylation of the *HTR2C* sequences was not associated with BMI or BMI changes; 3) *HTR2C* -759C/T polymorphism was associated with methylation of *HTR2C* promoter sequence; 4) *MTHFR* SNPs did not have an effect on methylation of *HTR2C* in both chronic and first episode patients.

The different findings between chronic and first episode patients and different findings between global *LINE-1* methylation and specific *HTR2C* methylation including; 1) there

was an age effect on DNA methylation of the *HTR2C* sequences in chronic but not first episode patients; 2) smoking and antipsychotic drugs influence global *LINE-1* methylation but not *HTR2C* methylation in chronic patients; 3) *FTO* and *MTHFR* SNPs did not influence methylation of *HTR2C* gene but there were effects on global methylation in chronic patients; 4) *FTO* SNP did not influence methylation of *HTR2C* in chronic patients but it has strong effect in first episode Spanish male patients.

3.4.4 Limitations in this study

In first episode schizophrenia patients, DNA methylation was determined at baseline but not after antipsychotic drug treatment. Therefore, comparison of the methylation changes between before and after antipsychotic drug treatment as well as the association of DNA methylation and weight gain cannot be investigated.

The small sample size leads to lack of statistical power in determining small effect of polymorphisms on DNA methylation, or DNA methylation on BMI gain or BMI.

DNA samples from peripheral blood of schizophrenia patients were used to determine DNA methylation of the *HTR2C* and global *LINE-1* methylation may be limitation in the present study to reflect the alteration of DNA methylation in brain. Because of the extent of global DNA methylation differs between tissues (Lokk *et al.,* 2014) and the antipsychotic drugs or the environmental factors may affect DNA methylation in a tissue-specific manner (Shimabukuro *et al.,* 2006). Although, there have recently been several studies reported that the DNA methylation changes in peripheral blood or saliva were well correlated with that of different brain regions and could serve as markers for disease diagnostic and/or therapeutic biomarkers for schizophrenia (Horvath *et al.,* 2012; Abdolmaleky *et al.,* 2014; Ota *et al.,* 2014; Mill and Petronis, 2007), further studies are required to compare DNA methylation of the *HTR2C* gene in peripheral blood and brain in animal models.

3.4.5 Conclusions

In this study, the association of the DNA methylation of the *HTR2C* promoter regions at baseline with increased weight groups (<7% vs >7%) was observed suggesting the DNA methylation status of the *HTR2C* promoter may account for the weight gain in first episode schizophrenia patients after receiving antipsychotic drugs. The *HTR2C* -759C/T

and the *FTO* rs9939609 associated with the extent of DNA methylation of the *HTR2C* promoter sequences indicating that the genetic-epigenetic interaction may influence the *HTR2C* -759C/T or the *FTO* rs9939609 polymorphism-associated weight changes in schizophrenia patients. In other words, the polymorphisms of the *HTR2C* and *FTO* may affect the body weight regulation through epigenetic modification. The findings give a novel insight into the mechanistic link between the genetic polymorphism effects and weight gain.

In chronic schizophrenia patients, the significant association of the *HTR2C* -759C/T polymorphism with the extent of methylation of the *HTR2C* promoter at CpG sites located near the SNP suggests that the effect of the polymorphism on epigenetic changes may strongly influence at nearby CpG sites rather than at distant CpG sites. The leptin -2458G/A polymorphism was associated with the methylation levels of the *HTR2C* at CpG sites near the transcription start site, although lack of the link between leptin and *HTR2C* methylation, these results indicate the interaction between leptin genotype and the *HTR2C* methylation that might be involved in the control of satiety.

Data in this chronic patient group also showed that smoking was significantly associated with higher global *LINE-1* methylation while olanzapine/clozapine treatment tended to have lower methylation. These results indicate that environmental factors impact on global DNA methylation. Furthermore, the association between the *MTHFR* 677C/T polymorphism and global *LINE-1* methylation suggests a genetic-epigenetic interaction and that the influence of the *MTHFR* polymorphism on global DNA methylation may be related to obesity in schizophrenia. The significant interaction between smoking and the *MTHFR* 677C/T polymorphism on global DNA methylation also indicates a genetic-environment interaction that may play an important role in body weight regulation in schizophrenia patients.

Therefore, the DNA methylation of the *HTR2C* promoter region may be used as an epigenetic marker for antipsychotic drug-induced weight gain. The genetic-epigenetic interactions provide more understanding of the molecular mechanism underlying antipsychotic drug-induced weight gain. However, further studies in a larger sample size are required to obtain confirmation of these findings.

Chapter 4: Effect of antipsychotic drugs on DNA methylation and expression of the HTR2C gene in SH-SY5Y cells

4.1 Introduction

This chapter describes experiments that aim to determine the effect of antipsychotic drugs on DNA methylation of the *HTR2C* promoter and 5-HT2C receptor mRNA expression. Two antipsychotics, clozapine and haloperidol, were chosen according to their affinity for the 5-HT2C receptor for these cell culture experiments. Clozapine has a high affinity to the 5-HT2C receptor with an equilibrium dissociation constant (Kd) = 4.8 nM whereas haloperidol has a markedly low affinity to the 5-HT2C receptor with Kd = 4,700 nM in human brain (Richelson and Souder, 2000). A few studies have reported a reduction of 5-HT2C receptor binding in rat brain (Kuoppamaki *et al.,* 1993, 1995), but no alteration in *HTR2C* mRNA expression after chronic clozapine and haloperidol treatment (Burnet *et al.,* 1996). However, some studies found clozapine and haloperidol treatment causes changes in *HTR2C* mRNA expression in specific regions of rat brain (Buckland *et al.,* 1997; Huang *et al.,* 2007). There have been no previous studies on the effect of antipsychotic drugs on *HTR2C* mRNA expression as well as on *HTR2C* DNA methylation in cell culture. As mentioned in the previous chapter, the -759C/T polymorphism of the *HTR2C* gene is the most consistently identified SNP associated with antipsychotic drug-induced weight gain (**see section 2.1**), however the nearby -697G/C SNP has also been implicated. Due to the repetitive nucleotides and high GC density of the sequence at and nearby the -759C/T it proved impossible to design suitable primers to amplify this sequence. Therefore, the effects of antipsychotic drug treatment on DNA methylation levels around the nearest SNP (-697G/C) were investigated.

In the present study 5-Aza-2-deoxycytidine, a demethylating agent inhibiting DNA methyltransferase, was also used in cell culture treatment to serve as a positive control for DNA methylation inhibition. These experiments use the SH-SY5Y neuronal

cell line and PCR techniques to investigate the effects of these drugs on receptor gene expression and specific promoter sequence DNA methylation around the -697G/C polymorphism of the *HTR2C* gene.

4.1.1 SH-SY5Y human neuroblastoma cell

SH-SY5Y human neuroblastoma cells (ATCC® CRL-2266™) are a neuronal-like cell line originally established from a bone marrow biopsy of a woman patient with sympathetic adrenergic ganglial neuroblastoma and deposited with the American Type Culture Collection (ATCC) by June L. Biedler in the early 1970's (Biedler *et al.*, 1973). SH-SY5Y cells are a sub-line of the parental line SK-N-SH which were subcloned three times (SK-N-SH to SH-SY, SH-SY to SH-SY5, and SH-SY5 to SH-SY5Y). SH-SY5Y cell culture properties are mixed type of adherent and floating cells and both of them are viable. The cells grow as clusters or clumps and pile on top of each other. For this reason, they slough off into the media. The cells attach better when they are more dilute; therefore, to facilitate cell attachment, subculturing at a high subculture ratio (1:20 to 1:50) for 100% confluence should be done. The SH-SY5Y cell line has been extensively used in various experimental neurological studies; for example: neurodegenerative and neuroadaptive processes, neurotoxicity, and neuroprotection (Xie *et al.*, 2010).

The SH-SY5Y cell line exhibits neuronal marker enzyme activity such as tyrosine hydroxylase and dopamine-β-hydroxylase, specific uptake for norepinephrine, as well as expressing neurofilament proteins, and opioid, muscarinic and nerve growth factor receptors (Ciccarone *et al.,* 1989). The SH-SY5Y cell line constitutively expresses *HTR2C* mRNA (Biedler *et al.,* 1978; Cavarec *et al.,* 2013). It also expresses *HTR1A*, *HTR1B* and *HTR2A* in addition to dopamine D1 and D2 receptors (Rohm *et al.,* 2013). Furthermore, SH-SY5Y cells release dopamine, norepinephrine and serotonin (Shaul *et al.,* 2003; Rohm *et al.,* 2013).

Due to its capability of proliferating in culture for long periods without contamination this SH-SY5Y cell line is suitable for *in vitro* study. In addition, SH-SY5Y cells exhibit stem cell properties because they are derived from immature neoplastic neural crest cells, and can be induced to differentiate to more mature neuron-like cells upon treatment with a variety of agents such as retinoic acid (Singh and Kaur, 2007). Other agents used to induce differentiation are phorbol ester 12-O-tetradecanoylphorbol-13-

acetate (TPA)(Pahlman *et al.,* 1981), brain-derived neurotrophic factor (BDNF) (Cernaianu *et al.,* 2008), dibutyryl cyclic AMP (dBcAMP)(Kume *et al.,* 2008), purine (Guarnieri *et al.,* 2009), or staurosporine (Mollereau *et al.,* 2007). However, both differentiated and undifferentiated SH-SY5Y cells have been widely used in neuroscience research.

4.1.2 Differentiation of SH-SY5Y human neuroblastoma cell by retinoic acid

Upon differentiation, cells become a functionally mature neuronal phenotype showing extensive outgrowth of neurites (Cheung *et al.,* 2009). Cells stop proliferating and become a stable population and have biochemical, ultrastructural, morphological, and electrophysiological similarity to neurons when differentiated. They express various neuronal-specific markers; for example: growth-associated protein (GAP-43), noradrenaline (NA), neuropeptides, neuronal nuclei (NeuN), receptors for neurotrophic factors, neuron-specific enolase (NSE), neurosecretory granula, vesicle proteins such as synaptophysin, and neuronal-specific cytoskeletal proteins such as microtubule associated protein (MAP), Tau, and neurofilament proteins (Cheung *et al.,* 2009; Fagerstrom *et al.,* 1996). GAP-43, MAO, NeuN, and synaptophysin are the classical markers of mature neurons.

Different agents used in differentiation induction can bring about various phenotypes of SH-SY5Y cells, including cholinergic, adrenergic, or dopaminergic phenotypes. For example, treatment with phorbol esters induces differentiation to an adrenergic neuronal phenotype, whereas retinoic acid treatment results in cholinergic (Pahlman *et al.,* 1984) and dopaminergic phenotypes (Korecka *et al.,* 2013).

Retinoic acid (RA) is the most common method used for inducing differentiation of SH-SY5Y cells (Kovalevich and Langford, 2013). The effects of RA on SH-SY5Y cells are well described; RA exerts its effect by acting at two classes of non-steroid nuclear hormone receptors, including the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs). Although RA can bind only to the RAR receptors, activated RAR is able to heterodimerize with RXR and then RAR/RXR heterodimers can bind to the RA response element (RARE) giving rise to transcriptional activation (Joshi *et al.,* 2006).

4.1.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is the most powerful laboratory technique used in molecular biology to amplify a huge number of copies of a specific segment of DNA. The key components to enable selective and repeated amplification are the primers and DNA polymerase. Primers are oligonucleotides or short DNA fragments containing sequences complementary to the target region. DNA primers are required for initiation of DNA synthesis at a specific region. DNA polymerase used in PCR is a heat-stable DNA polymerase, such as Taq polymerase which is an enzyme originally isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from the nucleotides by using single stranded DNA as a template.

The PCR is performed in a thermal cycler and relies on thermal cycling which consists of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of DNA. There are three major steps in a PCR cycle (**Figure 4.1**), which are repeated for 25-50 cycles.

- **Denaturation or DNA melting at 94°C**

  During this denaturation step, the double stranded DNA template is heated to 94-95°C for separating to single-stranded DNA, the hydrogen bonds which hold the two strands together are broken. In the first step, this may also serve to activate the DNA polymerase.

- **Annealing or connecting at annealing temperature**

  After separating the double stranded DNA, each single-stranded is then used as the template in DNA synthesis. The temperature is decreased to the annealing temperature, around 54-60°C so primers can bind to a specific point on the single-stranded DNA template. The DNA polymerase can then attach and start copying the DNA template. The annealing temperature (Ta) depends on the primers used in the PCR reaction, usually 5°C lower than the melting point of the primers.

- **Extension at 72°C**

  This temperature is the ideal working temperature for the DNA polymerase. In this final step, the DNA polymerase enzyme synthesizes the complementary

strand of DNA. In other words, the bases that are complementary to the DNA template are coupled to the primer on the 3' side.

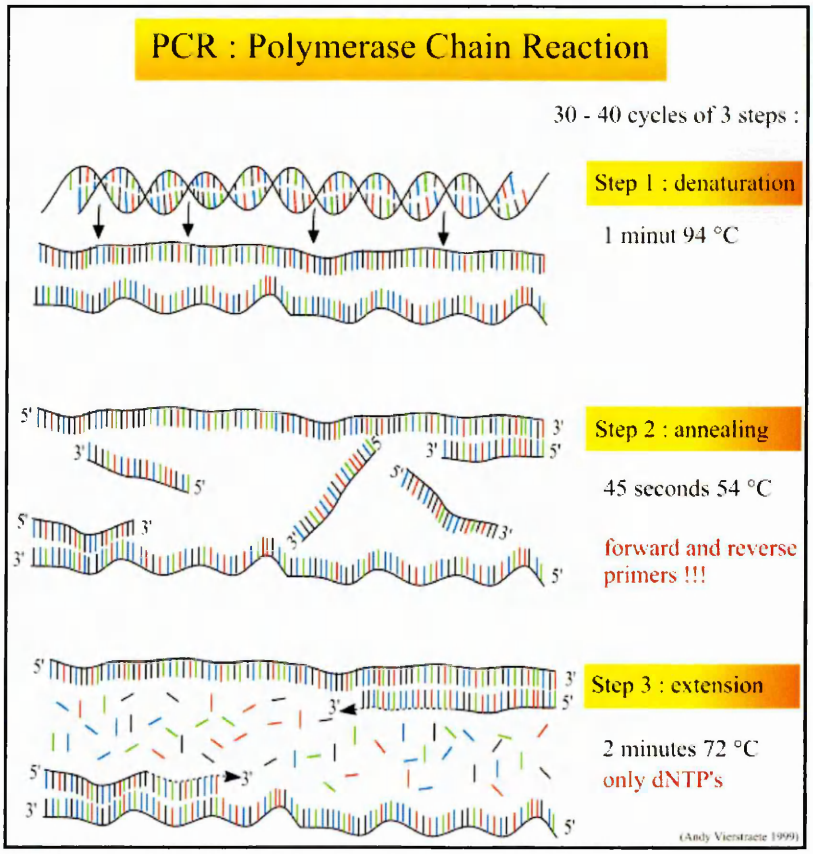


Figure 4.1: Cycling reaction of PCR

(http://users.ugent.be/~avierstr/principles/pcr.html)

As PCR progresses, the amplified DNA products act as a template for amplification in the next cycle. Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene.

At the end of PCR, the PCR products are checked by gel electrophoresis to see whether there is product amplified and whether it is the expected size.

4.1.4 Real-time PCR

Real-Time PCR is a valuable technique used extensively in biological research nowadays in order to quantify nucleic acid amplification and detection. In real-time PCR, the accumulation of amplified PCR product is detected and measured as the

reaction progresses. The detection of PCR product is made by including fluorescent molecules in the PCR reaction. The fluorescence used includes DNA-binding dyes and fluorescently labelled sequence specific primers or probes. In addition, the fluorescent dye, the components of real-time PCR are similar to traditional PCR. The measured fluorescence reflects the amount of amplified product in each cycle.

During the progression of the real-time PCR assay, the fluorescence which is proportional to the amount of amplified product is detected and can be used to generate an amplification plot (**Figure 4.2**). In this plot, the PCR cycle number is shown on the x-axis and the fluorescence is shown on the y-axis. The plot shows two phases, an exponential phase followed by a plateau phase. In the first phase, the amount of PCR product approximately doubles in each cycle. However, the reaction components are consumed as the reaction proceeds, and ultimately one or more of the components becomes limiting. So the reaction slows and enters the plateau phase.

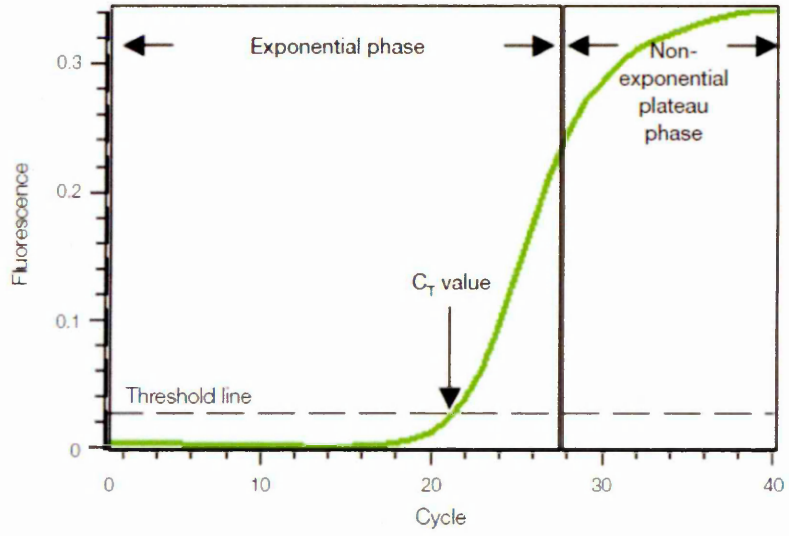


Figure 4.2: Amplification plot from RT-qPCR
(http://www.gene-quantification.de/real-time-pcr-guide-bio-rad)

Initially, an increase in fluorescence cannot be detected, so it remains at the background levels (cycle 1-18) even though the PCR products are accumulated exponentially. Eventually, the amplified PCR products are accumulated enough to yield a detectable fluorescent signal. The cycle number at which the amplified products are detectable is called the threshold cycle, or C_t (**Figure 4.2**). This C_t value is measured

in the exponential phase in which the reagents are not limited. The C_t value is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of DNA template is present at the start of the reaction, the reaction will have an early or low C_t value, meaning that relatively few PCR cycles are required to accumulate enough PCR products to give a fluorescent signal above background. In contrast, if a small amount of DNA template is present at the start of the PCR reaction this will result in late or high C_t value, meaning that more PCR cycles are required for the fluorescent signal to rise above background. This relationship is the basis for the quantitative aspect of real-time PCR.

Real-time PCR results can either be qualitative (the presence or absence of a sequence) or quantitative (number of copies of DNA) which is also known as qPCR. In addition, real-time PCR data can be evaluated without gel electrophoresis, therefore, the experimental time is reduced.

4.1.5 Quantitative Reverse Transcription PCR (RT-qPCR)

Quantitative reverse transcription PCR (RT-qPCR) is the combination of techniques to determine mRNA expression level. It is a combination of reverse transcription PCR and real-time PCR. The principle of RT-PCR is that the starting material is mRNA that is reverse transcribed into complementary DNA (cDNA) by reverse transcriptase which is then used as a template for amplification using PCR.

The mRNA expression level of the gene of interest can be determined by two methods; standard curve and relative methods. The standard curve (or absolute quantification) method is used to determine the absolute target quantity of transcripts in samples. The amplification of samples and a standard dilution series containing a range of known quantities of transcripts are measured. Data from the standard dilution series are used to generate a standard curve, then the absolute quantity of target in the samples are obtained.

The relative quantification method uses the house-keeping gene transcripts which act as reference transcripts used to normalize the target transcripts of samples. The best reference genes should be expressed at a constant level between the cells of different tissues and under different experimental conditions (Thellin *et al.,* 1999). Selection of

the housekeeping genes is a crucial requirement for real-time RT-PCR experiments because they are highly specific for a particular experimental model. Experimental conditions can induce an alteration in gene expression of some housekeeping genes causing the high variability and affecting the reliable normalization (Dheda *et al.,* 2004). The house keeping genes with high variability can lead to increased noise or erroneous results (Bustin, 2000). Therefore, the appropriate validation of internal reference genes is necessary to avoid misinterpretation of gene expression results.

Once normalized, the relative transcripts of mRNA among multiple samples or treatments or different gene transcripts can be compared. By this relative method, the quantification of the results is analyzed by comparing the linear range of both target and internal control amplification; therefore, it is essential to determine the amplification efficiency prior to starting the analysis. The details of calculations of the relative expression and amplification efficiency are described in the methods (**see section 4.2.6**).

4.1.6 Aims

1. To determine the effect of antipsychotic drugs on 5-HT2C receptor mRNA expression using reverse-transcription real-time PCR to quantify 5-HT2C receptor mRNA levels.

2. To determine the effect of antipsychotic drugs on DNA methylation of the *HTR2C* promoter sequences around the -697G/C polymorphism using bisulfite pyrosequencing to quantify DNA methylation levels.

4.2 Materials and methods

4.2.1 Materials

All solutions for cell culture were purchased from Life Technologies including fetal bovine serum (FBS), trypsin/EDTA, Dulbecco's modified Eagle's medium (DMEM), Penicillin-Streptomycin, Phosphate-Buffered Saline (PBS). 3-(3, 4-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), retinoic acid (RA), clozapine, haloperidol, 5-Aza-2-deoxycytidine, 2-mercaptoethanol (2-ME), ethidium bromide, ethanol, and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich, UK. Agarose was purchased from Invitrogen, UK. Bisulfite conversion kit, DNA extraction kit, all solutions for pyrosequencing, RNA extraction kit and RT kit were purchased from QIAGEN, UK.

4.2.2 Cell culture

The human neuroblastoma SH-SY5Y cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin in a humidified, 5% CO_2, 37°C incubator. Cells were subcultured before confluence was reached. Cell passage numbers of less than 15 were used in the experiments.

4.2.3 Cell line stocks

SH-SY5Y cells were suspended in 1 ml of fetal bovine serum containing 10% (v/v) of DMSO. Cells were stored in cryovial tubes immersed in liquid nitrogen until required for use.

4.2.4 Drug Treatment

The drug treatments were designed using three models; 1) treatment in undifferentiated SH-SY5Y cells, 2) treatment in differentiated SH-SY5Y cells, and 3) treatment in undifferentiated cells and then induced differentiation after treatment.

4.2.4.1 Treatment in undifferentiated SH-SY5Y cells

The SH-SY5Y cells were plated into 6-well plates at a density of $5x10^5$ cells per well and cultured for 24 hours before drug treatment. Cells were treated with clozapine at

doses of 2 μM and 10 μM and haloperidol at doses of 10 nM and 10 μM for 48 hours. Cells were also treated with 5-Aza-2-deoxycytidine at the doses of 0.5, 1, and 2 μM for 72 hours. The freshly prepared medium containing drugs was replaced every 24 hours. The control (no drug) was also performed by treatment with DMSO at the same concentration as in the drug treatment (control 0.01% DMSO for clozapine and haloperidol treatment, and control 0.002% DMSO for 5-Aza-2-deoxycytidine treatment). The treated cells were harvested by trypsinization and washed with PBS before being stored at -80°C until used. DNA was extracted to determine DNA methylation of *HTR2C* gene, while RNA was extracted to determine mRNA expression of 5-HT2C receptor. The experiments were performed in triplicate and 3 independent experiments were carried out.

4.2.4.2 Treatment in differentiated SH-SY5Y cells

To induce differentiation of human neuroblastoma SH-SY5Y cells to become more mature neuronal cells, the SH-SY5Y cells were seeded into 75 cm^2 culture flask at the density of $2x10^4$ cells/cm^2 in the culture medium containing 10 μM retinoic acid (Cheung *et al.*,, 2009). The differentiation induction was carried out for 7-8 days prior to treatment.

In order to study the effect of antipsychotic drugs on gene expression and DNA methylation of the *HTR2C* gene in mature neuronal cells, differentiated cells at the density of $5x10^5$cells/well were seeded overnight into 6-well plates. Cells were treated with clozapine at the doses of 2 μM and 10 μM and haloperidol at the doses of 10 nM and 10 μM for 48 hours. Differentiated cells were also treated with 5-Aza-2-deoxycytidine at the doses of 0.5, 1, and 2 μM for 72 hours. The freshly prepared medium containing drugs was replaced every 24 hours. The control (no drug) was also performed by treatment with DMSO at the same concentration as drug treatment (control 0.01% DMSO for clozapine and haloperidol treatment, and control 0.002% DMSO for 5-Aza-2-deoxycytidine treatment). The treated cells were further processed to determine DNA methylation of *HTR2C* gene and mRNA expression of 5-HT2C receptor as described in **section 4.2.4.1**.

4.2.4.3 *Treatment in undifferentiated SH-SY5Y cells followed by differentiation induction*

The SH-SY5Y cells were plated into 6-well plate at a density of $5x10^5$ cells per well and cultured for 24 hours before drug treatment. Cells were treated with 5-Aza-2-deoxycytidine at a dose of 0.5 µM for 72 hours. The freshly prepared medium containing drugs was replaced every 24 hours. The control (no drug) was also performed by treatment with DMSO at the same concentration as drug treatment (control 0.002% DMSO). After 72 hours of treatment, the culture medium with drug was removed and replaced by culture medium containing retinoic acid (10 µM) to induce differentiation or replaced by 0.001% DMSO which served as a parallel control undifferentiation induction. At the end of differentiation period (7 days), the treated cells were further processed to determine DNA methylation of *HTR2C* gene and mRNA expression of 5-HT2C receptor as described in **section 4.2.4.1**.

4.2.5 Determination of cell viability by MTT assay

Cell viability was determined by a mitochondria enzyme dependent reaction of MTT (Datki *et al.,* 2003; Cheung *et al.,* 2009). Briefly, the SH-SY5Y cells at a density of 1×10^4 cells per well were cultured onto 96-well plates for 24 hours before treatment. After treatment, 10 µl of MTT stock solution (5 mg/ml) was added to each well (100 µl) and left to incubate at 37°C for 3 hours. Metabolically active cells cleaved the yellow tetrazolium salt MTT to purple formazan crystal. At the end of incubation time, the medium was removed and the formazan crystals were solubilised with 150 µl of DMSO. The absorbance was measured by a microplate reader at 570 nm. Results were expressed as percentage of the vehicle control. All MTT assays were performed in triplicate model.

4.2.6 Determination of *HTR2C* mRNA expression by real time RT-PCR

4.2.6.1 *RNA extraction*

The extraction of RNA from cultured cells was carried out with the RNeasy Mini Kit (QIAGEN) following the protocol provided by the manufacturer. This kit used spin columns to extract RNA.

Initially, cell lysis solution was prepared by addition of 10 µl of 2-mercaptoethanol (2-ME) per 1 ml of provided lysis buffer (Buffer RLT). 350 µl of 2-ME/lysis solution was added to each cell pellet and mixed thoroughly by vortexing to lyse the cells and inactivate RNases. The lysate was also homogenized and DNA sheared by passing at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. An equal volume (350 µl) of 70% ethanol was added to the homogenized lysate to prepare for RNA binding. The lysate/ethanol solution was transferred to an RNeasy spin column which was placed in a 2 ml collection tube. Then centrifugation was done at 8000xg for 15 seconds. RNA was bound to the spin column membrane. The flow-through was discarded and the collection tube was reused.

The spin column with bound RNA was washed by addition of 700 µl of wash buffer 1 (buffer RW1) followed by centrifugation at 8000xg for 15 seconds. The flow-through was discarded and the collection tube was reused. Then 500 µl of wash buffer 2 (buffer RPE) was run through the spin column by further centrifugation at 8000xg for 15 seconds. This step was repeated with a third column wash with buffer RPE and was again centrifuged at 8000xg for 2 minutes to dry the column. The spin column must be free of ethanol before the elution step; therefore, a further drying spin column was performed by centrifugation at maximum speed for 1 minute.

The RNA was eluted from the spin column by adding 40 µl of elution buffer directly to the centre of membrane. After incubation at room temperature for 1 minute, the spin column was centrifuged at 8000xg for 1 minute. The purified RNA was stored at -80 °C.

4.2.6.2 Determination of RNA quantity and quality

The yield of each total RNA sample was obtained from A_{260} measurements with Nanodrop-1000 UV-VIS spectrophotometer in RNA-40 nucleic acid mode. Absorbance at 260 nm, 1 absorbance unit (A_{260}) equals 40 µg of single-stranded RNA/ml. Initially, the spectrophotometer was initialised using nuclease-free water, and then blanked using the elution buffer from the kit or nuclease-free water. 1 µl of each RNA sample was used to obtain the accurate RNA concentration. RNA was stored at -80°C for use in cDNA synthesis.

Extracted RNA must be clear from any contaminants such as salt, protein, solvents and genomic DNA (gDNA). Poor quality RNA can interfere with downstream processing. The purity of RNA was estimated by spectrophotometry from the relative absorbance at 230, 260, and 280 nm i.e. A_{260}/A_{280} and A_{260}/A_{230} ratios. Pure RNA exhibits A_{260}/A_{280} ratios range of 1.7 to 2.1. A low RNA ratio refers to contamination by salt, solvent, protein, etc. A low A_{260}/A_{280} ratio is typically due to protein contamination while a low A_{260}/A_{230} ratio is typically due to salt or solvent contamination such as guanidine thiocyanate.

RNA integrity was determined using agarose gel electrophoresis. The ratio of 28S to 18S eukaryotic ribosomal RNAs should be approximately 2:1 by ethidium bromide staining, indicating that no gross degradation of RNA has occurred. In RNA samples that have been degraded, 28S:18S ratio will be reversed because the 28S rRNA characteristically is degraded to an 18S-like species.

4.2.6.3 Complementary DNA (cDNA) synthesis

The first strand complementary DNA (cDNA) synthesis was carried out using the QuantiTect Reverse Transcription Kit (QIAGEN). Complementary DNA was synthesized according to the manufacturer's protocol. In brief, the gDNA elimination reaction was initially set up which consisted of 1 µg of the total RNA adjusted to a volume of 12 µl using a variable amount of nuclease-free water and 2 µl of gDNA Wipeout Buffer (7x). The reaction was set up on ice in a 0.2 ml microcentrifuge tube. Then the reaction was incubated at 42°C for 2 minutes and placed immediately on ice. The reverse-transcription master mix was prepared on ice and consisted of 1 µl of Quantiscript Reverse Transcriptase, 1 µl of RT Primer Mix, and 4 µl of Quantiscript RT Buffer(5x). The master mix (6µl) was added to the template RNA from the previous gDNA elimination step and then the reaction mixture was mixed and stored on ice. After this the reaction mixture was incubated for 15 minutes at 42°C. The reverse-transcription reaction was inactivated by incubating the reaction mixture at 95°C for 3 minutes. The cDNA was stored on ice and preceded directly to real-time PCR or stored at or below -20°C for later use.

4.2.6.4 *Reference gene selection*

Twelve housekeeping (HK) genes including *ACTB, ATP5B, B2M,CYC1, EIF4A2, GAPDH, RPL13A, SDHA, TOP1, UBC, YWHAZ,* and *18S* from Primerdesign (Primerdesign, UK) were tested on SH-SY5Y cells across differentiated and undifferentiated cells under different treatment conditions such as clozapine and haloperidol treatment by using RT-qPCR to assess the stability of expression (**Table 4.1**). Each sample was carried out in duplicate. A negative control PCR reaction of each gene was performed in which no cDNA template was added. The expression stability of these HK genes were analysed using GeNorm qbasePLUS Software (Precision, Primer Design, UK), with stability defined by *M* value, where higher *M* value indicates less stability (Vandesompele *et al.*, 2002). The C_t values of HK genes obtained from real-time PCR were input into software which were $\log_2$ transformed and obtained the expression ratios calculated by the equation:

$$2^{(C_t \text{ of control} - C_t \text{ of sample})}$$

The control in this case was the lowest expressing sample (the highest C_t value) to have positive values for all expression ratios. The software calculated the most stable expressing HK genes by step-wise removal of genes after analysis by pair-wise variation of all possible combinations of all HK genes then the most stable expressing HK gene was left with a lowest stability value *M*. Two or three HK genes that were the most stable expressing genes selected from the GeNorm were used as reference control genes in determining relative expression of gene of interest.

Table 4.1: Housekeeping genes analysed for expression stability in SH-SY5Y cells using RT-qPCR

Gene symbol	Name and function
ACTB	beta-actin, cytoskeletal structural protein
ATP5B	ATP synthase subunit beta, H^+ transporting, mitochondrial F1 complex, beta polypeptide, ATP production
B2M	Beta-2-microglobulin, beta-chain of major histocompatibility complex class I molecules
CYC1	Cytochrome c-1, electron transport chain
EIF4A2	Eukaryotic translation initiation factor 4A2, ATP-dependent RNA helicase, mRNA binding to ribosome
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase, oxidoreductase in glycosis and gluconeogenesis
RPL13A	Ribosomal protein L13a, structural component of the large 60S ribosomal subunit
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp), electron transporter in the TCA cycle and respiratory chain
TOP1	Topoisomerase I, DNA repair
UBC	Ubiquitin C, protein degradation
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, signal transduction by binding to phosphorylated serine residues on a variety of signalling molecules
18S	18S ribosomal RNA, ribosome subunit

4.2.6.5 Determination of primer efficiency

The amplification efficiency of primers used in this expression study including 5-HT2C receptor, *GAPDH* and *CYC1,* was determined using RT-qPCR. To determine primer efficiency of 5-HT2C receptor, the cDNA template was diluted in dilution series (1:5, 1:10, 1:50 and 1:100) with nuclease-free distilled water and 4 µl of each dilution sample was a subject to real-time PCR reaction (**section 4.2.6.6**). The serial dilutions of 1:1, 1:10, 1:100, and 1:1000 were also performed to determine primer efficiency of *GAPDH* and *CYC1*. Each sample was carried out in duplicate. The primer efficiency of each primer set was calculated by the method described by Pfaffl (2001) as follows:

$$\text{Efficiency (E)} = 10^{(-1/\text{slope})}$$

$$\text{Percentage of Efficiency} = (10^{(-1/\text{slope})}-1)*100$$

The slope was calculated by plotting semi-log between cDNA concentration or dilution factor on the x axis of a scatter plot and C_t values on y axis using Microsoft Excel software. An ideal efficiency value 2.00 representing 100 percent efficiency was corresponded to a slope of -3.32 which indicated precisely double amplify the amount of PCR product during each cycle of PCR. Primer efficiencies between 90-110% were considered acceptable for use in subsequent analyses (Pfaffl, 2001).

4.2.6.6 Quantitative RT-PCR (RT-qPCR)

The expression of reference genes and the 5-HT2C receptor were assessed using the cDNA as the template. The reaction mixture volume of 20 µl was prepared containing cDNA, 20 ng, (4µl), 1 µl of 10 µM oligonucleotide forward and reverse primers, and 10 µl of (2x) qPCR mastermix (Precision, Primer Design) and nuclease-free distilled water. This mastermix contains 2x reaction buffer, 0.025 U/µl Taq Polymerase, 5 mM $MgCl_2$, dNTP mix (200 µM each dNTP), and SYBR®Green and ROX passive reference dye.

The primer sequences of 5-HT2C receptor were shown in **Table 4.2** which were purchased from Primerdesign.

Table 4.2: Oligonucleotide primer sequences for 5-HT2C receptor mRNA

Primer	Sequence (5'-3')	T_m (°C)	Product length
HTR2C_F	GCAAGTTGAGAATTTAGAGTTACCA	56.7	97 bp
HTR2C_R	CGTAGGAAAAGACTGTGCTGTT	57.4	

The PCR reactions were set up in a 96-well PCR plate, the reactions were then run in a real-time PCR system, StepOne Plus (Applied Biosystems, USA). The PCR cycles for determining 5-HT2C receptor mRNA expression were set as follows;

95°C for 10 minutes to activate hotstart enzyme

94°C for 15 seconds
64°C for 30 seconds with data collection
72°C for 30 seconds
} 40 cycles

Melt curve

95°C for 15 seconds

50°C for 60 seconds increasing in 1°C increment

The PCR cycles for determining mRNA expression of HK gene were set as follows;

95°C for 10 minutes to activate hotstart enzyme

94°C for 15 seconds
60°C for 1 minute with data collection
} 40 cycles

Melt curve

95°C for 15 seconds

50°C for 60 seconds increasing in 1°C increment

The final melt curve step was used to estimate the specificity of the primers in SYBR®Green real-time RT-PCR. One single peak of melt curve should be obtained to ensure only one PCR product was amplified. If the primers have low specificity binding to template sequences, more than one PCR products were amplified and resulted in multiple peaks showing on melt curve.

The expression of 5-HT2C receptor mRNA transcript was normalized to those of reference genes and relative to control treatment using the comparative C_t method ($2^{-\Delta\Delta Ct}$ method) according to formula below (Livak and Schmittgen, 2001).

$$\text{Expression ratio} = 2^{-[\Delta Ct \text{ sample} - \Delta Ct \text{ control}]}$$

$$\text{Expression ratio} = 2^{-\Delta\Delta Ct}$$

Where ΔC_t sample = $C_{t\ \text{sample}} - C_{t\ \text{reference}}$

and ΔC_t control = $C_{t\ \text{control}} - C_{t\ \text{reference}}$

4.2.7 Determination of DNA methylation of *HTR2C* gene

The promoter sequence of the *HTR2C* gene at and nearby the -697G/C SNP site was used to investigate the effect of antipsychotic drug treatment on the extent of DNA methylation of CpGs in this region. Initially, the CpGs at and nearby the -759C/T SNP site were targetted but there was a problem with pyrosequencing as mentioned in **section 4.1**. Instead, the nearby region (at -697G/C and nearby CpG sites) was investigated.

The extraction of DNA from cultured cells was carried out using QIAamp® DNA Mini and Blood Mini kit (Qiagen) following the protocol provided by the manufacturer. Initially, cell lysis solution was prepared by resuspending the cell pellet with 200 µl of PBS, and then 20 µl of proteinase was added into the mixture followed by the addition of 200 µl of lysis buffer (Buffer AL) and mixing by pulse-votexing for 15 seconds. The lysate was incubated at 56°C for 10 minutes. 200 µl of 70% ethanol was added to the lysate and mixed by pulse-vortexing for 15 seconds to prepare for DNA binding. Then the mixture was transferred to a QIAamp Mini spin column which was placed in a 2 ml collection tube. Then centrifugation was done at 6,000xg (8,000 rpm) for 1 minute. DNA was bound to the spin column membrane. The flow-through was discarded and then the QIAamp Mini spin column was placed in a new 2.0 ml collection tube. The spin column with bound DNA was washed by addition of 500 µl of wash buffer 1 (buffer AW1) followed by centrifugation at 6,000xg for 1 minute. The flow-through was discarded and the spin column was placed in a new 2.0 ml collection tube. Then 500 µl of wash buffer 2 (buffer AW2) was run through the spin column by further centrifugation at full speed 20,000xg (14,000 rpm) for 3 minutes. The column was

again centrifuged at full speed for 1 minute to dry the column. The DNA was eluted from the spin column by adding 50 µl of elution buffer (buffer AE) or distilled water directly to the centre of membrane. After incubation at room temperature for 1 minute, the spin column was centrifuged at 6,000xg for 1 minute. The purified DNA was stored at -20°C.

Genomic DNA was extracted and used in bisulfite pyrosequencing. The methods for bisulfite treatment, PCR, gel electrophoresis, and pyrosequencing were mentioned earlier in a previous chapter (see **chapter 3, section 3.2**). All methods for determining DNA methylation, analysing CpG sites, PCR primers and conditions, sequencing primer, and the pyrosequencing protocol were the same as described in the previous chapter (**see section 3.2**).

4.2.8 Statistical analysis

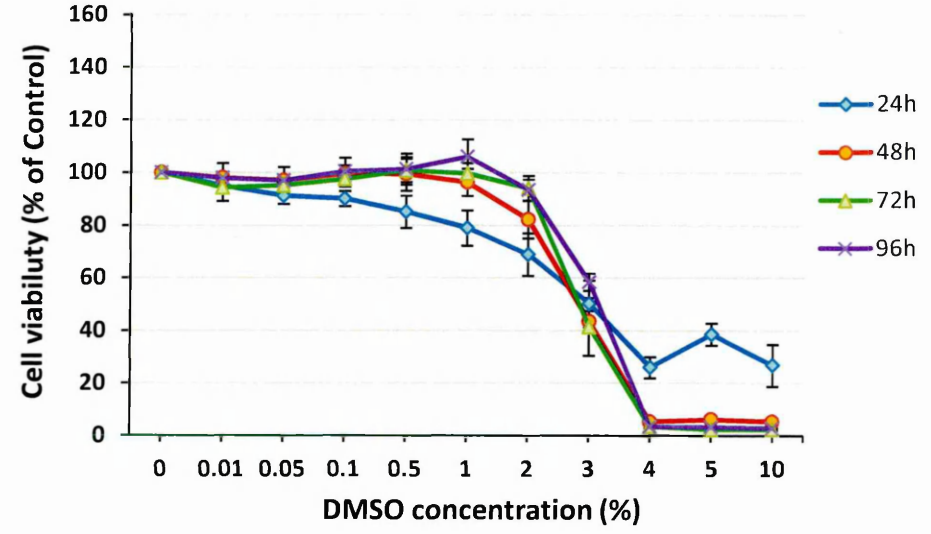
The effects of antipsychotic drugs on *HTR2C* mRNA expression and DNA methylation of *HTR2C* promoter sequence were tested using SPSS for Windows. The expression of *HTR2C* mRNA was expressed as fold change comparing to control untreated group. DNA methylation of *HTR2C* promoter sequence was expressed as mean percentage methylation of all CpGs. The normal distribution was tested by using the Kolmogorov–Smirnov test. One-way ANOVA was used to compare means between multiple groups when the values had a normal distribution with Bonferroni post hoc test. The Kruskal-Wallis test was used for non-parametric tested when the values did not have normal distribution. Statistical significance was considered when P value was less than 0.05.

4.3 Results

4.3.1 Effect of antipsychotic drug treatments on cell viability

The cell viability of various drug treatments in SH-SY5Y cells at 24, 48, 72, and 96 hours determined by the MTT assay is shown as percentage of control at each time period. The highest concentration of DMSO that can be used as a drug solvent and the highest concentration of drugs that can be used in the experiment were determined as when the cell viabilities were more than 80% of their untreated controls. The results showed that the highest concentration of chemicals that can be used in the experiments were up to 1% for DMSO, 20 μM for 5-Aza-2-deoxycytidine (within 72h incubation period), 50 μM for haloperidol, and 25 μM for clozapine (**Figure 4.3 and Figure 4.4**). The cell viability of some cell treated with DMSO (1%) at 96h was increased above 100% of the control. In addition, treatment with 5-Aza-2-deoxycytidine (≤5μM) for 24-72h resulted in increased cell viability.

(A)



(B)

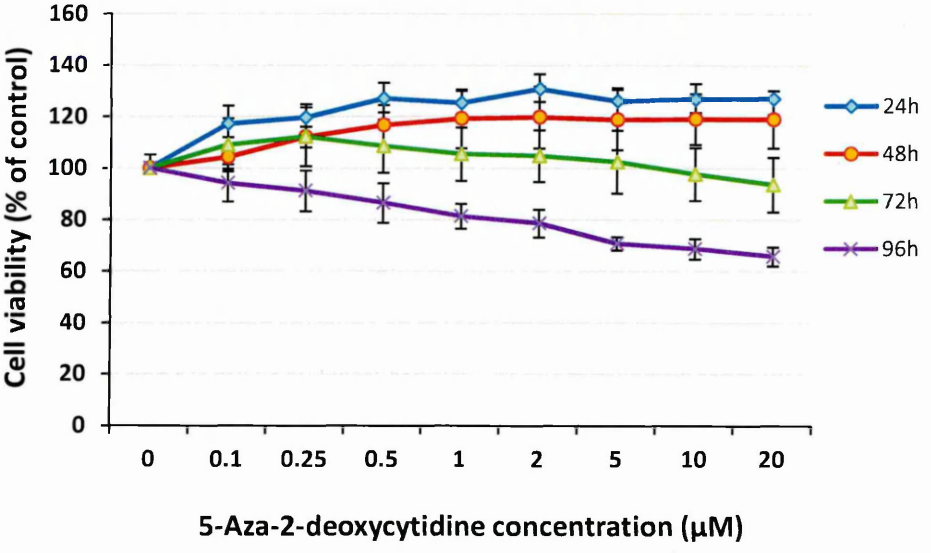


Figure 4.3: The percentage of cell viability when treated with (A) DMSO (n=4), and (B) 5-Aza-2-deoxycytidine (n=3) for 24, 48, 72, and 96 hours compared to control untreated cells (0% DMSO).

Data presented as mean±SEM.

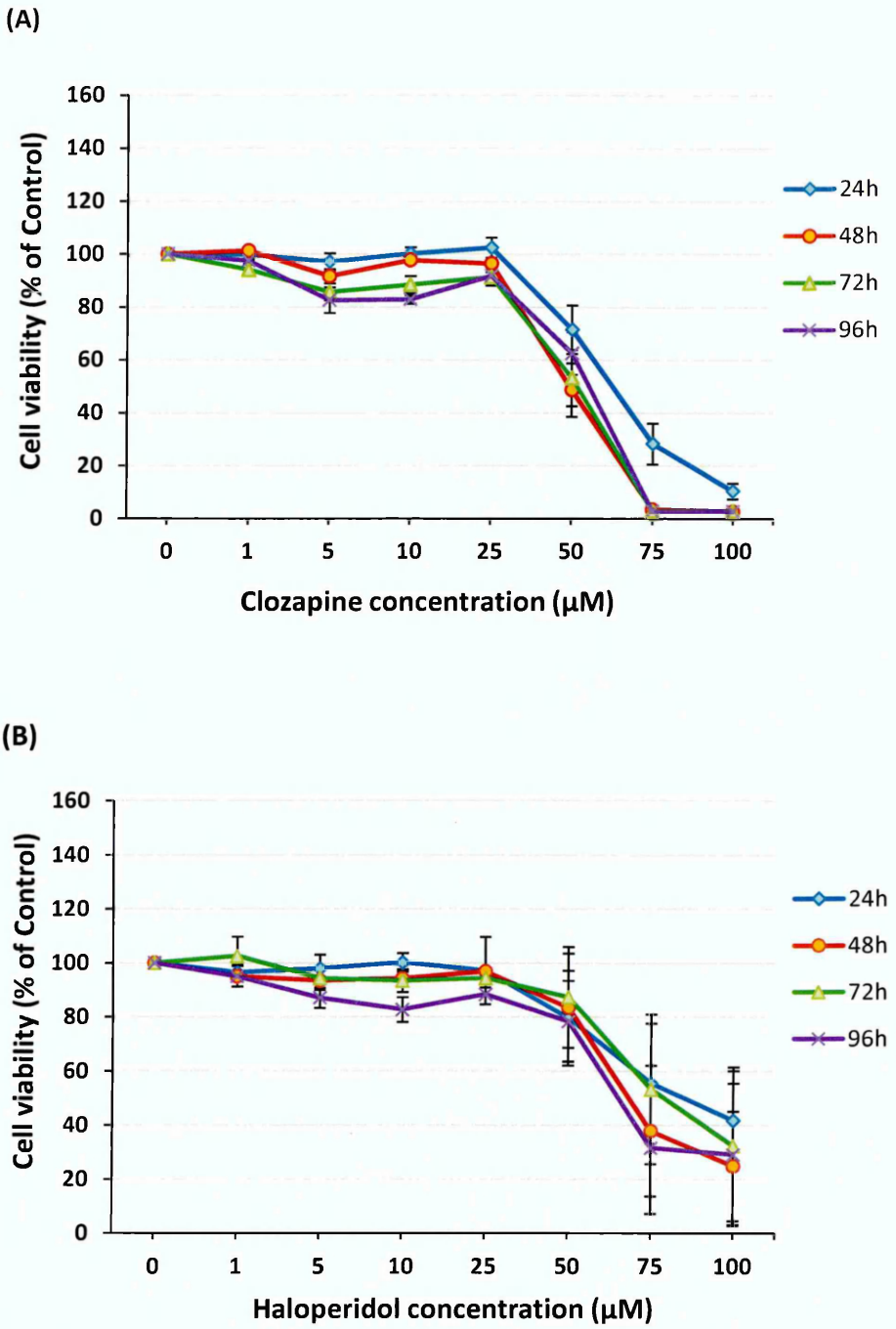


Figure 4.4: The percentage of cell viability when treated with (A) clozapine and (B) haloperidol for 24, 48, 72, and 96 hours compared to control untreated cells.
Data presented as mean±SEM, n=4.

4.3.2 Effect of antipsychotic drug treatments on *HTR2C* mRNA expression

4.3.2.1 RNA extraction

The total RNA was extracted from cultured cells and its concentration was determined using the Nanodrop-1000 spectrophotometer. The absorbance ratio of A_{260}/A_{280} and A_{260}/A_{230} were between 1.8 to 2.2 (data not shown) that reflected the purity of extracted RNA. In addition, the integrity of RNA was also determined by running on agarose gel electrophoresis. The ratio of 28S to 18S ribosomal RNAs showed approximately 2:1 by ethidium bromide staining, indicating that no gross degradation of RNA occurred (**Figure 4.5**).

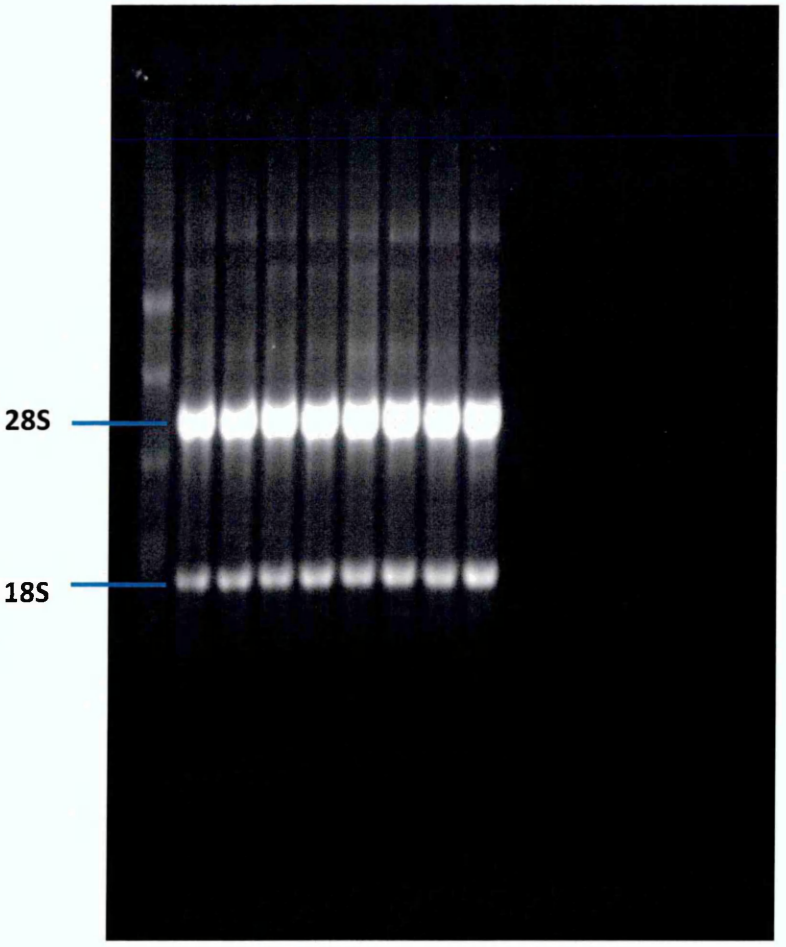


Figure 4.5: RNA on gel electrophoresis determining integrity of RNA

4.3.3 Selection of housekeeping genes as internal control genes

The internal reference control genes for normalizing the expression of the gene of interest were selected from 12 HK genes. Initially, the expression of 12 HK genes was analysed in undifferentiated SH-SY5Y cells treated with clozapine compared to untreated control. The melt curve of each reference gene showed a single peak indicating that single specific PCR product was amplified; the amplification plots and melt curves of reference genes were illustrated in **Figure 4.6**.

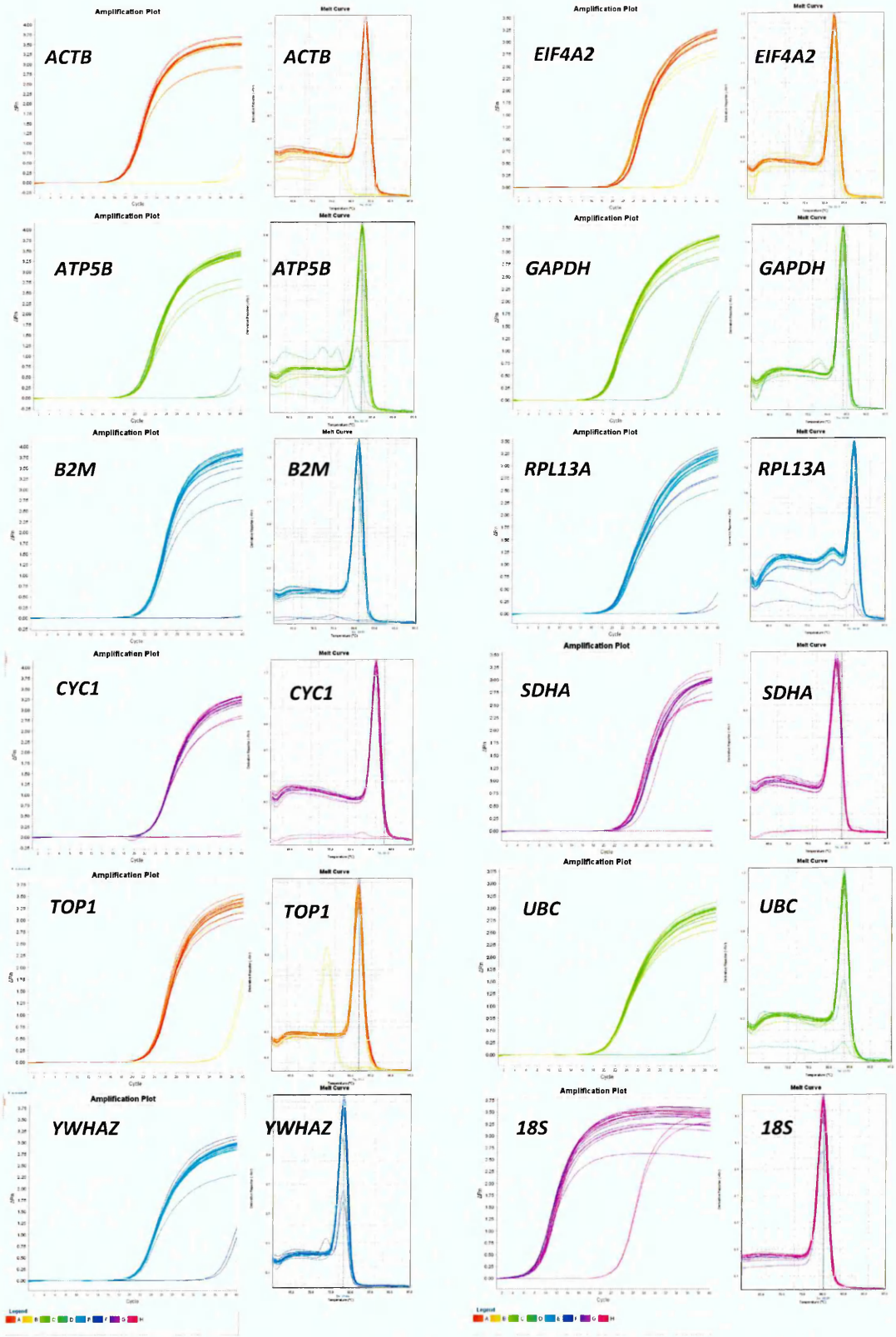


Figure 4.6: Amplification plots and melt curves of the house keeping genes tested for transcript expression stability

The results from qPCR were evaluated by GeNorm software to select HK genes with the most stable expression for using as internal reference control genes. The results from the GeNorm software indicated several HK genes that had high average expression stability values (*M* value or geNorm *M* <0.2) (**Figure 4.7**). The program eliminated the worst-scoring HK gene that is the one with the highest *M* value and recalculated of new *M* values for the remaining genes. Therefore, genes with the lowest *M* values have the most stable expression. The software suggested *GAPDH* and *CYC1* as the most stable reference genes. In addition, the minimum required number of HK gene was suggested by a geNorm *V* value which should be lower than 0.15 (**Figure 4.8**) indicating that at least two reference genes should be used in subsequent analyses. This *V* value indicates the systemic variation for repeated RT-qPCR experiments on the same gene reflecting the variation of the machine, enzymatic reaction and pipetting error. In summary, the most stable expressing HK genes observed across undifferentiated and differentiated cells and all treatments were found to be *GAPDH* and *CYC1*. Two HK genes were used as reference internal control genes for subsequent quantitative reverse transcription PCR analyses.

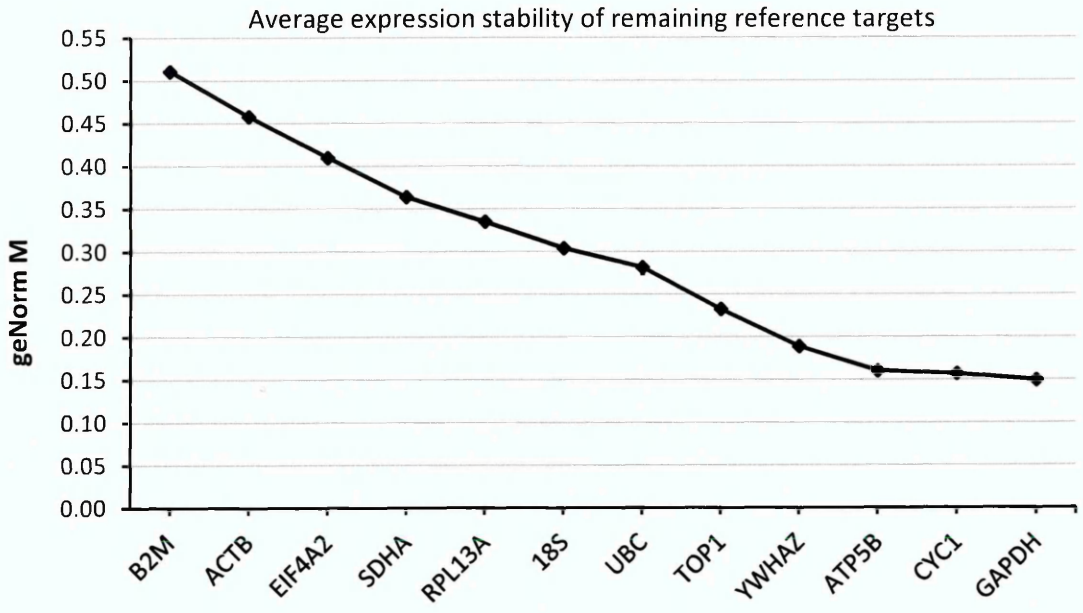


Figure 4.7: The geNorm *M* of 12 housekeeping genes of undifferentiated and differentiated cells treated with clozapine, haloperidol, and untreated control samples.

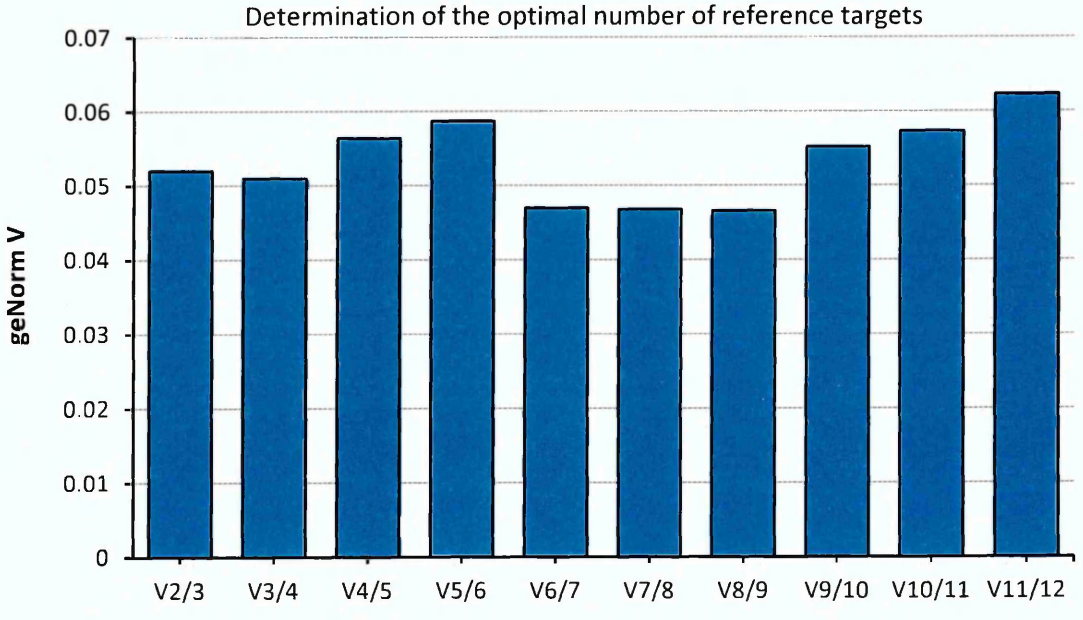


Figure 4.8: The pair-wise variation (geNorm *V*) of reference genes of undifferentiated and differentiated cells treated with clozapine, haloperidol, and untreated control samples.

4.3.4 The primer efficiency of *HTR2C*, *GAPDH* and *CYC1*

The amplification efficiency of primers including *HTR2C*, *GAPDH*, and *CYC1* were determined. The slopes that were obtained from semi-log plots between cDNA concentration and C_t values of each primer set were used to calculate primer efficiency (**Figure 4.9**). The slope values for *HTR2C*, *GAPDH*, and *CYC1* were -3.1418, -3.375, and -3.4457, respectively. It should be noted that the narrow range of *HTR2C* dilution series compared to HK genes indicated the low quantity of transcripts. The percentage of primer efficiency were calculated as described in **section 4.2.6.5**; $E(\%) = (10^{(-1/slope)} - 1)*100$, and shown in **Table 4.3**.

In addition, the specificity of each primer set was also assessed by melt curves as shown in **Figure 4.10**. Every primer set amplified only one single product indicating that no non-specific PCR product was amplified.

Table 4.3: The amplification efficiencies of primers used in expression study in SH-SY5Y cells

Primers	Efficiency (%) (mean ± SD) (n=3)
HTR2C	108.12 ± 1.40
GAPDH	97.86 ± 1.90
CYC1	95.11 ± 2.15

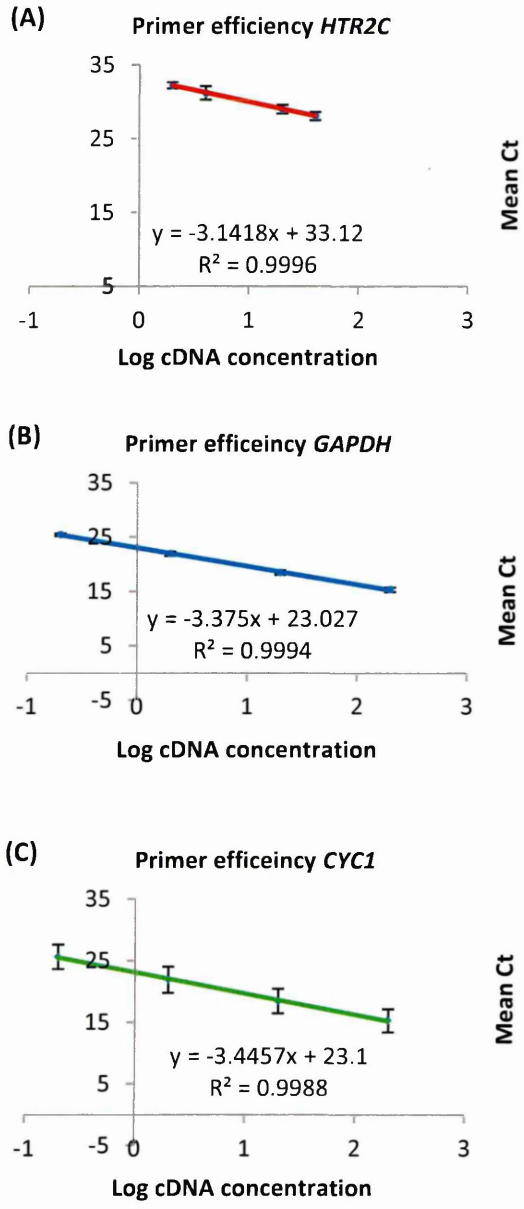


Figure 4.9: Semi-log plots between cDNA concentrations and Ct values of each primer set using for primers efficiency calculation

Efficiencies of all primer sets were determined including *HTR2C*, *GAPDH*, and *CYC1*. The C_t values of each dilution series of each transcript were used to calculated slope of the trend line. Data presented as mean±SD (n=3). The percentage of efficiency was calculated using $E= [10^{(-1/slope)}-1] *100$. The slope values for *HTR2C*, *GAPDH*, and *CYC1* were -3.1418, -3.375, and -3.4457, respectively. The means calculated primer efficiency (n=3) of *HTR2C*, *GAPDH*, and *CYC1* were 108.12%, 97.86%, and 95.11%, respectively.

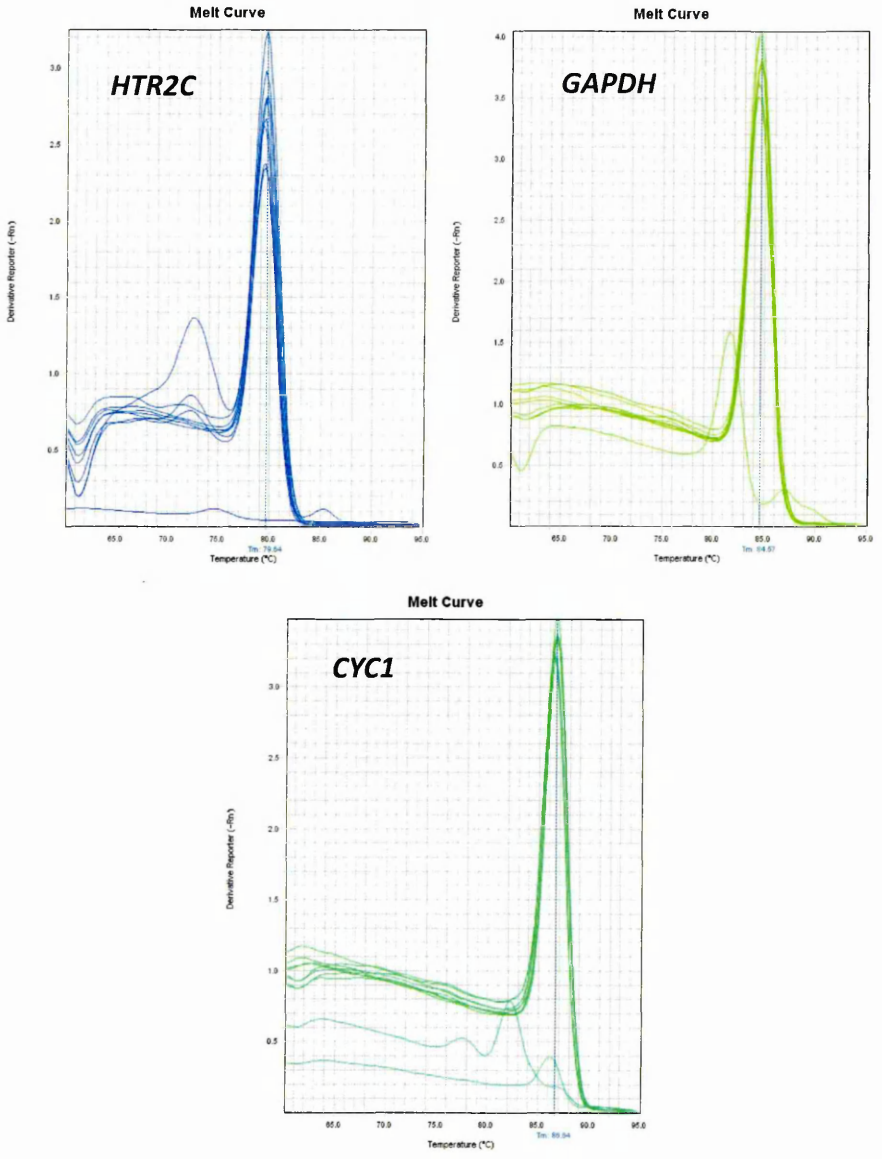


Figure 4.10: Melt curve of *HTR2C*, *GAPDH*, and *CYC1* transcripts when amplified by primers which used in determining of primer efficiency in SYBR® Green real-time RT-PCR in SH-SY5Y cells

4.3.5 Effect of drug treatment on *HTR2C* mRNA expression in undifferentiated SH-SY5Y cells

The effect of antipsychotic drug treatment on the expression of the *HTR2C* transcript in undifferentiated SH-SY5Y cells was determined by quantitative RT-PCR. Treatment with clozapine for 48 hours in undifferentiated SH-SY5Y cells at doses of 2 μM and 10 μM as well as treatment with haloperidol at doses of 0.01 and 10 μM had no effect on the

expression of the *HTR2C* transcript relative to control untreated cells (DMSO 0.01%) at day 0 as shown in **Figure 4.12**. However, treatment with 5-Aza-2-deoxycytidine, a demethylating reagent, at doses of 0.5, 1, and 2 µM resulted in an increase in the expression of the 5-HT2C receptor transcript, but this did not reach significant levels ($p>0.05$) after 72 hours of treatment (**Figure 4.13**). In addition to observing the melt curve, the PCR products from RT-qPCR were run using gel electrophoresis which confirmed that only one PCR product was amplified without any non-specific PCR product, indicating the specificity of primer sets used in SYBR®Green RT-qPCR (**Figure 4.11**).

4.3.6 Effect of drug treatment on *HTR2C* mRNA expression in differentiated SH-SY5Y cells

The expression of the *HTR2C* transcript in differentiated cells was also determined by quantitative RT-PCR and results were similar to previous experiments in undifferentiated SH-SY5Y cells. Treatment with clozapine for 48 hours in differentiated SH-SY5Y cells at doses of 2 µM and 10 µM as well as treatment with haloperidol at doses of 0.01 and 10 µM had no effect on the expression of *HTR2C* transcript relative to control untreated cells at day 0 (Kruskal-Wallis test) as shown in **Figure 4.14**. However, treatment with 5-Aza-2-deoxycytidine, a demethylating reagent, at doses of 0.5, 1, and 2 µM resulted in an increase in the expression of the *HTR2C* transcript but this did not reach significant levels ($p>0.05$) after 72 hours of treatment (**Figure 4.15**).

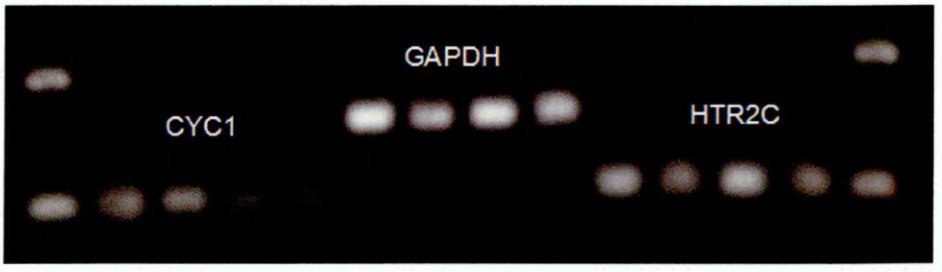


Figure 4.11: PCR products resulted from RT-qPCR were run on agarose gel electrophoresis to assess the specificity of primer sets used in SYBR®Green RT-qPCR

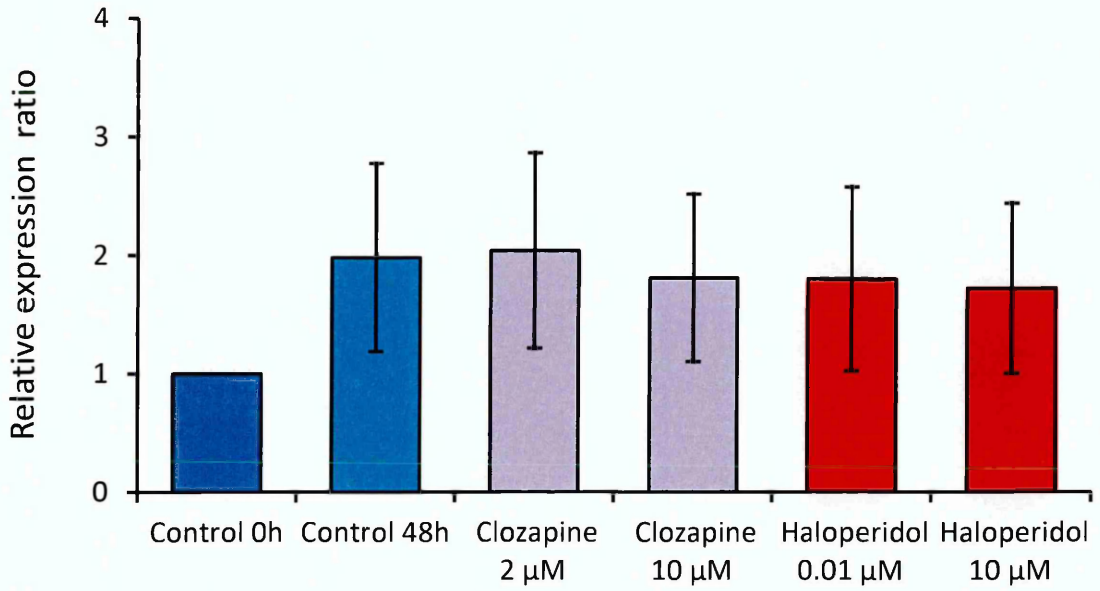


Figure 4.12: *HTR2C* mRNA expressions of various concentrations of clozapine and haloperidol treatment in undifferentiated SH-SY5Y cells. Expression was normalized to *GAPDH* and *CYC1*.

Data presented as mean±SEM.

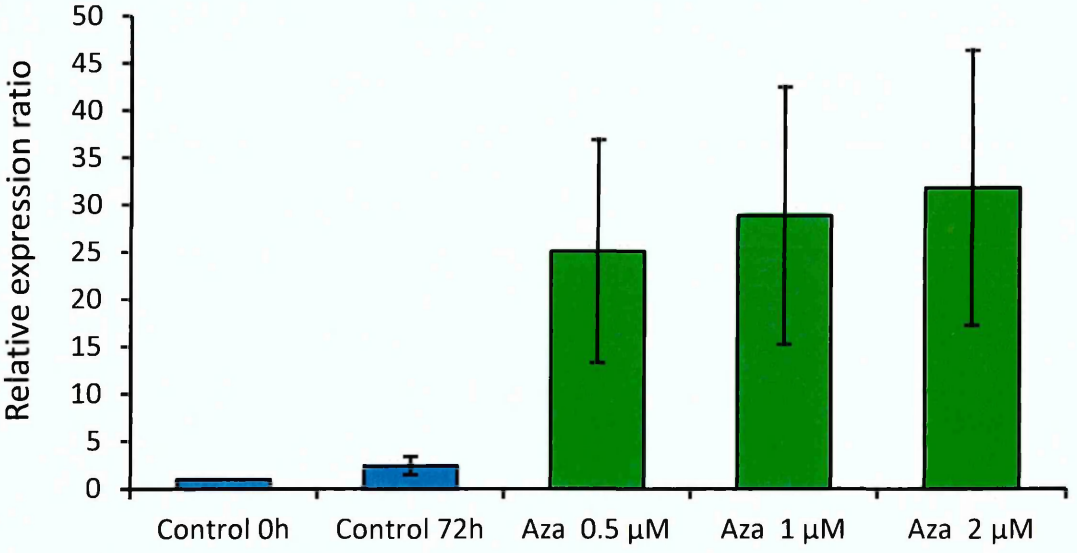


Figure 4.13: Expression of *HTR2C* transcript when treatment with 5-Aza-2-deoxycytidine 0.5, 1, and 2 µM in undifferentiated SH-SY5Y cells for 72 hours. Expression was normalized to *GAPDH* and *CYC1*.

Data presented as mean±SEM.

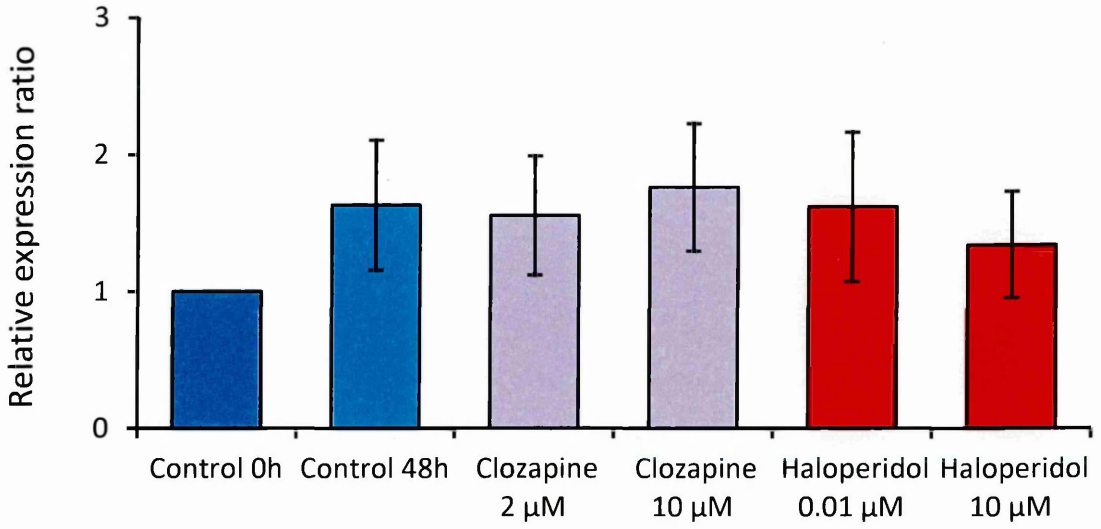


Figure 4.14: *HTR2C* mRNA expressions of various concentrations of clozapine and haloperidol treatment in differentiated SH-SY5Y cells. Expression was normalized to *GAPDH* and *CYC1*.

Data presented as mean±SEM.

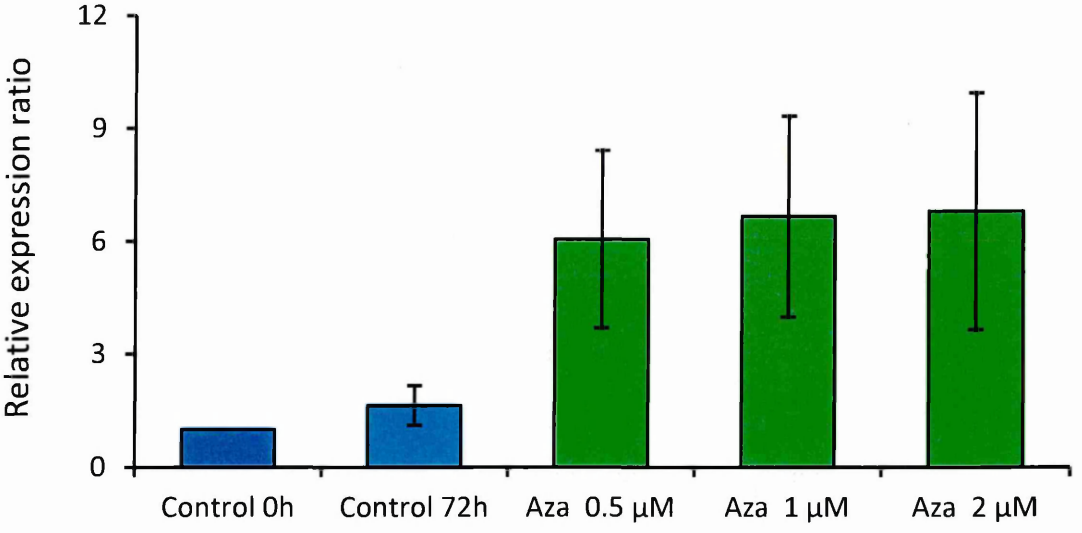


Figure 4.15: *HTR2C* mRNA expressions of various concentrations of 5-Aza-2-deoxycytidine treatment in differentiated SH-SY5Y cells. Expression was normalized to *GAPDH* and *CYC1*.

Data presented as mean±SEM.

4.3.7 Effect of 5-Aza-2-deoxycytidine treatment on *HTR2C* mRNA expression in undifferentiated SH-SY5Y cells followed by differentiation induction

The results from previous sections showed that the antipsychotic drugs, clozapine and haloperidol, did not have an effect on *HTR2C* mRNA expression whereas 5-Aza-2-deoxycytidine treatment led to increased *HTR2C* mRNA expression in both undifferentiated and differentiated SH-SY5Y cells. Therefore, in this experiment only 5-Aza-2-deoxycytidine treatment was carried out to assess the effect of 5-Aza-2-deoxycytidine on *HTR2C* mRNA expression in undifferentiated cells followed by differentiation induction using retinoic acid for 7 days. The results show that 5-Aza-2-deoxycytidine had a long lasting effect on *HTR2C* mRNA expression even if it was removed before induction of differentiation (**Figure 4.16B**). This effect was observed in undifferentiated cells when cells were grown for the same period of time with no retinoic acid treatment, as well as differentiated cells; 5-Aza-2-deoxycytidine resulted in an increase in 5-HT2C receptor transcript under both conditions (**Figure 4.16A**).

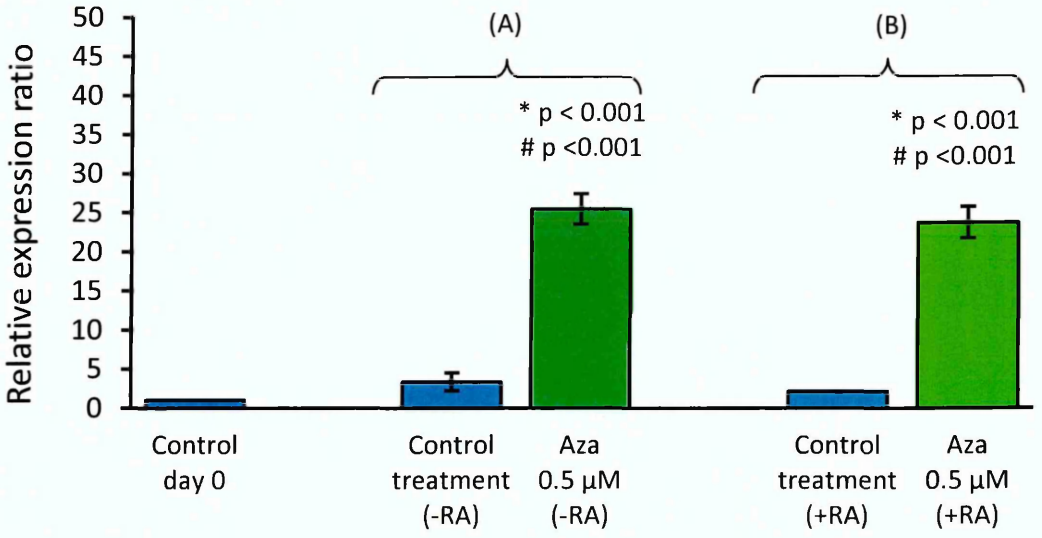


Figure 4.16: *HTR2C* mRNA expression with 0.5 µM 5-Aza-2-deoxycytidine treatment for 72 hours in undifferentiated SH-SY5Y cells followed by with (B) or without (A) differentiation induction for 7 days.

Expression was normalized to *GAPDH* and *CYC1*. Data presented as mean±SEM. * indicates statistical significant difference compared to control (DMSO 0.002%) at day 0, # indicates statistical significant difference compared to their control treatments (DMSO 0.002% (-RA) or retinoic acid (+RA) for same experimental periods).

4.3.8 Effect of drug treatment on DNA methylation extent of *HTR2C* gene in undifferentiated SH-SY5Y cells

The effect of antipsychotic drug treatment on DNA methylation of the 5-HT2C receptor gene (*HTR2C*) in undifferentiated SH-SY5Y cells was determined by bisulfite pyrosequencing. Treatment with clozapine for 48 hours in undifferentiated SH-SY5Y cells at doses of 2 μM and 10 μM as well as treatment with haloperidol at doses of 0.01 and 10 μM had no effect on DNA methylation of *HTR2C* promoter sequences compared to that of control untreated cells at day 0 (treated with DMSO 0.01% as same as treatment solution) as shown in **Figure 4.17**. However, treatment with 5-Aza-2-deoxycytidine, a demethylating reagent, at doses of 0.5, 1, and 2 μM significantly decreased DNA methylation of *HTR2C* ($p<0.001$) when compared to control (DMSO 0.002%) at 72 hours of treatment period (**Figure 4.18**).

4.3.9 Effect of drug treatment on DNA methylation extent of *HTR2C* in differentiated SH-SY5Y cells

The effect of antipsychotic drug treatment on DNA methylation of *HTR2C* gene in differentiated SH-SY5Y cells was also determined by bisulfite pyrosequencing and the results were similar to undifferentiated cells. Treatment with clozapine for 48 hours in differentiated SH-SY5Y cells at doses of 2 μM and 10 μM as well as treatment with haloperidol at doses of 0.01 and 10 μM had no effect on DNA methylation of *HTR2C* promoter sequences compared to that of control untreated cells at day 0 (treated with DMSO 0.01% as same as treatment solution) as shown in **Figure 4.19**. However, treatment with 5-Aza-2-deoxycytidine at doses of 0.5, 1, and 2 μM significantly decreased DNA methylation of *HTR2C* ($p<0.001$) when compared to control (DMSO 0.002%) at 72 hours of treatment period (**Figure 4.20**).

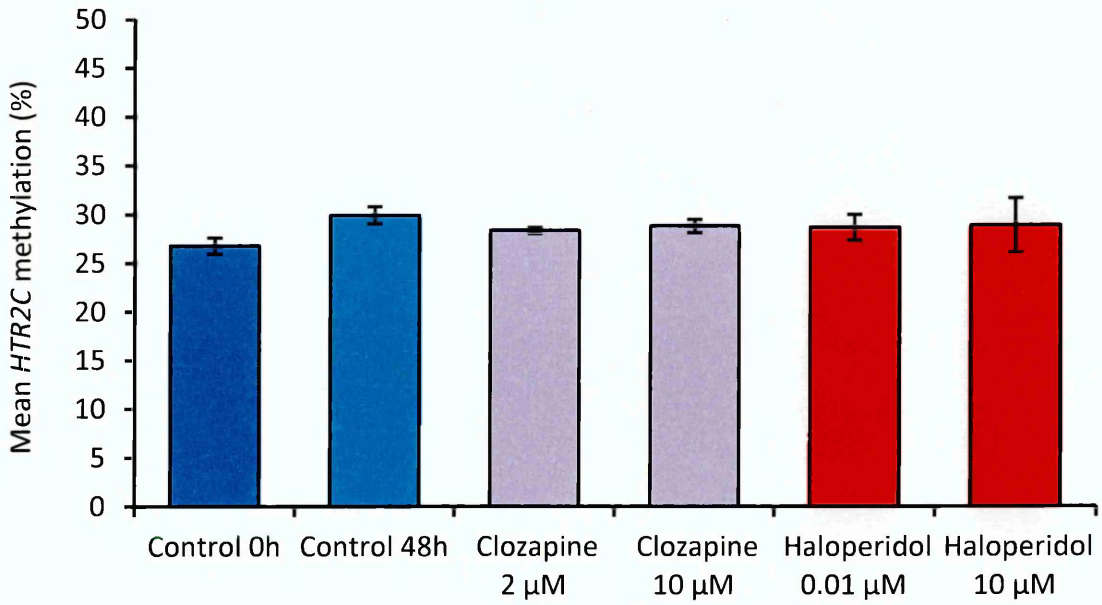


Figure 4.17: Mean DNA methylation levels of 5 CpGs of *HTR2C* promoter sequences treatment with clozapine 2 µM and 10 µM and haloperidol 0.01 µM and 10 µM compared to control (DMSO 0.01%) at 0h and 48h in undifferentiated SH-SY5Y cells.
Data presented as mean±SEM.

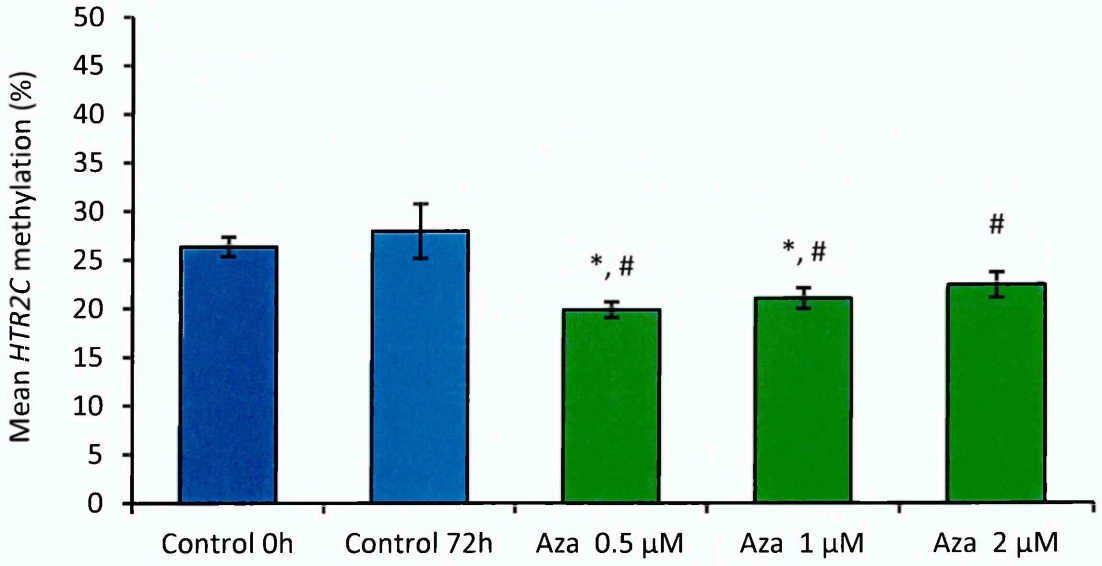


Figure 4.18: Mean DNA methylation levels of 5 CpGs of *HTR2C* promoter sequences treatment with 5-Aza-2-deoxycytidine 0.5, 1 and 2 µM compared to control (DMSO 0.002%) at 0h and 72h in undifferentiated SH-SY5Y cells.
Data presented as mean±SEM. * indicates statistical significant difference compared to control (DMSO 0.002%) at 0h, # indicates statistical significant difference compared to control (DMSO 0.002% at 72h.

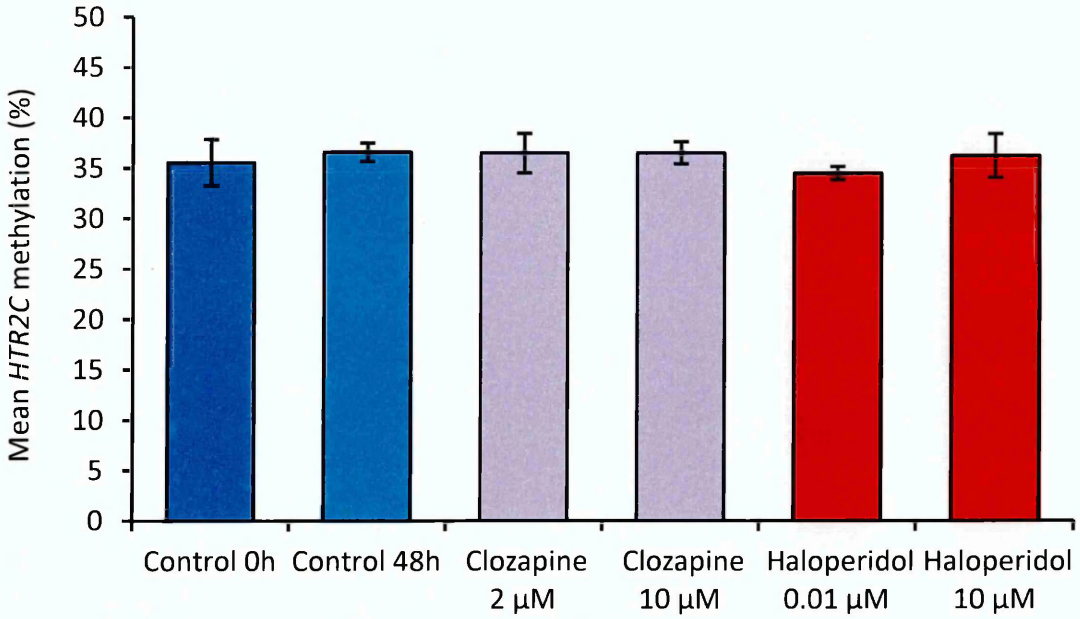


Figure 4.19: Mean DNA methylation levels of 5 CpGs of *HTR2C* promoter sequences treatment with clozapine 2 µM and 10 µM and haloperidol 0.01 µM and 10 µM compared to control (DMSO 0.01%) at 0h and 48h in differentiated SH-SY5Y cells.
Data presented as mean±SEM.

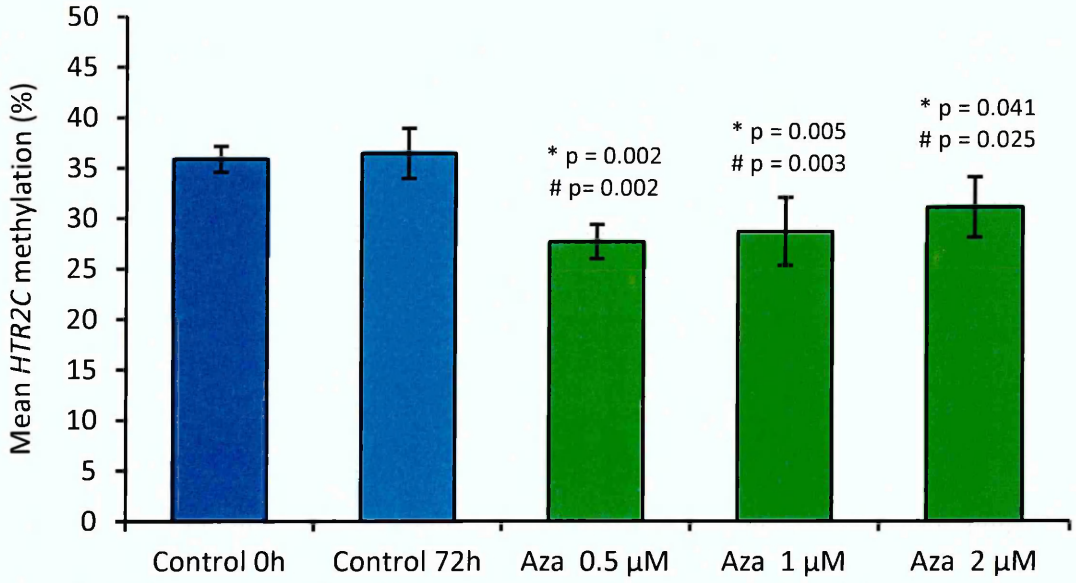


Figure 4.20: Mean DNA methylation levels of 5 CpGs of *HTR2C* promoter sequences treatment with 5-Aza-2-deoxycytidine 0.5, 1 and 2 µM compared to control (DMSO 0.002%) at 0h and 72h in differentiated SH-SY5Y cells.
Data presented as mean±SEM. * indicates statistical significant difference compared to control (DMSO 0.002%) at 0h, # indicates statistical significant difference compared to control (DMSO 0.002% at 72h.

4.3.10 Effect of 5-Aza-2-deoxycytidine treatment on DNA methylation extent of *HTR2C* in undifferentiated SH-SY5Y cells followed by differentiation induction

The results from previous sections showed that the antipsychotic drugs, clozapine and haloperidol, did not have an effect on DNA methylation of *HTR2C* promoter sequences around the -697G/C polymorphism whereas 5-Aza-2-deoxycytidine treatment led to a decrease in DNA methylation in both undifferentiated and differentiated SH-SY5Y cells. Therefore, this experiment aimed to assess the effect of 5-Aza-2-deoxycytidine treatment on undifferentiated cells followed by differentiation induction using retinoic acid for 7 days. The results showed that 5-Aza-2-deoxycytidine had a long lasting effect on DNA methylation even if it was removed before induction of differentiation (**Figure 4.21B**). This effect was observed in undifferentiated cells when cells were grown for the same period of time with no retinoic acid treatment, as well as differentiated cells; 5-Aza-2-deoxycytidine resulted in a decrease in DNA methylation of the *HTR2C* promoter sequences (**Figure 4.21A**).

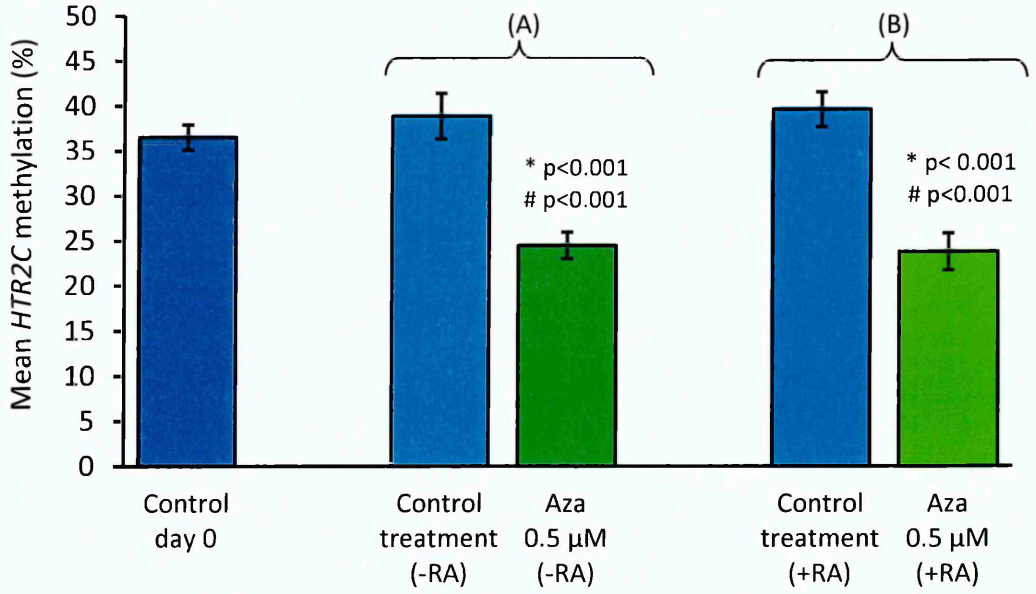


Figure 4.21: Mean DNA methylation levels of 5 CpGs of *HTR2C* promoter sequences treatment with 0.5 µM 5-Aza-2-deoxycytidine for 72 hours in undifferentiated SH-SY5Y cells followed by with (b) or without (a) differentiation induction for 7 days.
Data presented as mean±SEM. * indicates statistical significant difference compared to control (DMSO 0.002%) at day 0, # indicates statistical significant difference compared to their control treatments (DMSO 0.002% (-RA) or retinoic acid (+RA) for same experimental periods).

4.4 Discussion

Previous findings reported the association of *HTR2C* -759C/T and -697G/C polymorphisms and antipsychotic drug-induced weight gain (Reynolds *et al.,* 2002; Shao *et al.,* 2008; Godlewska *et al.,* 2009); however, the mechanisms underlying this association were unknown. In addition, the -759T and -697C allele of the *HTR2C* polymorphisms are associated with lower promoter activity (Hill and Reynolds, 2007). Antipsychotic drugs might cause the alteration in *HTR2C* expression via, in part, epigenetic modification. Therefore, the experiments in this chapter were carried out with the aim of investigating the effect of antipsychotic drugs on *HTR2C* gene expression and DNA methylation of *HTR2C* promoter sequences at and near -697G/C SNP site, (but not at and near -759C/T site due to technical difficulties (**see section 4.1**) by using RT-qPCR and bisulfite pyrosequencing in SH-SY5Y human neuroblastoma cell line.

4.4.1 Validation of methodology

4.4.1.1 MTT assay

Clozapine, haloperidol, 5-aza-2-deoxycytidine, and DMSO which was used as the drug solvent were tested for cytotoxicity using the MTT assay. The drugs used in the experiments did not affect cell viability of SH-SY5Y cells.

4.4.1.2 Reverse-transcription real-time PCR

4.4.1.2.1 RNA

RNA used in this study had a good quality observed from the clear sharp bands of 28S and 18S on the agarose gel whereby the 28S/18S rRNA ratio was approximately 2:1. The assessment of RNA quality was determined by quantification of 28S and/or 18S ribosomal RNA on ethidium bromide gels (Sambrook and Russel, 2001). This method is a common and traditional method. RNA quality/integrity affects downstream applications such as RT-qPCR and microarrays (Copois *et al.,* 2007); therefore, it is essential to assess the integrity of extracted RNA prior to perform downstream applications to ensure the reliability of the subsequent analysis.

4.4.1.2.2 Housekeeping gene validation

In the present study *GAPDH* and *CYC1* were selected as the most stably expressed housekeeping genes using GeNorm software in order to use them as internal reference control genes to normalise the expression of the gene of interest. These reference genes had the least M value determined by GeNorm software under various experimental conditions.

4.4.1.2.3 Amplification efficiency of primers

The amplification efficiency of the primer pairs used in this study was determined to quantify the relative gene expression. The primer efficiencies of all primer sets were within the acceptable range, 90-110%. At 100% efficiency in PCR 1 cycle corresponds to a 2-fold change. The analysis of gene expression was calculated using the comparative Ct method according to the Livak and Schmittgen (Livak and Schmittgen, 2001).

4.4.2 Validation of experimental procedure in determining *HTR2C* DNA methylation and mRNA expression using 5-aza-2-deoxycitidine

The present study demonstrates that treatment with 5-Aza-2-deoxycytidine, a demethylating agent inhibiting DNA methylation, increased mRNA expression of *HT2CR* whereas it decreased DNA methylation levels of the *HTR2C* promoter sequences. These results not only verified the experimental model and procedure that indicated the change in mRNA expression level of the *HTR2C* in response to treatment in SH-SY5Y cells, but also confirmed the role of DNA methylation in the regulation of *HTR2C* gene expression. It should be noted that the effect of 5-Aza-2-deoxycytidine on the expression of *HTR2C* mRNA in undifferentiated cells was higher than in differentiated cells (approximately 5 fold higher). In addition, the effect of 5-Aza-2-deoxycytidine on methylation showed levels approximately 10% lower in undifferentiated cells compared to differentiated cells. This may be due to the high potential for changes in DNA methylation in undifferentiated cells and/or that the differentiated cells have a much less dynamic transition in gene expression pattern when comparing the differentiated state to pluripotency (reviewed by Meissner, 2010).

5-aza-2-deoxycytidine and 5-azacytidine are epigenetic drugs that inhibit DNA methylation and have been widely studied especially in cancer research. 5-aza-2-deoxycytidine and 5-azacytidine were first synthesized about 50 years ago and recently used in clinical as standards of care for patient with myelodysplastic syndromes because of its antimetabolic activities which can lower malignant progression to acute myeloid leukemia and increase survival of patients (Estey, 2013). There are many other diseases treated with 5-Azacytidine such as beta-thalassemia, sickle cell anemia, leukemias, metastatic lung cancer, androgen insensitive prostate cancer, cervical cancer, testicular cancer, colorectal, head and neck, renal malignant melanoma, ovarian cancer (Santini *et al.*, 2001; Wongtrakoongate, 2015). Since these drugs are already in clinical use, and this study has shown that 5-aza-2-deoxycytidine can modify DNA methylation and change mRNA expression of *HTR2C* in this study, it might be useful in the future for alleviation of weight gain side effects following antipsychotic drug treatment.

4.4.3 Effect of antipsychotic drugs on *HTR2C* mRNA expression

Clozapine, an atypical antipsychotic drug having a high affinity for the 5-HT2C receptor was expected to have an effect on 5-HT2C receptor comparing to haloperidol which has a low affinity to 5-HT2C receptor. The results in the present study show no alteration in the *HTR2C* mRNA expression after clozapine and haloperidol treatment for 48 hours in both undifferentiated and differentiated SH-SH5Y cells. The lack of an effect of clozapine at the mRNA level suggests that clozapine may affect the 5-HT2C receptor at the level of translational or post-transcriptional regulation, whereas unaltered expression of the *HTR2C* mRNA after haloperidol treatment might be due to its low affinity for this receptor (Leysen *et al.*, 1993).

The results in this study are consistent with previous studies in rats; an early study reported that chronic treatment with clozapine and haloperidol for 14 days did not change *Htr2c* mRNA levels in choroid plexus or other areas (Burnet *et al.*, 1996). Another study did not find a change in the levels of mRNA encoding the 5-Ht2c receptor in midbrain (containing choroid plexus cells) and also in whole brain of rats after chronic clozapine treatment for 32 days (Buckland *et al.*, 1997). However, chronic

treatment with clozapine for 14 days caused down-regulation of the 5-HT2C receptor binding in the choroid plexus, while no change was found with haloperidol, chlorpromazine or risperidone treatment (Kuoppamaki *et al.,* 1993, 1995). It is has been suggested that the decrease in 5-HT2C receptor binding can be explained by translational or post-transcriptional regulation (Burnet *et al.,* 1996).

However, the results in the present study are not in line with several previous studies that reported an effect of antipsychotic drug treatment on mRNA expression of *Htr2c* in rat brain. One study found a reduction of mRNA levels of *Htr2c* in hippocampus, cerebellum and cortex after chronic treatment of clozapine whereas chronic haloperidol treatment elicited a decrease in mRNA levels of *Htr2c* in midbrain, cerebellum and cortex (Buckland *et al.,* 1997). Another study found changes of *Htr2c* mRNA levels in many brain areas after chronic clozapine treatment for 36 days, after 2 hours of last drug administration there was a decrease of *Htr2c* mRNA levels in posteromedial cortical amygdaloid nucleus (PMCo)(limbic system) and substantia nigra (SN)(brainstem), while at 48 hours after the drug withdrawal there was a decrease mRNA levels in PMCo, SN, superior colliculus and ventral tegmental area; haloperidol caused mRNA reduction only in SN at both 2 hours and 48 hours drug withdrawal (Huang *et al.,* 2007). These findings indicate the region-specific and complex pattern of changes of *Htr2c* mRNA expression following antipsychotic treatment.

The effect of acute treatment of clozapine also has been reported by Buckland's group, acute treatment with clozapine (4 days) caused increased *Htr2c* mRNA levels in whole brain and also midbrain whereas *Htr2c* mRNA levels decreased in cerebellum and cortex. The transient rise in *Htr2c* mRNA levels may be explained by a classical compensation in response to initial antagonism of the receptor by clozapine leading to up-regulation of 5-Ht2c receptor by increasing transcription (Buckland *et al.,* 1997).

The different findings between the present study and these studies may be due to the difference of experimental model. Those studies were conducted in animal models that may have many other factors that affect the expression of the *Htr2c* gene for example female hormones can influence *Htr2c* mRNA (Zhou *et al.,* 2002; Rivera *et al.,* 2012) and protein expression (Henderson and Bethea, 2008). The neuroblastoma SH-SY5Y cell line used in the current study may provide a good model to study the direct

effect of antipsychotic drugs on *HTR2C* expression because of its relevance to human neurons and the reproducible level of *HTR2C* mRNA expression reported in these cells (Biedler *et al.,* 1978; Cavarec *et al.,* 2013); but, 5-HT2C receptor protein expression in this cell line has not been reported so far. However, because it is a neuroblastoma cell line, the properties of the cells may differ from normal neurons and may cause different response to antipsychotic drugs. Further studies are required to confirm this finding.

4.4.4 Effect of antipsychotic drugs on DNA methylation of *HTR2C* promoter sequences

Clozapine and haloperidol treatments resulted in unaltered levels of DNA methylation of the *HTR2C* promoter sequences. These results were in accordance with the mRNA expression results showing that these tested antipsychotic drugs did not exert their effect on the expression of *HTR2C* mRNA. Lack of influence on DNA methylation and mRNA expression suggests that clozapine may have an effect on post-transcriptional regulation rather than transcriptional regulation, while haloperidol did not have any effect due to its low affinity to 5-HT2C receptor. Post-transcriptional modification of *HTR2C* mRNA editing in SH-SY5Y by adenosine deaminases acting on RNA was reported by Cavarec *et al.,* (2013); this regulation was altered in depressed suicide victims. This adenosine deamination editing mRNA of 5-HT2CR brings about a substantial increase in the functional plasticity of the receptor and is thought to contribute to homeostatic mechanisms in neurons (Di Narzo *et al.,* 2014). No other studies have looked at the effect of antipsychotic drugs on DNA methylation of the *HTR2C* gene. Further studies are required to confirm the effect of antipsychotic drugs on *HTR2C* methylation.

4.4.5 Limitations

The limitation in this study is the low expression levels of the *HTR2C* mRNA. Although it has been shown that there is constitutive expression of the *HTR2C* in this cell line, SH-SY5Y human neuroblastoma cells (Biedler *et al.,* 1978; Flomen *et al.,* 2004; Cavarec *et al.,* 2013), it may limit the magnitude of alteration in both DNA methylation and mRNA expression. However, the SH-SY5Y cells response to treatment at least with the 5-aza-2-deoxycytidine and the changes in methylation and mRNA expression of *HTR2C* gene can be detected.

4.4.6 Conclusions / further work

In summary, the results of this chapter have shown no alteration of *HTR2C* mRNA expression and DNA methylation of the *HTR2C* promoter under treatment with clozapine and haloperidol. However, the results linked the increased mRNA expression of *HTR2C* and induced DNA hypomethylation after treatment with 5-aza-2-deoxycytidine. This observation might be relevant to further therapeutic development to increase the receptor numbers in patients with schizophrenia.

Chapter 5: Effect of antipsychotics on leptin secretion and Htr2c mRNA expression in 3T3-L1 adipocytes

5.1 Introduction

Weight gain is an important side effect of antipsychotic medication, which increase a risk for developing chronic diseases or obesity-related complications such as type 2 diabetes and cardiovascular diseases (Allison *et al.,* 1999; Casey *et al.,* 2004). Little is currently known about the mechanisms of antipsychotic drug-induced weight gain. One common hypothesis is an increase in appetite and food intake (Casey and Zorn, 2001). In this context, many studies have investigated the role of leptin in regulating appetite and adiposity during treatment with antipsychotic drugs.

Leptin is primarily secreted by adipocytes (Hamilton *et al.,* 1995) which is a major source of plasma leptin. The plasma leptin level is strongly and positively correlated with adiposity or BMI (Klein *et al.,* 1996; Haupt *et al.,* 2005; Venkatasubramanian *et al.,* 2010). The plasma leptin level is gender dependent with females normally having higher leptin levels than males (Wang *et al.,* 2007b; Sentissi *et al.,* 2009). This may explain the relationship of adiposity and leptin levels because females have a higher percentage of adipose mass than men (Ranasinghe *et al.,* 2013). Another explanation may be due to the effect of sex hormones on leptin production. There is evidence that estrogen stimulates leptin expression in females, whereas testosterone levels in males are correlated with decreased leptin levels (Wabitsch *et al.,* 1997; Machinal *et al.,* 1999; Machinal-Quelin *et al.,* 2002). However, this gender difference in leptin has been found in groups treated with conventional antipsychotics but not in the olanzapine or clozapine treatment groups (Melkersson and Hulting, 2001).

Increased plasma leptin levels during treatment with atypical antipsychotic drugs, particularly olanzapine and clozapine have been reported in several studies. For example, olanzapine treatment causes increased BMI, plasma leptin, adiponectin, insulin, lipids, and neuropeptides (Melkersson *et al.*, 2000; Amano *et al.,* 2012; Ak *et al.,* 2013). Plasma leptin levels and body weight increase rapidly in the first 1-2 weeks

after treatment with SGAs, but not FGAs haloperidol (Kraus *et al.,* 1999; Monteleone *et al.,* 2002; Sentissi *et al.,* 2008), and was also observed after 6 weeks of treatment (Atmaca *et al.,* 2003). Increased plasma leptin levels correlate with increased BMI after treatment with clozapine or olanzapine for 6 weeks (Kluge *et al.,* 2009). Increased leptin levels, body weight, BMI, body fat mass, and lean body mass after 10 weeks of clozapine treatment has been reported (Bromel *et al.,* 1998).

Risperidone is one of the second generation antipsychotic drug that cause weight gain following treatment in schizophrenia patients (Goeb *et al.,* 2010) and it also has been related to the increase in plasma leptin levels (Zhang *et al.,* 2003, 2004). Risperidone treatment for 4 weeks increases BMI and plasma leptin in psychotic patients (Yanik *et al.,* 2013). It has been hypothesized that antipsychotic drugs including risperidone induced weight gain may be in part, due to its action through 5-HT antagonism that alter hypothalamic neuropeptides regulating appetite and food intake (Kursungoz *et al.,* 2015).

Although an early increase in plasma leptin levels is observed after antipsychotic treatment, it has been suggested that the increased leptin levels are most likely due to weight gain during antipsychotic drug treatment rather than a direct effect of antipsychotic drugs on leptin production and release from adipocytes (Bromel *et al.,* 1998; Kraus *et al.,* 1999; Melkersson *et al.*, 2000). The correlation of plasma leptin and antipsychotic treatment does not appear after controlling for BMI or comparing to BMI-matched controls (Haupt *et al.,* 2005; Jin *et al.,* 2008). In this study, it has also been hypothesized that antipsychotic drugs including clozapine and risperidone treatment might alter leptin secretion from adipocytes comparing to haloperidol.

Genetic polymorphisms have also been reported to be associated with elevation of plasma leptin levels during antipsychotic medications. The SNP in the promoter region of the leptin gene, -2548A/G, is associated with obesity and plasma leptin levels (Mammes *et al.,* 2000; Hinuy *et al.,* 2008). The polymorphisms of leptin -2548A/G and the *HTR2C* -759C/T are associated with antipsychotic drug-induced weight gain (Templeman *et al.,* 2005; Wu *et al.,* 2011) and influence plasma leptin levels in schizophrenia patients (Templeman *et al.,* 2005).

The expression of 5-HT receptors including 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1F, 5-HT2A, 5-HT2C, 5-HT5A, 5-HT6, and 5-HT7 have been detected in mouse preadipocyte 3T3-L1 cells and treatment with 5-HT enhances adipocyte differentiation while treatment with the 5-HT2A antagonist (ketanserin) and the 5-HT2C receptor antagonist (SB 242084) result in inhibited adipocyte differentiation (Kinoshita *et al.,* 2010). This study identified 5-HT in adipose tissue as a novel autocrine factor that is required for adipocyte differentiation (Kinoshita *et al.,* 2010). The role of the 5-HT2C receptor in adipocyte differentiation and a decrease of this process when treated with a 5-HT2C receptor antagonist indicate the role of the 5-HT2C receptor in adipogenesis. The functional system for serotonin synthesis, reuptake, and receptors including 5-HT2C and 5-HT2A receptors are also expressed in rat adipocytes and peripheral serotonin administration for 5 days or longer treatment for 4 months results in decreased plasma leptin (Stunes *et al.,* 2011); however, this reduction of leptin levels could be secondary effect of serotonin decreasing insulin secretion from pancreatic β cells (Zhang *et al.,* 2013c). The role of the 5-HT2C receptor on leptin secretion and the direct effect of antipsychotic drugs on the expression of the 5-HT2C receptor in adipocytes as well as on leptin secretion in adipocytes have not been studied yet. The present study hypothesizes that treating adipocytes with antipsychotic drugs may change the mRNA expression of the *HTR2C* and also alters leptin secretion. The results of this study may provide data about the peripheral effect of atypical antipsychotic drugs on weight gain.

5.1.1 Principle of ELISA in leptin secretion determination

An enzyme-linked immunosorbent assay (ELISA) is a technique used in this study for determining leptin concentration in culture medium. It is a quantitative sandwich enzyme immunoassay using two polyclonal antibodies specific to mouse/rat leptin. The plate surface is pre-coated with the polyclonal antibody specific for mouse/rat leptin. The leptin in samples is bounded by the immobilized antibodies (**Figure 5.1, step 1**). The unbound substances arewashed away before adding the enzyme-linked polyclonal antibody specific for mouse/rat leptin (**Figure 5.1, step 2**). The enzyme used in this assay kit is horseradish peroxidase (HRP). The unbound enzyme-linked antibodies have been washed away before adding the substrate solution to the wells. The substrate solution contains tetramethylbenzidine and hydrogen peroxide. The enzyme reaction yields a blue product of tetramethylabenzidine diimine which turns

yellow when adding stop solution containing diluted hydrochloric acid (**Figure 5.1, step 3**).

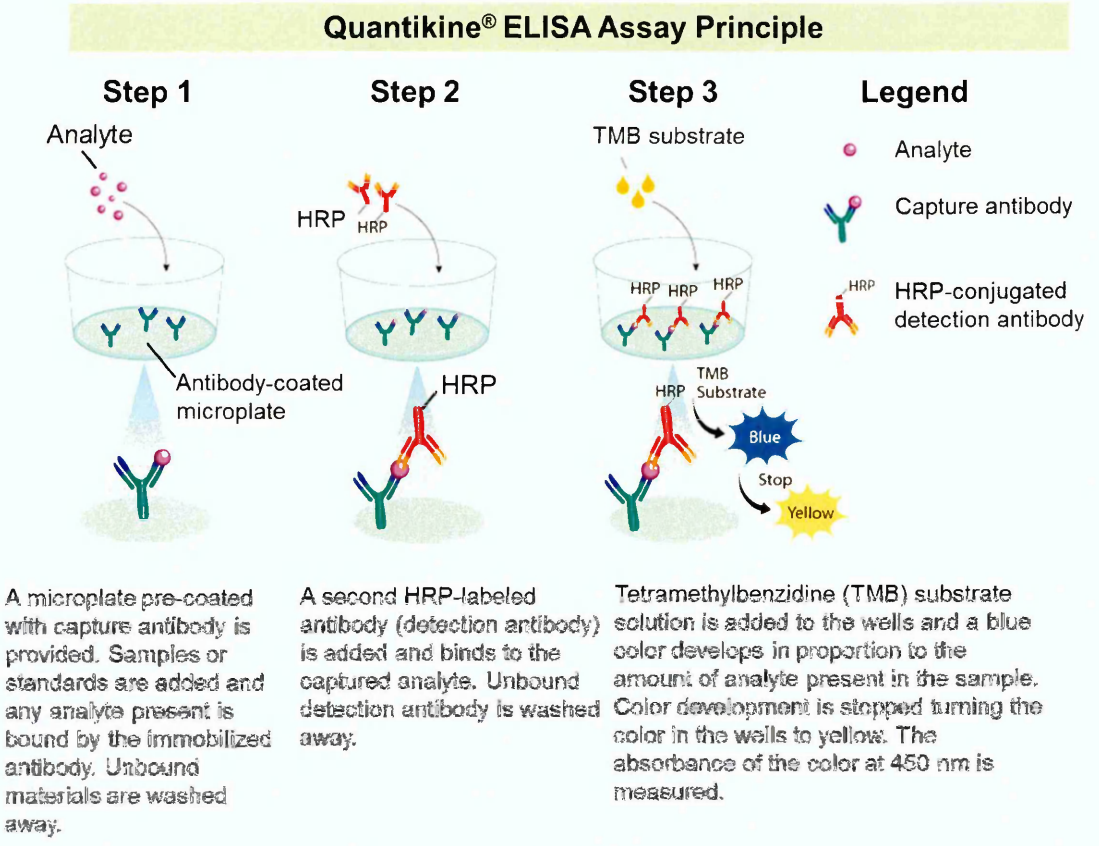


Figure 5.1: ELISA assay principle

(modified from R&D Systems™ website; www.rndsystems.com/product_detail_objectname_quantikineelisaassayprinciple.aspx) HRP, horseradish peroxidase; TMB, tetramethylbenzidine.

5.1.2 Aims

1. To investigate the influence of antipsychotic drugs on leptin secretion from 3T3-L1 adipocytes.

2. To investigate the influence of antipsychotic drugs on *Htr2c* mRNA expression in 3T3-L1 adipocytes.

5.2 Materials and methods

The mouse 3T3-L1 preadipocyte cell line was cultured and induced by differentiation to be mature adipocyte cells for using in antipsychotic drug treatment experiments. Sterile cell culture techniques were performed to avoid the contamination throughout the experimental period. The leptin concentration in the culture media was measured using ELISA and the mRNA expression of the *Htr2c* was determined using RT-qPCR. The experiments were carried out at least 3 times with two replicates for all experimental conditions.

5.2.1 3T3-L1 preadipocyte cell line

The mouse 3T3-L1 preadipocyte cell line obtained from the American Type Culture Collection (ATCC, UK), was maintained in DMEM high glucose (Gibco®, UK) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (Complete Medium or CM) in a humidified, 5% CO_2, 37°C incubator. The medium was changed every 2-3 days. Cells were subcultured before 70-80% confluency was reached. Cell passage numbers of less than 6 were used in the experiments.

Cells were cultured in 75 cm^2 culture flasks in 15 ml medium until 70-80% confluency was reached, cells then were trypsinized using 2-3 ml of trypsin/EDTA solution (0.05% trypsin in 0.53mM EDTA, Gibco®, UK) after removing medium and briefly rinsing the cell layer with trypsin/EDTA solution. The flask was incubated at 37°C for 3-5 minutes or until cells have detached from the surface. Once the cells' detachment was checked under a microscope, 6-8 ml of complete medium was added to inhibit the action of trypsin. The cell suspension was transferred to a sterile 50 ml Falcon tube and centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 2-5 ml fresh complete medium. The cells were counted by mixing 10 µl of cell suspension with 10 µl of 0.4% trypan blue solution (Gibco®, UK) and the cells were allowed to stain at room temperature for 1 minute. The mixture (10 µl) was loaded into a Countess™ chamber slide (Countess™, Invitrogen, UK). Viable and dead cells were counted using an automated cell counter (Countess™, Invitrogen, UK). Live cells are not coloured whereas dead cells are stained blue. Total cell count, live cells, dead cells, cell viability, were obtained from the instrument. An average of live cell

number was calculated from two slides and the viable cells were used in plating for the experiments, subculturing, or freezing.

5.2.2 Freezing and thawing

Mouse 3T3-L1 preadipocytes (T75 cm^2 flask) were trpysinized, and resuspended in 3 ml of freezing medium containing 90% fetal bovine serum and 10% DMSO. Then 1 ml of cell suspension (1-1.5x10^6 cells) was aliquoted into cryovial tubes. The tubes were wrapped with tissue and plastic bubble wrap then stored at -80°C for several days before transferring the tubes into a liquid nitrogen tank where the cells were immersed in liquid nitrogen vapour until required for use.

All pieces of equipment for cell culture, including a tube containing culture medium was prepared (9-10 ml in 50 ml-Falcon tube) in a laminar flow tissue culture hood before taking the frozen cells from liquid nitrogen. The cryovial tube of cells was agitated gently in a water bath at 37°C to thaw cells quickly, within 2-3 minutes. Once the ice crystals were melted, the tube was decontaminated by spraying 70% ethanol. Cells were transferred into the prepared tube containing medium, gently mixed and centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded to remove DMSO and the cells were resuspended in 1-2 ml of complete medium before transferring to a culture flask (T75 cm^2) containing complete medium and mixing thoroughly by gentle rocking. The cultures were observed after 24 hours.

5.2.3 Differentiation induction of 3T3-L1 cells

To differentiate 3T3-L1 cells from fibroblastic phenotype to adipocytes, the first stage is growth arrest which is achieved by contact inhibition at post confluence. The growth-arrested postconfluent 3T3-L1 preadipocytes re-enter the cell cycle (called mitotic clonal expansion) immediately after induction by the differentiating reagents, and start the adipocyte differentiation (Rosen and Spiegelman, 2000). The most commonly used reagents are insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX). Rosiglitazone was also added in the induction medium in the present study. These chemicals were purchased from Sigma-Aldrich, UK. The differentiation induction was performed according to the methods described by Eseberri *et al.* (2013) with slight modifications. Briefly, cells were seeded into 6-well plate at a density of 5x10^4

cells/well (in 2 ml). The complete medium was replaced every 48 hours until cells reached confluency which took about 5-7 days. On the day that cells reached confluency, the medium was replaced and the cells further grown for 48 hours. After 48 hours (day 0), differentiation was induced by changing the medium to DMEM containing 10% FBS, 1% Penicillin/Streptomycin, 0.5 mM IBMX, 1 µM dexamethasone, 10 µg/ml insulin, and 2 µM rosiglitazone (differentiation medium, DMI). After 48 h (day 2), the medium was changed to DMEM containing 10% fetal bovine serum, 1% Penicillin/Streptomycin, and 10 µg/ml insulin (DMII) and this medium was refreshed every 48 hours until drug treatment on day 14, which represents the usual time required to acquire a fully differentiated phenotype (Sertie *et al.,* 2011). The protocol for differentiation induction was shown in **Figure 5.2**.

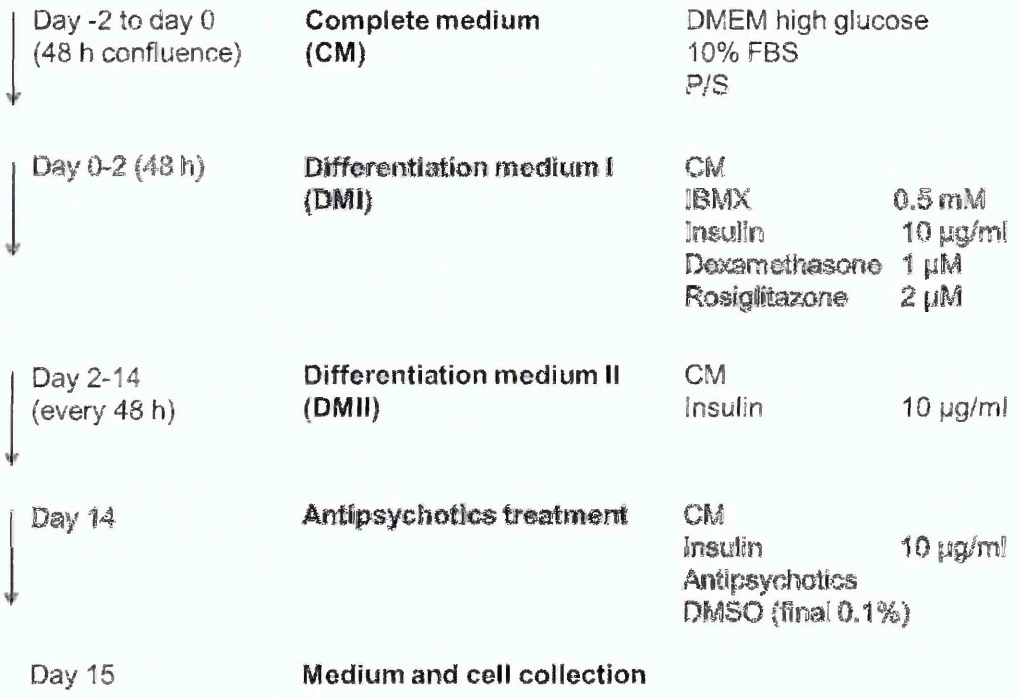


Figure 5.2: Flowchart of 3T3-L1 differentiation protocol throughout the experimental period

5.2.4 Oil Red O staining

In this study, oil Red O staining was performed to illustrate the presence of lipids in the 3T3-L1 differentiated adipocytes which is used as a marker of differentiation, for further details, see **section 5.3.1**. The oil red O is an oil-soluble dye which has high solubility in fatty substances. The staining results in bright red of lipid droplets in

adipocytes when observed under microscope. Staining protocol was carried out according to the method described elsewhere (Oh *et al.,* 2012; Mukherjee and Yun, 2013) with slight modifications. In brief, 0.35% Oil Red O stock solution was prepared by stirring 0.7g Oil Red O in 200 ml isopropanol overnight. The overnight solution was filtered through a 0.2-µM filter paper and stored at 4°C. Oil Red O working solution was freshly prepared by mixing stock solution with distilled water (6:4), followed by incubation at room temperature for 10 min and further filtration. The working solution was stable in 2 hours. Cells were washed twice with PBS and fixed with 4% formaldehyde in PBS at room temperature for 1 hour. Cells were washed 3 times with distilled water, followed by 60% isopropanol, and then stained with Oil Red O working solution for 1 hour at room temperature. Cells were then washed 4 times or more with distilled water and photographed.

5.2.5 Antipsychotic treatment

Differentiated cells were treated with antipsychotic drugs on day 14 after differentiation induction when the full differentiation was obtained. The antipsychotic drugs were dissolved in complete medium containing insulin 10 µg/ml. The treatment volume was 1 ml/well. The control treatment group was carried out in each experiment using the same amount of DMSO (0.01%) that has been used as a solvent of drug dissolves. The amount of DMSO in all drug concentrations was equal concentration (at 0.1%) to eliminate the effect of unequal DMSO levels in different drug concentrations.

Antipsychotics used in this study were composed of clozapine, SB 242084 (serotonin 2C receptor antagonist), haloperidol, and risperidone which were purchased from Sigma-Aldrich, UK. The cells were treated for 24 hours before the medium was collected. Then the collected medium was centrifuged at 3,000 rpm for 30 minutes to remove remaining particles or cell debris. The supernatants were assayed to determine leptin level immediately or stored at -20° C for later use, in which case repeated freeze-thaw cycles were avoided.

5.2.5.1 *Dose response curve of antipsychotic treatment on leptin secretion*

The adipocyte cells were treated with various concentrations of antipsychotics including clozapine, SB 242084, haloperidol, and risperidone as shown in **Table 5.1**. The chosen concentrations covered the therapeutic plasma levels and the inhibition constant (Ki). All of the cell culture experiments were performed in duplicate.

Table 5.1: Concentrations of antipsychotic drugs used in this study

Antipsychotics/chemicals	Concentrations
Clozapine	0.3, 1, 10 µM
SB 242084	0.01, 0.1, 1 µM
Haloperidol	0.03, 0.1, 1 µM
Risperidone	0.01, 0.03, 0.3 µM

5.2.6 Determination of leptin secretion

The concentrations of leptin secreted from adipocytes into the cell culture medium were measured by using the mouse leptin immunoassay (Mouse Leptin Quantikine® ELISA Kit, from R&D SYSTEMS, UK). The assay procedures were performed following the manufacturer's instructions. In brief, all reagents and samples were allowed to warm at room temperature before performing the assay, and then the standard dilutions, wash buffer, and microplate strips were prepared. The serial dilutions of mouse leptin standard was performed at concentrations 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/ml with calibrator diluent RD5-3.

The assay was started by adding 50 µl of assay diluent RD1W to each well followed by adding of 50 µl of standards, control, or samples. The mixture was gently mixed by tapping the plate frame for 1 minute, and then the plate was covered by an adhesive strip before incubating for 2 hours at room temperature. The mixture was aspirated from the wells and the wells were washed five times with wash buffer using a squirt bottle. After the last wash, the remaining liquid or wash buffer was completely removed using aspiration pump followed by inverting the plate and blotting it against clean paper towels. Then 100 µl of mouse leptin conjugate was added into each well before covering with a new adhesive strip, and the plate was incubated for 2 hours at

room temperature. The mixture was aspirated from the wells and the wells were washed five times with wash buffer using a squirt bottle. Then 100 µl of substrate solution was added into each well followed by incubation at room temperature for 30 minutes protecting from light. 100 µl of stop solution was added into each well. The plate was mixed thoroughly by gently tapping before the optical density was read at 450 nm within 30 minutes using microplate reader. The readings at 450 nm were corrected using 570 nm readings which corrected for optical imperfections in the plate. All samples were assayed in duplicate.

5.2.7 Determination of *Htr2c* mRNA expression

5.2.7.1 RNA isolation and cDNA synthesis

After the culture medium was collected to determine leptin level, cells were harvested by trypsinization and washed with PBS before extracting total RNA using RNeasy Mini kit (Qiagen). The extraction protocols were carried out following the manufacturer's protocol as described in the previous chapter (**see section 4.2.6.1**).

RNA concentrations were quantified by Nanodrop-1000 UV-VIS spectrophotometer and A_{260}/A_{280} as well as A_{260}/A_{230} ratios were recorded. RNA quality was checked on 1.5% agarose gel as described before (**see section 4.2.6.2**).

Total RNA, 1000 ng, was reverse-transcribed using oligodT and random primers (QuantiTech Reverse Transcription kit, Qiagen) according the manufacturer's protocols as described in the previous chapter (**see section 4.2.6.3**). The minus RT (-RT) controls were performed by replacing reverse transcriptase with RNase-DNase free water during the RT step to serve as negative control to assess to contamination of genomic DNA in RNA samples. In general, cDNA samples were diluted 1:10 in RNase-DNase free water before using in qPCR.

5.2.7.2 Reference control gene(s) selection

Reference control gene selection was carried out using 8 housekeeping genes from the mouse geNorm™ housekeeping gene selection kit (PrimerDesign Ltd, UK) and 11 samples from different antipsychotic drug treatments and control untreated group. Candidate genes are listed in **Table 5.2**. The genes from this geNorm kit were assessed

because the primer sets were guaranteed by the company to have high efficiencies. The genes included members from distinct cellular pathways and they are groups of a number of classical housekeeping genes which are in common use in expression studies.

Table 5.2: geNorm housekeeping gene candidates for selecting of the most stable expressing gene(s) to use as a reference control gene(s)

Gene symbol	Gene name	Function/Pathway/Process
Actb	β-actin	Cytoskeletal protein
Gapdh	Glyceraldehide-3-phosphate dehydrogenase	Glycolysis
Canx	Calnexin	Protein folding
Atp5b	ATP synthase subunit 5B	Mitochondrial ATP synthesis
18s	18S ribosomal RNA	Protein synthesis
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	Signal transduction
Rpl13a	Ribosomal protein L13A	Protein synthesis
Ubc	Ubiquitin C	Protein turnover

The PCR reactions were set up in a 96-well PCR plate. The reactions were then run in a real-time PCR system, StepOne Plus (Applied Biosystems, USA). The PCR reaction mixture consisted of 10 ng of cDNA template (2 µl of 1:10 dilution), 1 µl of 10 µM forward and reverse primers, 10 µl of 2x qPCR Mastermix (Precision, PromerDesign) and nuclease-free distilled water to a final volume of 20 µl. The precision Mastermix contains 2x reaction buffer, 0.025 U/µl Taq Polymerase, 5 mM $MgCl_2$, dNTP mix (200 µM each dNTP), and SYBR®Green and ROX passive reference dye. The PCR condition was set as shown in **Table 5.3**. The stability of expression of candidate reference genes were compared using geNorm software Demo version 2.6.1.

Table 5.3: PCR condition for selecting the most stable expressing housekeeping gene(s)

Step	Time	Temperature	
Enzyme activation	10 min	95°C	
Denaturation	15 s	95°C	Cycling x40
Data collection	60 s	60°C	
Melt curve	15 s	95°C	
	30 s	60°C increasing in 0.3°C increment till 95°C	

5.2.7.3 Determination of primer efficiency

The amplification efficiencies of primers used in this expression study including *Htr2c* and the most stable expressing housekeeping genes identified by geNorm including *Actb*, *Ywhaz*, and *Canx* were determined. Serial dilutions of cDNA samples were used to determine primer efficiency as described in the previous chapter (**section 4.2.6.5**). The primer sequences for *Htr2c*, *Actb*, *Ywhaz*, and *Canx* are listed in **Table 5.4**.

Table 5.4: Nucleotide sequences of primers used in *Htr2c* expression in 3T3-L1 cells

Gene	Primer sequences	Product length
Htr2c	Forward 5'-TCTCCCTTCCTTCCGTATTCC-3' (21bp) Reverse 5'-ACATCAACTTTTCCACATTCACAA-3' (24bp)	95 bp
Actb	Forward 5'-CCTGTGCTGCTCACCGAGGC-3' (20bp) Reverse 5'-GACCCCGTCTCTCCGGAGTCCATC-3' (24bp)	174 bp
Ywhaz	Forward 5'-AAAAACAGCTTTCGATGAAGCC-3' (22bp) Reverse 5'-GCCGGTTAATTTTCCCCTCC-3' (20bp)	168 bp
Canx	No data	

5.2.7.4 Determination of Htr2c gene expression

The PCR reaction and PCR condition were set as described in the previous section in determining of reference genes. The PCR conditions for determination of *Htr2c* expression were slightly different (**Table 5.5**). Following qPCR, melt curve analysis and agarose gel electrophoresis were carried out to identify the PCR product.

Table 5.5: PCR condition for determining of the *Htr2c* expression

Step	Time	Temperature	
Enzyme activation	10 min	95°C	
Denaturation	15 s	95°C	Cycling x40
Annealing	30 s	62°C	
Data collection	30 s	72°C	
Melt curve	15 s	95°C	
	30 s	60°C increasing in 0.3°C increment till 95°C	

5.2.8 Statistical Analyses

The effects of antipsychotic drugs on leptin secretion and *Htr2c* gene expression were tested using SPSS for Windows. Leptin secretion was expressed as a percentage of control (untreated) group. The expression of *Htr2c* was expressed as fold change comparing to control untreated group. The normal distribution was tested by using the Kolmogorov–Smirnov test. One-way ANOVA was used to compare means between multiple groups when the values were normally distributed with either Bonferroni post hoc test when variances were equal or Tamhane's T2 when variances were not equal. Kruskal-Wallis test was used for non-parametric data when the values were not normally distributed. Statistical significance was considered when P value was less than 0.05.

5.3 Results

5.3.1 Differentiation induction of 3T3-L1 preadipocytes to mature adipocytes

The morphology of a 3T3-L1 preadipocyte cell line was changed after inducing differentiation from fibroblast-like cells to mature adipocytes. The process of differentiation was easily observed under the microscope both with and without oil Red O staining. The differentiated cells show characteristics of lipid droplets. The intracellular lipid droplets started to be visible at around day 5 and increased in both number and size throughout the following days of induction. After day 12 of differentiation induction, the cells contained lipid droplets of different sizes and this differentiation state was not changed until at least day 16 of observation (**Figure 5.3**). The differentiated 3T3-L1 adipocyte cells at day 14 were chosen in the subsequent experiments of antipsychotic drug treatments to ensure the cells had been fully differentiated.

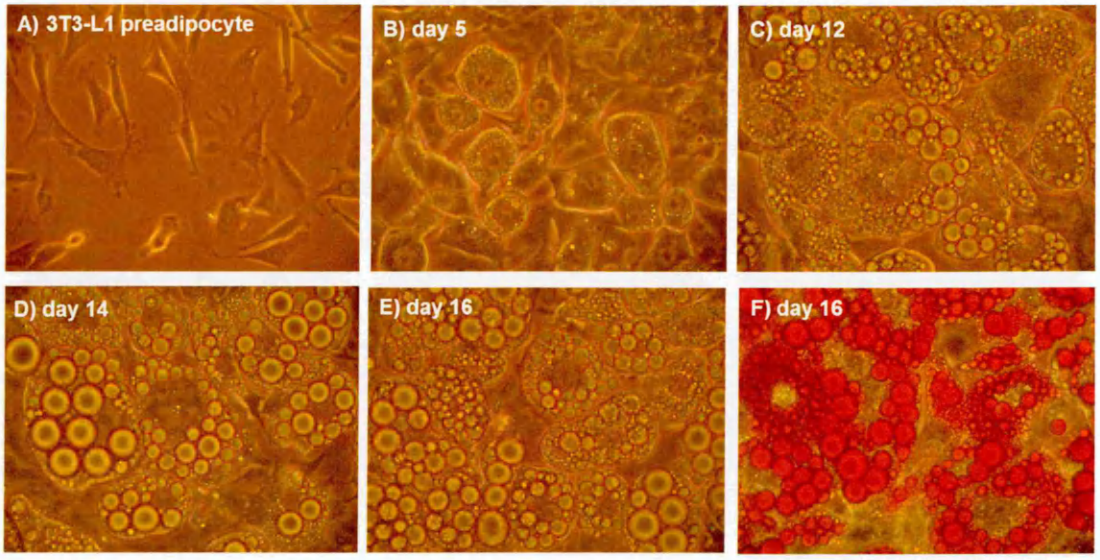


Figure 5.3: Morphology of 3T3-L1 cells

Figure shows the morphology of the 3T3-L1 preadipocyte cells before differentiation induction (A, x100), after differentiation induction at day 5 (B, x200), day 12 (C, x200), day 14 (D, x200), day 16 (E, x200), and oil red O staining at day 16 (F, x200).

5.3.2 Effect of antipsychotic treatment on leptin secretion from 3T3-L1 adipocytes

The effects of clozapine, SB 284104, risperidone, and haloperidol at various concentrations on the amount of leptin secreted from mature adipocyte cells were determined by ELISA assay. The medium containing the same amount of DMSO (0.1%DMSO) was carried out as a control group.

The clozapine treatment at concentrations of 0.3, 1, and 10 μM did not show significant change in leptin levels compared to the control group ($p>0.05$) (**Figure 5.4A**). Similar results were observed when treatment with SB 242084 at concentrations of 0.01, 0.1, and 1 μM (**Figure 5.4B**), risperidone treatment at concentrations of 0.03, 0.1, and 1 μM (**Figure 5.4C**), and haloperidol treatment at concentrations of 0.01, 0.03, and 0.3 μM(**Figure 5.4D**).

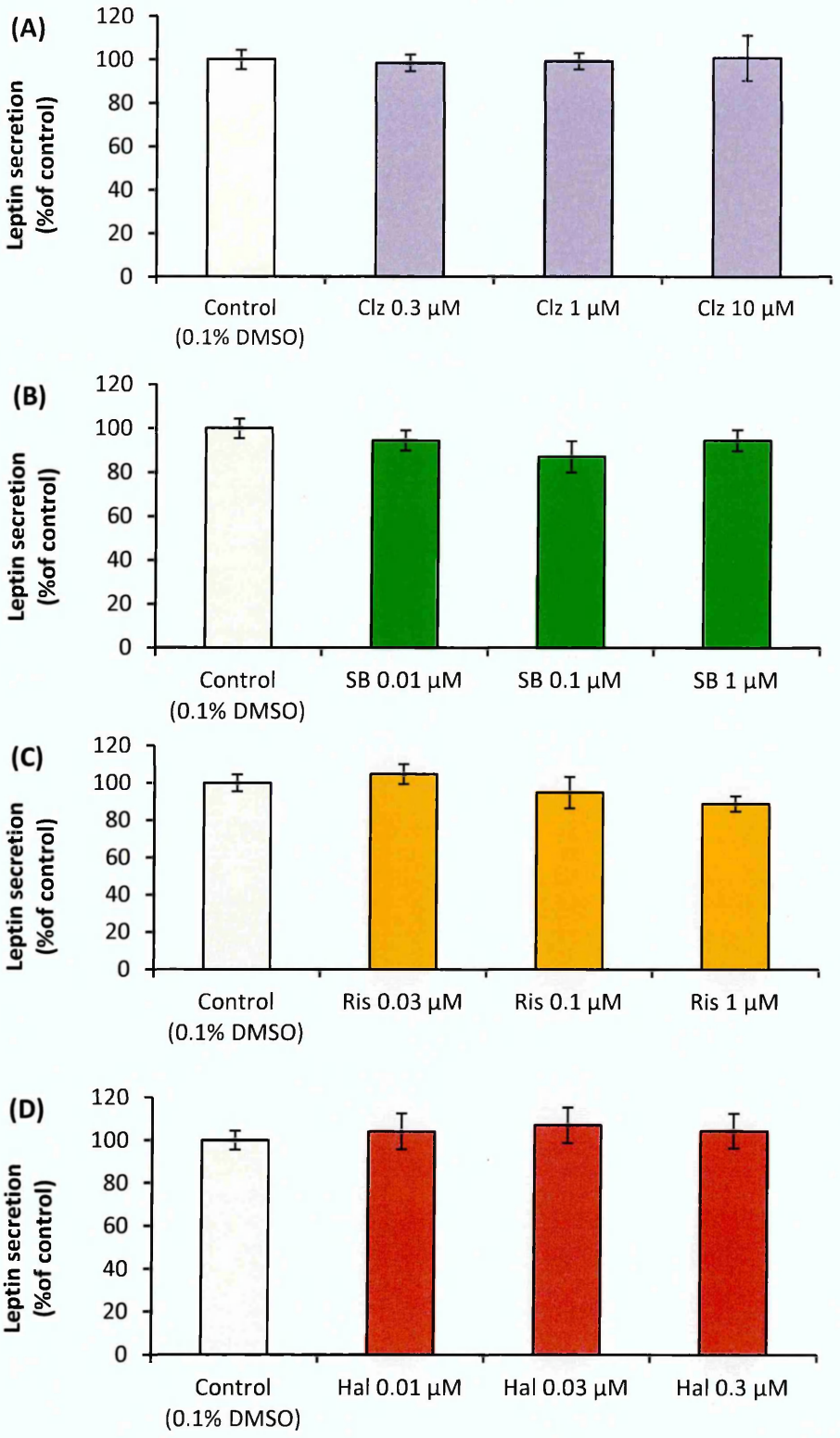


Figure 5.4: Effect of antipsychotic drug treatment on leptin secretion in differentiated 3T3-L1 cells

The figure shows the effect of antipsychotic drugs; clozapine (A), SB 242084 (B), risperidone (C), and haloperidol (D) on leptin amount in mature adipocyte 3T3-L1 cells. Data are expressed as mean percentage of control (±SEM), n=3-4.

5.3.3 Effect of antipsychotics treatment on *Htr2c* mRNA expression in 3T3-L1 adipocytes

5.3.3.1 RNA extraction

RNA was extracted from adipocyte 3T3-L1 cells at the end of antipsychotic drug treatment experiments. Extracted RNA concentration and purity were determined using Nanodrop spectrophotometry. The gel electrophoresis was performed to investigate the integrity of extracted RNA which presents both 28s and 18s rRNA bands indicating the good quality of RNA (**Figure 5.5**).

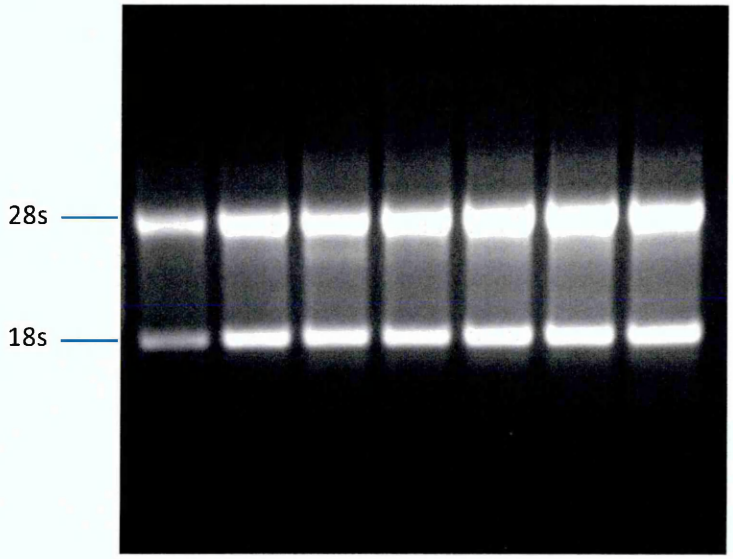
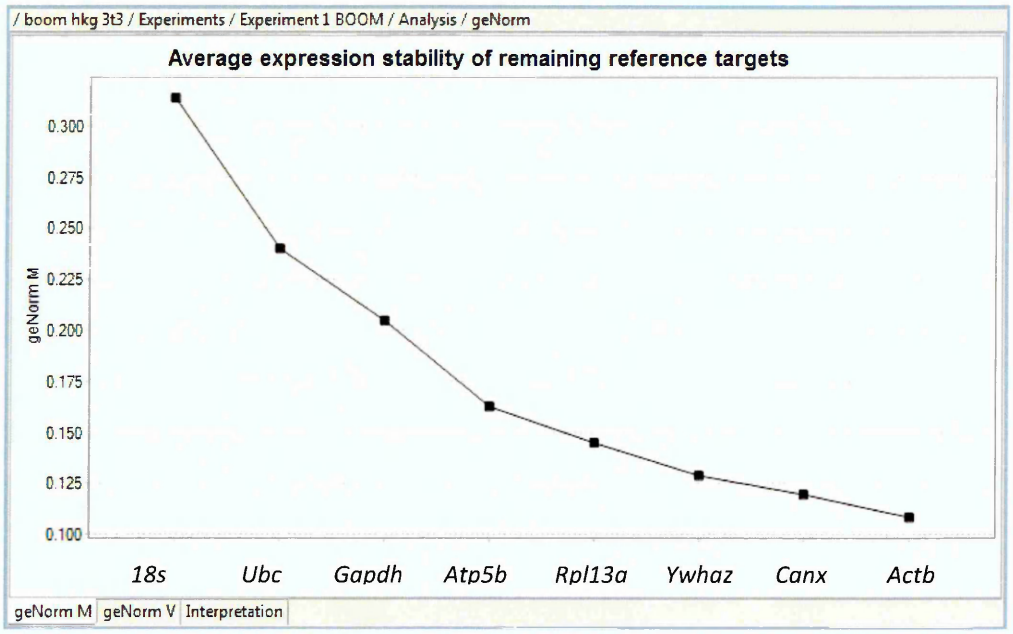


Figure 5.5: Agarose gel electrophoresis of RNA extracted from 3T3-L1 adipocytes
Figure shows clearly visible bands of 28s and 18s ribosomal RNA with the 2:1 ratio of 28s:18s indicating the good integrity of RNA extracted from 3T3-L1 adipocytes.

5.3.3.2 Reference gene(s) selection

The stability of expression of eight housekeeping genes was determined using GeNorm software. Eleven cDNA samples from different experimental conditions including control, clozapine, SB 242084, risperidone, and haloperidol treatments were used to determine reference genes. The GeNorm software calculates the expression ratio of each housekeeping gene from input Ct values. The M value calculated by the software indicates the stability of each housekeeping gene. The lowest M value is the most stable expressing gene. As shown in **Figure 5.6**, the *Actb*, *Canx*, and *Ywhaz* were the

most stably expressed housekeeping genes. The optimal number of housekeeping genes was represented as V value. The result of GeNorm V value indicates that 2 or 3 most stable genes (GeNorm V <0.15) were optimal number of selected housekeeping genes for using as the reference genes in this experimental situation (**Figure 5.7**).



Gene	*18s*	*Ubc*	*Gapdh*	*Atp5b*	*Rpl13a*	*Ywhaz*	*Canx*	*Actb*
M value	0.314	0.24	0.205	0.163	0.145	0.129	0.12	0.108

Figure 5.6: GeNorm M value of 8 housekeeping genes represented the stability of expression of each housekeeping gene

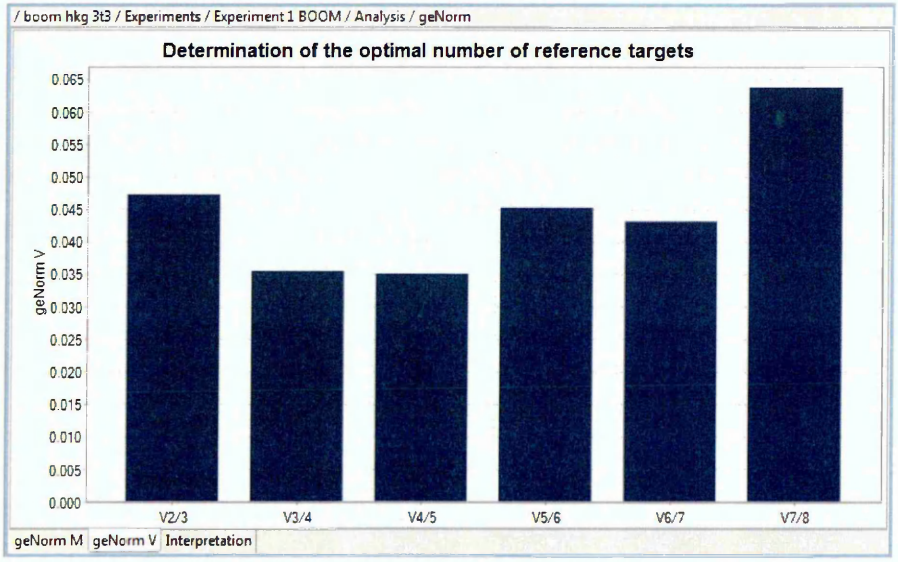


Figure 5.7: GeNorm V value indicated optimal number of reference genes

5.3.3.3 *Primer efficiency and specificity*

Serial dilutions of cDNA were amplified with the primer sets of the *Htr2c* and selected reference genes, *Actb*, *Canx*, and *Ywhaz* to determine their primer efficiencies. The Ct values of dilutions of each primer set were used to calculate the slope of the trend line when plotting Ct values against log cDNA concentrations (**Figure 5.8**). Then the primer efficiency of each primer set was calculated using the equation $\%E = (10^{(-1/slope)}-1) \times 100$. The percentage efficiencies of the *Htr2c, Actb*, *Canx*, and *Ywhaz* were 107.48%, 102.77%, 106.70%, and 102.49%, respectively.

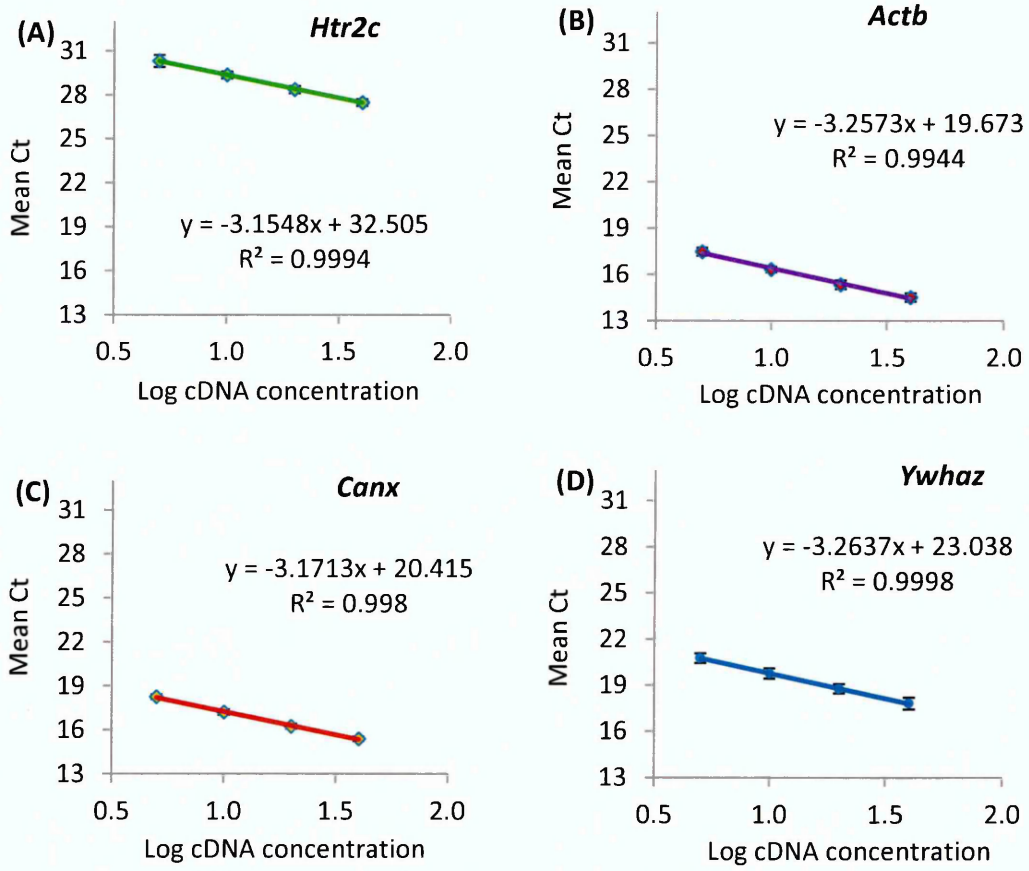


Figure 5.8: Efficiency of primer used in SYBR®Green RT-PCR in adipocyte 3T3-L1 cells
The Ct values of each dilution series of each transcript were plotted against log cDNA concentrations to obtain slope of the trend line of the *Htr2c* (A), *Actb* (B), *Canx* (C), and *Ywhaz* (D). The slope has been used in primer efficiency calculation. Data are expressed as mean ±SEM (n=3-4).

The specificity of each primer set was observed by melt curve analyses following RT-qPCR as shown in **Figure 5.9** and it was also observed by agarose gel electrophoresis (**Figure 5.10**). Each primer sets amplified one single product indicates that no non-specific PCR product was amplified.

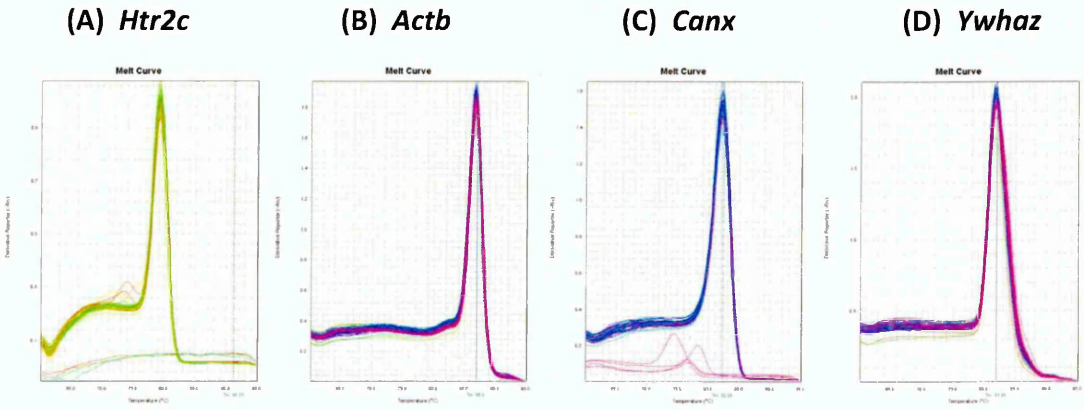


Figure 5.9: Melt curve of transcripts when amplified by different primer sets in adipocyte 3T3-L1 cells

Single peak of each transcript was obtained when amplified by primer sets of the *Htr2c* (A), *Actb* (B), *Canx* (C), and *Ywhaz* (D) in SYBR®Green real-time RT-PCR in adipocyte 3T3-L1 cells indicating the specificity of the primers used in this study.

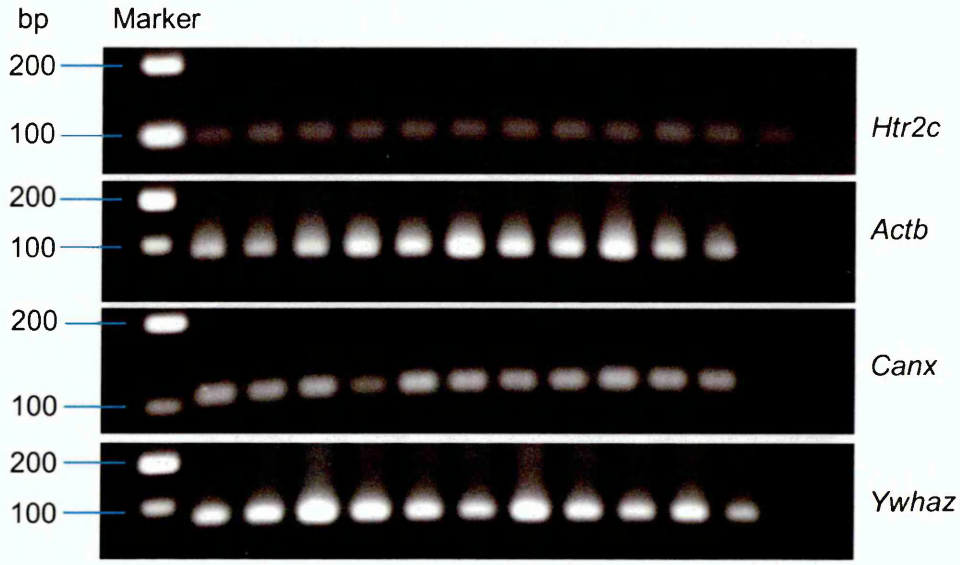


Figure 5.10: PCR products of the *Htr2c, Actb, Canx,* and *Ywhaz* transcripts

Single PCR product of each transcript was amplified indicating high specificity of each primer set used in this RT-PCR experiment.

5.3.3.4 *Effect of antipsychotics treatment on Htr2c mRNA expression*

The effect of antipsychotic drug treatment on the expression of the *Htr2c* mRNA in adipocyte 3T3-L1 cells was determined by RT-qPCR. Treatment with clozapine for 24h at concentrations of 0.3, 1, and 10 μM produced no significant changes in expression of *Htr2c* mRNA relative to control group ($p>0.05$) as shown in **Figure 5.11A**. However, the expression levels of the *Htr2c* mRNA treated with clozapine seems slightly decreased at or below the therapeutic concentration (0.3 and 1 μM).

Adipocyte 3T3-L1 cells treated with SB 242084 at the concentrations of 0.01, 0.1, and 1 μM did not have a significant change in *Htr2c* mRNA expression ($p>0.05$), although it seems to slightly increase the expression at all three concentration compared to control (**Figure 5.11B**).

Treatment with risperidone at concentrations of 0.03, 0.1, and 1 μM as well as haloperidol at concentrations of 0.01, 0.03, and 0.3 μM produced no significant changes the *Htr2c* mRNA expression in adipocyte 3T3-L1 cells ($p>0.05$), although treatment with both drugs at all concentrations slightly decreased the expression (**Figure 5.11C and D**).

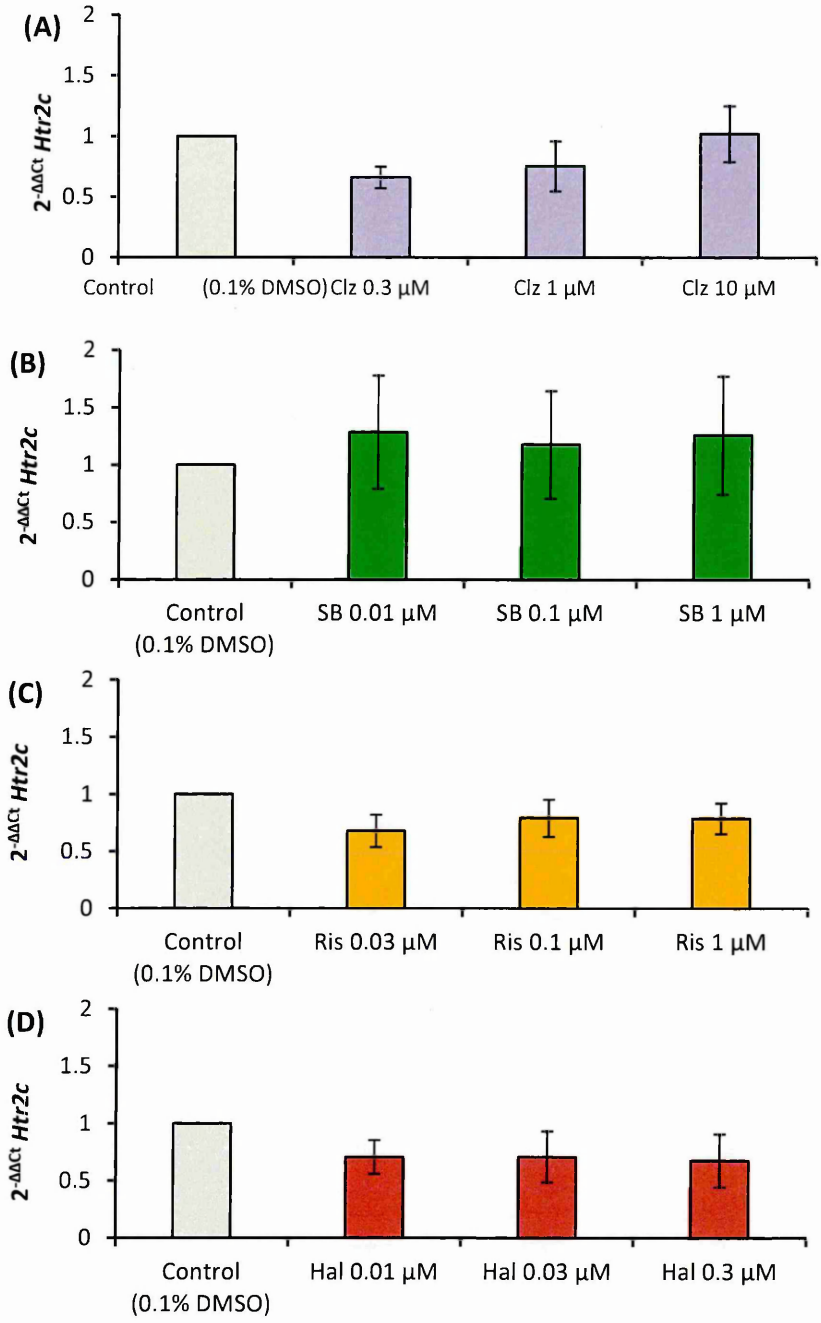


Figure 5.11: Effect of antipsychotic drug treatment on *Htr2c* mRNA expression in differentiated 3T3-L1 cells

The figure shows the effect of antipsychotic drugs; clozapine (A), SB 242084 (B), risperidone (C), and haloperidol (D) on *Htr2c* mRNA expression in mature adipocyte 3T3-L1 cells. Data are expressed as mean percentage of control (±SEM), n=3-4.

5.4 Discussion

Plasma leptin level is strongly and positively correlated with adiposity or BMI (Klein *et al.,* 1996; Haupt *et al.,* 2005; Venkatasubramanian *et al.,* 2010). Increased plasma leptin levels during atypical antipsychotic treatment, particularly olanzapine and clozapine (and risperidone), but not typical antipsychotic, haloperidol have been reported in several studies (Sentissi *et al.,* 2008; Kluge *et al.,* 2009; Kraus *et al.,* 1999; Yanik *et al.,* 2013; Zhang *et al.,* 2004). It has been suggested that the increased leptin levels are most likely due to weight increased during antipsychotic drug treatment rather than a direct effect of antipsychotic drugs on leptin production and release from adipocytes. The 5-HT2C receptor is expressed in mature adipocytes and it plays role in adipocyte differentiation (Kinoshita *et al.,* 2010). No previous study has investigated the role of 5-HT2C receptor in regulating leptin secretion from mature adipocytes. In addition, the effects of antipsychotic drugs on expression of the 5-HT2C receptor in adipocytes and leptin secretion from adipocytes have not been studied yet. The present study hypothesizes that antipsychotic drugs treatment may change the mRNA expression of the *HTR2C* and also alter the leptin secretion from adipocytes. The experiments in this chapter were carried out with the aim of investigating the effect of antipsychotic drugs on leptin secretion and mRNA expression of the *Htr2c* in adipocytes. The experiments were carried out in the 3T3-L1 adipocyte cell line that was induced by differentiation to become mature adipocytes for testing the effect of antipsychotic drugs.

5.4.1 Validation of methodology

5.4.1.1 Differentiation of 3T3-L1 cells

Full differentiation of the preadipocyte 3T3-L1 was obtained from day 12 after induction of differentiation. When observed under a microscope, the cell size and the accumulation of lipid droplets in the cells indicating the phenotype of an adipocyte cell were not increased after day 12. Differentiated cells at day 14 were used in antipsychotic drugs experiments in the present study to ensure that the cells reached full differentiation. Other studies have reported that by about day 10 to day 14 following differentiation induction by the standard method (without rosiglitazone), 90%-95% of the 3T3-L1 cells differentiated into adipocytes (Fu *et al.,* 2005; Yang and

Kim, 2015; Vestri *et al.,* 2007). The detected leptin secretion from 3T3-L1 cells into culture medium after differentiation induction also indicates the phenotype of adipocyte cells.

5.4.1.2 Housekeeping gene selection, primer efficiency and specificity

RNA extracted from cell cultures was intact and had a good quality observed from the clear sharp bands of 28s and 18s on an agarose gel whereby the 28S/18S rRNA ratio was approximately 2:1.

The analysis of a selection of the most stably expressed reference genes to be used in relative quantification of mRNA expression by using GeNorm software identified the *Actb*, *Canx*, and *Ywhaz* as the most stably expressed transcripts in 3T3-L1 adipocytes under the experimental conditions in this work.

The amplification efficiencies of the *Htr2c*, *Actb*, *Canx*, and *Ywhaz* primers used in the present study were within the acceptable range. The $2^{-\Delta\Delta Ct}$ method was therefore used in the calculation of the relative expression of the transcript.

The melt cure analysis in SYBR®Green real-time PCR and also the gel electrophoresis of the PCR product showed one single product was amplified indicating the specificity of the primers.

5.4.2 Effect of antipsychotic drugs on leptin secretion from 3T3-L1 adipocytes

The effect of antipsychotic drugs on leptin secretion from adipocytes was investigated using differentiated 3T3-L1 adipocytes. Cells were exposed to clozapine, SB 242084, risperidone, and haloperidol for 24h. Leptin levels in the culture medium were measured using ELISA kits.

5.4.2.1 Clozapine treatment and leptin secretion from 3T3-L1 adipocytes

There was no effect of clozapine at the concentrations of 0.3, 1, and 10 μM on leptin secretion. The therapeutic plasma concentration of clozapine is 1.07-1.84 μM (Hiemke *et al.,* 2011), thus clozapine even at a concentration about 10-fold higher than the typical plasma concentrations did not affect leptin secretion from adipocytes. This

result is consistent with previous findings in human differentiated adipocytes treated with clozapine at concentrations of 10^{-9}-10^{-5} M for 24h which showed no clozapine effect on leptin production and leptin mRNA expression (Hauner *et al.,* 2003). The authors suggested that the clozapine may cause weight gain via central pathways rather than a peripheral action. However, there has been a study in 3T3-L1 cells that reported that clozapine at doses as low as 5 μM were able to directly impair insulin actions by reduced glucose transport, increased lipogenesis, and decreased lipolysis (Vestri *et al.,* 2007). In addition, impaired insulin induced glucose uptake and insulin signaling has been reported both *in vitro* and *in vivo* (Panariello *et al.,* 2012). Furthermore, clozapine has been reported to enhance adipogenesis in white adipocytes, and 3T3-L1 cells (Yang *et al.,* 2009; Hu *et al.,* 2010), whereas it inhibited the differentiation accompanied by decreased mRNA expression of leptin and other lipogenic genes in brown adipocytes (Oh *et al.,* 2012). One study reported that clozapine treatment did not change intracellular triglyceride content during adipogenic differentiation of the human adipose-derived stem cells compared to the control DMSO group (Sertie *et al.,* 2011). However, a study in human adipocytes showed that clozapine treatment enhanced differentiation (Hemmrich *et al.,* 2006). The different findings of clozapine on adipogenesis may be due to the different cell types or different species, as well as the method for inducing differentiation. Taken together, clozapine may affect insulin-stimulated glucose uptake enhancing lipogenesis and adipogenesis during differentiation rather than an acute effect on leptin secretion from mature adipocytes. However, clozapine at the lowest concentration under this investigation (0.3μM) exceeds the binding affinities for many receptors in addition to 5-HT2C including 5-HT2A, α1-, α2- adrenergic, H1, H2 receptors which are expressed in 3T3-L1 adipocytes (Correll 2008; Morrow *et al.,* 2010), thus these receptors may mediate a clozapine effect on leptin secretion by way of antagonist action that may result in unchanged leptin levels. Further studies are required to elucidate the effect of antipsychotic drugs on leptin secretion.

5.4.2.2 SB 242084 treatment and leptin secretion from 3T3-L1 adipocytes

3T3-L1 adipocytes cells were treated with SB 242084, a selective 5-HT2C receptor antagonist to investigate whether the 5-HT2C receptor is involved in leptin secretion. SB 242084 treatment at concentrations of 0.01, 0.1, and 1 μM did not change leptin

levels. The binding affinity (Ki) of SB 242084 for 5-HT2C receptor is 9 nM (Kennett *et al.,* 1997), thus the lowest concentration in this study was at the Ki, the second concentration is 10-fold, and the third concentration is 100-fold higher than the concentration at the Ki. The result did not observe any significant effect of SB 242084 on leptin secretion from adipocyte 3T3-L1 cells. This is the first study that investigates the role of 5-HT2C receptor in leptin secretion in adipocytes. The action of SB 242084 on adipogenesis has been reported in previous studies; SB 242084 treatment at high doses (40 μM and 80 μM) during induction of differentiation significantly inhibited adipocyte differentiation in 3T3-L1 cells (Kinoshita *et al.,* 2010). This study indicates the role of the 5-HT2C receptor in adipogenesis, although the effect is only detectable at very high doses and they did not determine the cytotoxicity of drug treatment. Therefore, SB 242084 may have a direct effect on adipogenesis or cytotoxicity and at very high doses of the substance may cause the reduction of differentiation rate. In the present study, adipocytes were induced to differentiate before treatment with antipsychotic drugs, however drug concentrations were not as high as in Kinoshita and colleague's work, thus any change in leptin secretion may not be detectable. Further treatment with higher concentration may cause detectable changes. Further studies are needed to elucidate the role of 5-HT2C receptor in leptin secretion as well as in adipogenesis.

5.4.2.3 Risperidone treatment and leptin secretion from 3T3-L1 adipocytes

The effect of risperidone treatment on leptin secretion from adipocytes 3T3-L1 cells was investigated in this study. The result did not show any significant change of leptin levels when the adipocytes were treated with risperidone at concentration of 0.03, 0.1, and 1 μM compared to control group. The therapeutic plasma level of risperidone is 50-150 nM (Hiemke *et al.,* 2011) and the Ki of risperidone for 5-HT2C receptor is 32 nM (Correll, 2008) to 112 nM (Kuoppamaki *et al.,* 1995). Thus the drug concentrations used in this study cover the therapeutic plasma levels and Ki for 5-HT2C receptor, as well as 10-time higher than plasma level. The present study is the first study that investigated the direct effect of risperidone on leptin secretion from adipocytes. Risperidone has been reported to impair insulin-stimulated glucose uptake and insulin-mediated antilipolysis in primary cultured rat adipocytes (Vestri *et al.,* 2007). Another study reported that risperidone treatment at high doses (25μM and 50μM) showed

significantly enhanced adipogenesis in 3T3-L1 cells without drug-induced cytotoxicity (Hu *et al.*, 2010). The current study, investigated the drug effect on leptin secretion from mature adipocytes but not adipogenesis. The drug concentrations were not very high, although they covered therapeutic plasma levels. The lack of a significant effect of risperidone in this study may be due to the relatively low concentrations of drug in treatment of the cells. Further studies with supra-physiological concentration may result in detectable changes.

5.4.2.4 Haloperidol treatment and leptin secretion from 3T3-L1 adipocytes

The effect of haloperidol treatment on leptin secretion from adipocytes 3T3-L1 cells were investigated in this study which aimed to investigate the effect of typical antipsychotic drugs compared to atypical antipsychotic drugs. Adipocyte 3T3-L1 cells' treatment with haloperidol at concentrations of 0.01, 0.03, and 0.3 μM had no effect on leptin secretion into the culture medium. Haloperidol has very low 5-HT2C receptor binding affinity compared to other tested drugs. The Ki of haloperidol for 5-HT2C receptor is 4,700 nM and 2.6 nM for Dopamine D2 receptor (D2R) (Correll, 2008). The therapeutic plasma concentration of haloperidol is 2.66-26.6 nM (Hiemke *et al.,* 2011) to 50 nM (Coryell *et al.*, 1998). Thus drug concentrations used in this study are the therapeutic plasma concentration and about 10-fold higher than plasma level; however, all concentrations are lower than the Ki value of haloperidol for the 5-HT2C receptor but cover the D2R. D2 receptors are expressed in 3T3-L1 adipocytes (Mukherjee and Yun, 2013). No significant change of leptin secretion after treatment with haloperidol was observed in this study indicating that D2R may not be associated with regulating leptin secretion.

The present study is the first study that investigated the direct effect of haloperidol on leptin secretion from adipocytes. A previous study in human *in vitro* adipocyte-derived stem cells has reported that haloperidol had an effect on the control of the adipocyte differentiation program by increasing the gene expression levels of peroxisome proliferator-activated receptor gamma 2 (*PPARγ2*) and lipoprotein lipase (*LPL*) but only at high doses (20 μM and 40μM), the doses at therapeutic plasma concentrations and 10-fold higher than this level had no effect; however, these high concentrations had no effect on triglyceride accumulation (Sertie *et al.,* 2011). The authors also reported that

haloperidol at 40 and 100 μM decreased insulin-stimulated lipogenesis (Sertie *et al.,* 2011). However, these studies may not reflect the results of the present study because as mentioned above, the drugs were added to the differentiated cells instead of investigating adipogenesis, and also the relative low concentrations of drugs may contribute to the lack of change in leptin secretion. Further studies are required to elucidate the role of haloperidol on leptin secretion from adipocytes.

5.4.3 Effect of antipsychotic drugs on *Htr2c* mRNA expression in 3T3-L1 adipocytes

The detection of *Htr2c* mRNA expression in this study confirmed the expression of the 5-HT2C receptor in differentiated 3T3-L1 adipocytes (Kinoshita *et al.,* 2010). The expression of *Htr2c* mRNA levels of adipocyte 3T3-L1 cells treated with antipsychotic drugs were determined using real-time RT-qPCR. Treatment of 3T3-L1 adipocytes with clozapine, SB 242084, risperidone, and haloperidol at the same concentrations as tested in the leptin secretion study did not alter the levels of *Htr2c* mRNA expression compared to the control (DMSO) group. A previous study investigated the expression of *Htr2c* mRNA in 3T3-L1 adipocytes and found an increase in the *Htr2c* mRNA levels during adipocyte differentiation. Treatment with the 5-HT2C receptor antagonist SB 242084 inhibited adipocyte differentiation, although this inhibitory effect is only detectable at high doses (Kinoshita *et al.,* 2010). These authors also identified 5-HT in adipose tissue as a novel autocrine factor that is required for adipocyte differentiation (Kinoshita *et al.,* 2010). The role of 5-HT and the 5-HT2C receptor in adipocyte differentiation, suggest it is possible that the 5-HT2C receptor may also play a role in regulating leptin production and secretion. This is the first study that investigated the influence of antipsychotic drugs on mRNA expression of *HTR2C* in adipocytes that might affect the secretion of leptin. The lack of significant changes of the *HTR2C* mRNA expression as well as leptin secretion following antipsychotic treatment in this study may be due to the low concentrations of drugs that might not be high enough to induce intracellular changes at both mRNA or protein levels. Further studies are required to elucidate the role of antipsychotic drugs on the expression of gene-related to leptin secretion that may provide more understanding about the effect of antipsychotic drugs on weight gain side effect through peripheral actions.

To date, there have been several studies that reported the effect of antipsychotic drug treatment at gene expression levels. Treatment with atypical antipsychotic drugs increased mRNA expression of proinflammatory and adipogenic genes in human adipocytes *in vitro* (Sarvari *et al.,* 2014). Olanzapine treatment at high dose (50 μM) induced adipogenesis and triglyceride accumulation in 3T3-L1 cells, increased sterol regulatory element-binding protein 1 (*SREBP-1*) mRNA and protein levels upon treatment for 24h (50μM), and also increased mRNA expression of adiponectin and fatty acid synthase (*FAS*) upon treatment at 10μM for 48h (Yang *et al.,* 2007). Clozapine treatment in 3T3-L1 cells inhibited mRNA and protein expression of *LPL* and increased mRNA expression of adipocyte determination and differentiation factor1/SREBP$_{1C}$ (*ADD1/SREBP$_{1C}$*) (Yang *et al.,* 2009). Risperidone (50μM) treatment in 3T3-L1 cells for 8 days during differentiation resulted in increased mRNA expression of gene involved in SREBP-1 pathway (*SREBP-1*, *PPARγ*, *C/EBPα*, low density lipoprotein receptor (*LDLR*), adiponectin, and *FAS*), similar to clozapine (15μM) treatment which also increased mRNA expression of these genes except *FAS* (Hu *et al.*, 2010). A study in human adipocyte-derived stem cells showed that treatment with clozapine, olanzapine, or haloperidol increased mRNA expression of *PPARγ2* and *LPL* ((Sertie *et al.,* 2011). A further study in rat adipocytes treated with olanzapine for 5 weeks showed decreased lipolytic activity, decreased mRNA expression of hormone-sensitive lipase (*HSL*), and increased *FAS* mRNA expression (Minet-Ringuet *et al.,* 2007). The effect of antipsychotic drugs on these adipogenesis-related genes indicates the influence of drugs in cellular processes of adipocytes that might include the production and secretion of adipokines.

In addition, the expression of other receptors that are the targets of antipsychotic drugs has also been detected in adipocytes. The mRNA expression of histamine H1 receptor has been reported in both preadipocytes and fully differentiated adipocytes whereas the expression of the H2 receptor was found only after differentiation (Kawazoe *et al.*, 2004). This study also showed that knockdown of the H1 receptor by small interfering RNA impaired insulin-induced adipogenesis (Kawazoe *et al.*, 2004). Melanocortin MC2 and MC5 receptors are expressed in 3T3-L1 adipocytes and may mediate the ACTH and alpha-MSH actions in inhibiting leptin expression and secretion (Norman *et al.,* 2003) as well as actions in stimulating lipolysis (Moller *et al.,* 2011).

Adrenergic receptors are also expressed in adipocytes (Fain *et al.*, 1983); for examples, α2-adrenergic (Mukherjee and Yun, 2013), and β3-adrenergic receptors (Mottillo *et al.*, 2010). Dopamine receptors are expressed in adipocytes (Borcherding *et al.*, 2011; Pasqualini *et al.*, 2009). Therefore, treatment with antipsychotic drugs may mediate their effect on leptin secretion through some of these receptors; there may be antagonistic actions that may result in a lack of change in the net amount of leptin. Further studies are needed to elucidate the specific role of the 5-HT2C receptor in leptin secretion and also the influence of antipsychotic drugs on 5-HT2C receptor-mediated leptin secretion.

5.4.4 Limitations of this study

The 3T3-L1 cell line is derived from Swiss 3T3 mouse embryo fibroblasts. This, together with the differentiation induction protocol to induce 3T3-L1 preadipocyte to become mature adipocytes, may not represent human adipocyte cells. The drug concentrations used in the present experiments may not be specific to only the 5-HT2C receptors; therefore, the effect of antipsychotic drugs on leptin secretion may be the outcome of the drug effect on many receptors as well as affecting the expression of other receptors.

5.4.5 Conclusions

Antipsychotic drugs, both second generation including clozapine and risperidone, the selective 5-HT2C receptor antagonist SB 242084, and first generation haloperidol did not change the levels of leptin secretion and the *HTR2C* mRNA expression in 3T3-L1 adipocytes.

Chapter 6: General discussion

6.1 Introduction

Weight gain following antipsychotic treatment, particularly second generation antipsychotic drugs, is the most common and important adverse effect that increase a schizophrenic patient's risk of many consequent diseases, such as cardiovascular disease and type 2 diabetes mellitus, as well as increasing the risk of relapse because of a lack of compliance to the medication. The mechanism underlying this adverse effect is not fully understood. There is high inter-individual variability of weight gain and there are many factors that contribute to this variation. The influence of types of antipsychotic drugs, genetic factors, epigenetic factors, and other environmental factors may in part contribute to the aetiology of weight gain. Therefore an increased understanding of the mechanism involved in antipsychotic drug-induced weight gain is important and may be useful in recommending a therapeutic approach which brings about the least adverse effects. Study of genetic factors as well as epigenetic factors in antipsychotic drug-induced weight gain may provide genetic or epigenetic markers for predicting the adverse effect of an individual to design personal medication.

The common hypothesis used to explain antipsychotic drug-induced weight gain is that they induce an abnormality in the regulation of appetite leading to an increase in energy intake and eventually weight gain. The target receptors for antipsychotic drugs have been of interest and have been investigated. One of the most consistent findings to be associated with antipsychotic drug-induced weight gain is the genetic polymorphism of the *HTR2C* receptor -759C/T. However, other SNPs in many receptor genes of antipsychotic drugs such as *HTR2A* and *ADRA2A*, or the genes that encode neuropeptides involved in regulation of food intake such as *MC4R*, *GNB3*, *BDNF*, and *FTO* have been also reported, but there is still a lack of replication of these studies. The aim of this study was, therefore, to investigate the influence of genetic polymorphisms of these genes on weight gain following antipsychotic treatment in schizophrenia patients. In addition, the SNPs in the genes relating to DNA methylation including *MTHFR* (and *FTO*) have been hypothesized to be associated with weight gain and were investigated in this study.

Although the *HTR2C* -759C/T polymorphism is the most consistently associated with antipsychotic drug-induced weight gain, the mechanistic link is still unclear. The influence of the polymorphism on the expression of the receptor may be the link. A previous study by Hill and Reynolds found an association of the T allele of the *HTR2C* -759C/T polymorphism and lower promoter activity in human neuroblastoma SH-SY5Y cells (Hill and Reynolds, 2007). It has been hypothesized that the difference in promoter activity might be due to an epigenetic regulation. The aim of this study was therefore to investigate the association between levels of DNA methylation of the *HTR2C* promoter sequences and antipsychotic drug-induced weight gain as well as the influence of genetic polymorphisms in patients with schizophrenia.

The effect of antipsychotic drugs on the expression of the *HTR2C* has not been studied yet. In addition to weight gain, increased plasma leptin during antipsychotic drug treatment has been reported, but it has been suggested that the increased leptin levels might be due to the increased body weight or adiposity. The present study also hypothesized that the antipsychotic drugs may have an effect on DNA methylation and expression of the *HTR2C* and they may also have a direct effect on leptin secretion and expression of the *HTR2C* in adipocyte cells. Therefore, the aims of this study were to investigate the role of antipsychotic drugs on DNA methylation and mRNA expression of the *HTR2C* gene in human neuroblastoma cells, as well as their role on leptin secretion and *Htr2c* mRNA expression in 3T3-L1 adipocytes.

Data of the patient groups, the number of patients, important characteristic of the patients, and the source of samples is summarized as shown in **table 6.1**.

Table 6.1: Summary of studied population groups

Groups	Number	Characteristic	Sample
Chinese Han patients	182	First episode, drug naïve schizophrenia	Blood DNA
Spanish patients	72	First episode, drug naïve schizophrenia	Blood DNA
Belfast patients	72	Chronic schizophrenia	Blood DNA

6.2 Genetic polymorphisms in antipsychotic drug-induced weight gain in patients with schizophrenia

6.2.1 First episode drug naïve schizophrenia patients

6.2.1.1 MTHFR 677C/T polymorphism is associated with antipsychotic drug-induced weight gain in first episode schizophrenia patients

This study identified *MTHFR* 677C/T as a genetic risk factor for antipsychotic drug-induced weight gain in the first episode drug-naïve schizophrenia in both Chinese Han and Spanish patients, and showed that the T allele was a protective allele for antipsychotic drug-induced weight gain. This *MTHFR* 677C/T polymorphism showed an additive effect to the well-established *HTR2C* -759C/T polymorphism. The MTHFR enzyme plays an important role in DNA methylation, DNA synthesis and repair. The T allele causes decreased enzyme activity (Weisberg *et al.*, 1998), via its role in DNA synthesis and methylation (Sugden, 2006) and may influence gene expression (Jirtle and Skinner, 2007). However, the *MTHFR* 677C/T polymorphism did not show an influence on DNA methylation of the *HTR2C* promoter sequences either in Chinese Han or Spanish patients. This may be due to the small sample sizes relative to the modest effect of this genetic factor on epigenetic change.

6.2.1.2 MC4R rs489693 polymorphism is associated with antipsychotic drug-induced weight gain in first episode Chinese Han schizophrenia patients

Another genetic risk factor, the *MC4R* rs489693 was also associated with antipsychotic drug-induced weight gain in first episode Chinese Han patients with schizophrenia; the C allele carriers had greater weight gain compared to the AA genotype. This finding is opposite to a previous finding that the AA genotype was associated with weight gain following second generation antipsychotic treatment (Malhotra *et al.*, 2012; Czerwensky *et al.*, 2013b). This study differs from the present study in ethnicity; in addition, other factors such as prior drug exposure, the difference in drug treatments, clinical factors (19 years of age or younger in Czerwensky's study patients), as well as environmental factors could influence antipsychotic drug-induced weight gain. Further studies are needed to elucidate the association between the *MC4R* rs489693 and

antipsychotic drug-induced weight gain. The exact mechanism underlying the association of the *MC4R* rs489693 and antipsychotic drug-induced weight gain is unknown. No functional relevance of this polymorphism has been reported. The influence of the rs489693 may relate to other remote regulatory sites (Espinoza and Ren, 2011), or another polymorphism which is in LD that has actual biological function in the regulation of body weight.

6.2.1.3 HTR2A -1438G/A and ADRA2A -1291C/G polymorphisms are associated with risperidone-induced weight gain in first episode Chinese Han schizophrenia patients

An interaction of drug and genetic polymorphism was observed in Chinese Han schizophrenia patients. There was a significant drug x *HTR2A* -1438G/A genotype interaction and a non-significant indication of drug x *ADRA2A* -1291C/G genotype interaction when patients were analysed as groups receiving either risperidone or chlorpromazine. The drug-genetic polymorphism interaction may be due to the high binding affinity of the risperidone at the HTR2A and ADRA2A receptors. Therefore, post hoc analysis in a subgroup of patients who had received risperidone was performed and found a significantly greater weight gain in the A allele carriers of the *HTR2A* -1438G/A compared to GG genotype and also found a significantly greater weight gain in patients who carried the G allele of the *ADRA2A* -1291C/G compared to CC genotype carriers.

6.2.2 Chronic patients with schizophrenia

6.2.2.1 MTHFR 677C/T and MTHFR 1298A/C polymorphisms are not associated with BMI in chronic schizophrenia patients

In chronic schizophrenia patients, the *MTHFR* 677C/T and 1298A/C were not associated with BMI. Previous findings did not observe associations between the polymorphisms of the *HTR2C* -759C/T and leptin -2548A/G with BMI in this chronic patient cohort (Yevtushenko *et al.*, 2008), whereas the *FTO* rs9939609 polymorphism was previously found to be associated with the measurements of obesity including BMI, waist circumference, waist-hip ratio, and central obesity (Reynolds *et al.*, 2013). It

has been suggested that the *FTO* polymorphism may be enhanced in patients with chronic treatment and may also interact with other polymorphisms relating to antipsychotic drug-induced weight gain (Reynolds *et al.*, 2013), whereas the *HTR2C* -759C/T polymorphism may have strong effects on initial weight gain but lesser effects on long-term weight gain (Yevtushenko *et al.*, 2008). Taken together, the results suggest that the *MTHFR* and *HTR2C* polymorphisms may influence early weight gain while *FTO* polymorphism may affect long-term weight gain following antipsychotic drug medication.

The findings of the association of genetic polymorphism and weight gain or BMI in first episode and chronic patients with schizophrenia were summarized as shown in **table 6.2**. Gender distribution between subgroups of genotype of each polymorphism was tested using chi-square and there was no significant gender distribution between each genotype group.

Table 6.2: Summary of results of genetic polymorphism associated with weight gain or BMI in each studied population groups

Groups	SNP	rs number	Result (finding)	p value
Chinese Han	*HTR2C*	rs3813929*	C allele -->greater WG	0.004
	MTHFR	rs1801133	C allele -->greater WG	0.003
	MTHFR	rs1801131	NA	NS
	ADRA2A	rs1800544	G allele-->greater WG in risperidone subgroup	0.027
	HTR2A	rs6311	- A allele-->greater WG in risperidone subgroup	0.047
			- genotype-drug interaction	0.017
	MC4R	rs17782313	NA	NS
	MC4R	rs489693	C allele -->greater WG	0.040
	BDNF	rs6265	NA	NS
	GNB3	rs5443	NA	NS
	FTO	rs9939609	NA	NS
Spanish	*MTHFR*	rs1801133	C allele -->greater WG	0.049
	MTHFR	rs1801131	NA	NS
	HTR2C	rs3813929*	C allele -->greater WG	0.012
	FTO	rs9939609*	NA	NS
Chronic (Belfast)	*MTHFR*	rs1801133	NA	NS
	MTHFR	rs1801131	NA	NS
	HTR2C	rs3813929*	NA	NS
	FTO	rs9939609*	A allele-->greater BMI	0.029
	LEP	rs7799039*	NA	NS

In first episode drug naïve schizophrenia patients (Chinese Han and Spanish corhorts), the SNPs were analyzed with weight gain (change in BMI), and with BMI in chronic patients.

* Previous genotyped SNPs (Reynolds *et al.*, 2002; Templeman *et al.*, 2005; Yevtushenko *et al.*, 2008; Reynolds *et al.*, 2013); WG, weight gain (change in BMI); NA, no association with weight gain or BMI; NS, non-significant statistical p value.

6.3 DNA methylation of the *HTR2C* promoter regions and antipsychotic drug-induced weight gain in patients with schizophrenia

6.3.1 First episode drug naïve schizophrenia patients

The present study is the first study to investigate DNA methylation of the *HTR2C* in relation to antipsychotic drug-induced weight gain as well as the influence of genetic polymorphisms of the *HTR2C*, *MTHFR*, and *FTO* on DNA methylation of the *HTR2C*. The results indicated that there was a gender difference of DNA methylation of *HTR2C* promoter sequences in both Chinese Han and Spanish patients; females have higher methylation levels than males. This is because of X-chromosome inactivation in females leading to higher methylation levels.

6.3.1.1 DNA methylation of the HTR2C promoter sequences may be used as a predictor for antipsychotic drug induced weight gain in first episode schizophrenia patients

In first episode drug naïve schizophrenia patients, the methylation of the *HTR2C* promoter sequences did not show any correlation with body weight and BMI. However, Chinese Han patients who have a weight increase by >7% had lower methylation levels of the *HTR2C* promoter sequences than the <7% weight increase group. Because the levels of DNA methylation were measured at baseline but not after treatment; therefore, DNA methylation should be initially used to predict the weight gain because other factors such as antipsychotic treatment, dietary, exercise, or smoking may contribute to alterations in DNA methylation during treatment of antipsychotic drugs. A similar effect was also observed in the Spanish cohort but it did not reach statistically significant levels which may be due to a small sample size or the difference in ethnicity that may contribute to the weak association. It should be noted that the methylation levels of the *HTR2C* at both regions in Chinese Han patients were slightly higher than those of Spanish patients (**Table 3.10-Table 3.13**).

6.3.1.2 The T allele of the HTR2C -759C/T polymorphism is associated with higher DNA methylation of the HTR2C promoter sequences

The influences of genetic polymorphisms on DNA methylation of the *HTR2C* promoter polymorphisms in first episode drug naïve schizophrenia patients illustrated the effect of the *HTR2C* -759C/T polymorphism on the methylation of the cytosine bases in the promoter region of the *HTR2C*; the T allele carriers had higher methylation levels of the *HTR2C* promoter region near the SNP site compared to C/CC genotype. This finding may explain the previous finding that the T allele of the *HTR2C* -759C/T had lower promoter activity (Hill and Reynolds, 2007) in that the higher methylation levels of the T allele lead to lower promoter activity, and this may result in decreased transcription and receptor expression, and finally influence antipsychotic drug-induced weight gain. The results from this study may provide evidence of a link between epigenetic modification and promoter activity and may also make the link between this genetic polymorphism and weight gain.

This effect of -759C/T polymorphism on DNA methylation of the *HTR2C* was observed at the CpG sites that are located near the SNP site, whereas the CpG sites located far away near the transcription start site (TSS) did not show a significant difference indicating that the polymorphism may influence locally near the SNP sites. A similar direction was observed in first episode Spanish schizophrenia patients but did not reach statistical significance.

In addition, a lack of a significant difference of DNA methylation at the CpG sites located near the TSS may be due to the CpG sites near the TSS maintaining low methylation levels in order to facilitate transcription initiation (Jones, 2012). As it has low levels and only slight changes of DNA methylation in this region, the influence of genetic polymorphisms was not observed. However, the influence of the *HTR2C* -759C/T polymorphism on DNA methylation of the *HTR2C* near the TSS was observed to be in a similar direction (but not statistically significant) in first episode drug naïve schizophrenia patients.

6.3.1.3 The FTO rs9939609 polymorphism is associated with DNA methylation of the HTR2C promoter sequences in Spanish male first episode schizophrenia patients

The *FTO* rs9939609 polymorphism shows a significant effect on DNA methylation of the *HTR2C* promoter sequence in Spanish male patients in that the T allele carriers had lower methylation levels of the *HTR2C* -697G/C region compared to the AA genotype. In this cohort, *FTO* rs9939609 did not show a significant association with weight gain (Reynolds *et al.*, 2013). The result of the influence of the *FTO* rs9939609 on methylation of the *HTR2C* promoter sequences suggests an indirect effect of the *FTO* polymorphism on weight gain mediated through *HTR2C* DNA methylation, and this may also further change the level of receptor expression. Here is the first study demonstrating the effect of the *FTO* polymorphism on DNA methylation of the *HTR2C* promoter sequence, although the finding was not replicated in the other first episode group of Chinese Han schizophrenia patients who have different ethnicity. However, further studies in larger sample sizes are required to confirm this finding.

6.3.2 Chronic schizophrenia patients

In chronic schizophrenia patients, global *LINE-1* DNA methylation levels as well as DNA methylation of *HTR2C* promoter sequences were determined to investigate the association of the methylation levels with BMI and antipsychotics, as well as the influence of genetic polymorphisms.

6.3.2.1 Global LINE-1 DNA methylation is not associated with BMI in chronic schizophrenia patients

The methylation levels of *LINE-1* did not correlate with BMI and also did not show a difference between BMI obesity groups (>30 vs <30 kg/m^2), central obesity, and metabolic syndrome in the chronic Belfast schizophrenia patients. This finding is similar to a recent study in postmenopausal overweight woman that reported no association of *LINE-1* methylation and weight loss at 12 months comparing a reduced-calorie weight-loss diet and/or exercise program intervention groups and control group (Duggan *et al.*, 2014). However, studies in the adult general population reported the association of weight gain and an increased *LINE-1* methylation (Martin-Nunez *et*

al., 2014; Perng *et al.*, 2014), whereas a study in school-age children reported the association of adiposity development and lower *LINE-1* methylation in boys (Perng *et al.*, 2013). A study in visceral adipose tissue of severely obese people found lower *LINE-1* methylation was negatively associated with fasting glucose, diastolic pressure, and metabolic status (Turcot *et al.*, 2012). In addition, global DNA methylation was inversely associated with plasma vitamin A, female gender, and C-reactive protein (a marker of chronic inflammation), while positively associated with maternal BMI and socioeconomic status (Perng *et al.*, 2012). An inconsistency of the association of *LINE-1* methylation and body weight may be due to influences of gender, age, diet, tissue-specific variability, and other factors. The information of the association of *LINE-1* methylation and body weight or BMI in schizophrenia patients is limited. The disease conditions, symptoms, medications, and patient's life style may also contribute to epigenetic modifications in both global and specific gene levels. Further studies are required to confirm this association.

6.3.2.2 Gender difference and age associated of global LINE-1 DNA methylation in chronic schizophrenia patients

Gender had an influence on *LINE-1* methylation in chronic schizophrenia patients. Females had lower levels of the global *LINE-1* methylation than males (0.55% on average). This finding is consistent with previous studies in normal populations (Huen *et al.*, 2014; Perng *et al.*, 2013; Wilhelm *et al.*, 2010; El-Maarri *et al.*, 2011; Zhang *et al.*, 2011; Burris *et al.*, 2012). This gender-specific effect on *LINE-1* methylation is likely due to the association of *LINE-1* with X-chromosome inactivation (Singer *et al.*, 2012).

Age was not correlated with global *LINE-1* DNA methylation in the chronic patients studied here which confirmed several previous studies that found a weak inverse relationship or no association of age with *LINE-1* methylation (Bollati *et al.*, 2009; Jintaridth and Mutirangura, 2010; El-Maarri *et al.*, 2011). The weak or no association may be due to the modest age effect on *LINE-1* methylation and other factors such as life style, smoking, dietary, exercise, disease conditions, and antipsychotic drug treatment that may have a stronger effect on global DNA methylation.

6.3.2.3 Smoking is associated with higher global LINE-1 DNA methylation in chronic schizophrenia patients

Tobacco smoking was significantly associated with higher global *LINE-1* DNA methylation at CpG3 and a similar trend at CpG2. The effect of smoking on global DNA methylation is still controversial in which studies in lung cancer found hypermethylation (Piyathilake *et al.*, 2001; Lin *et al.*, 2007); in contrast to studies in head and neck cancer that found hypomethylation (Smith *et al.*, 2007; Hsiung *et al.*, 2007). Smoking associated with higher global *LINE-1* methylation may be due to the effect of nicotine which has been reported to cause DNA hypermethylation and histone acetylation (Abdolmaleky *et al.*, 2013).

6.3.2.4 Olanzapine or clozapine treatment trends to decrease global LINE-1 DNA methylation in chronic schizophrenia patients

No significant differences of global DNA methylation levels were observed between patients receiving olanzapine or clozapine and patients receiving all other antipsychotic drugs, although patients on olanzapine or clozapine treatment had slightly lower levels of global DNA methylation (0.46% on average, p=0.059 at CpG3) than all other drugs. This finding is in line with previous studies that reported the association of decreased methylation of reelin and *GAD67* promoters and antipsychotic drugs, particularly clozapine and the benzamide sulpiride, but not haloperidol and risperidone (Guidotti and Grayson, 2014; Melas *et al.*, 2012; Guidotti *et al.*, 2009; Melka *et al.*, 2014; Dong *et al.*, 2008). Melas *et al.* (2012) found global hypomethylation in schizophrenia patients and also reported that haloperidol treatment increased (normalized) this global hypomethylation. However, the role of antipsychotic drugs such as clozapine or olanzapine on global DNA methylation needs further studies.

6.3.2.5 The MTHFR 677C/T and FTO rs9939609 polymorphisms and global LINE-1 DNA methylation in chronic schizophrenia patients

MTHFR 677C/T polymorphism was significantly associated with global *LINE-1* DNA methylation in chronic patients with schizophrenia (Belfast cohort) in that the T allele carriers had higher methylation levels at CpG3 than the CC genotype carriers. At

another SNP, the *FTO* rs9939609 T allele carriers had a trend of lower methylation levels of *LINE-1* than AA genotype at CpG2 and CpG3. The polymorphisms of the *MTHFR* and *FTO* are involved in methylation processes that may influence the global DNA methylation. There was no *MTHFR x FTO* interaction on *LINE-1* methylation, but interactions of the *MTHFR* x smoking, and *MTHFR* x gender (correcting for smoking) were observed in that the T allele of the *MTHFR* 677C/T polymorphism in smokers or in females had higher methylation than in other groups. The relationship of the *MTHFR* 677C/T polymorphism and smoking has been reported in previous studies in that the TT genotype of the *MTHFR* 677C/T polymorphism had higher frequency in smokers (Johnson *et al.*, 2001; Linnebank *et al.*, 2012). The alteration in plasma homocysteine may be a link between genetic factors and smoking. Linnebank *et al.* (2012) found increased levels of plasma homocysteine in smokers as well as in the T allele carriers of the *MTHFR* 677C/T, and the smoking *MTHFR* 677TT carriers had the highest plasma homocysteine levels, the non-smoking 677CC carriers the lowest. A recent study reported the association of an increment of homocysteine and higher *LINE-1* methylation (Perng *et al.*, 2014). However, the present study did not obtain patient's plasma homocysteine levels.

6.3.2.6 *The methylation of the HTR2C promoter sequences is gender difference in chronic schizophrenia patients*

Females have higher levels of DNA methylation in both regions of the *HTR2C* (*HTR2C* -697G/C and Hs_*HTR2C*_01_PM). Taken with results in first episode schizophrenia patients, gender has a strong effect on the extent of DNA methylation of the *HTR2C* promoter sequences, both before and after long-term antipsychotic treatment.

6.3.2.7 *Smoking and antipsychotic treatment do not change the methylation levels of the HTR2C promoter sequences in chronic schizophrenia patients*

Although tobacco smoking significantly increases global *LINE-1* DNA methylation in this chronic schizophrenia cohort, it does not influence the methylation of the *HTR2C* promoter sequences; in contrast, it is likely to decrease the methylation. These results suggest a gene specific influence of smoking on DNA methylation.

The same direction of decreased DNA methylation levels of *LINE-1* in patients receiving antipsychotic drugs (olanzapine or clozapine) was also observed in the *HTR2C* promoter sequences indicating that not only the global DNA methylation but also the specific *HTR2C* gene was modified by antipsychotic drugs.

6.3.2.8 The methylation of the HTR2C promoter sequences is not associated with BMI in chronic schizophrenia patients

The methylation of the *HTR2C* promoter sequences is not correlated with BMI in chronic (Belfast) schizophrenia patients. Levels of DNA methylation of the *HTR2C* promoter sequences of patients who have BMI obesity (>30 kg/m^2), central obesity, and metabolic syndrome were not different (slightly higher methylation levels) compared to patients who have BMI <30 kg/m^2, absence of central obesity and absence of metabolic syndrome. The lack of association between *HTR2C* methylation and BMI or obesity may be due to small sample size. Further studies in larger sample sizes are required to elucidate this association.

6.3.2.9 The genetic polymorphisms and the methylation of the HTR2C promoter sequences in chronic schizophrenia patients

The genetic polymorphisms of the *HTR2C* -759C/T and the leptin -2548G/A are associated with the methylation levels of the *HTR2C* promoter sequences, whereas *FTO* rs9939609, *MTHFR* 677C/T and *MTHFR* 1298A/C polymorphisms are not associated with methylation levels of the *HTR2C* promoter sequences. A similar finding has been observed with first episode schizophrenia patients in that an association of the T allele of the *HTR2C* -759C/T polymorphism with higher levels of the *HTR2C* methylation was also observed in chronic patients (at CpG-670, CpG-661, and trend at CpG-644). A similar direction was observed in the Hs-*HTR2C*_01_PM region but it did not reach significant levels. The result indicates the local effect of the *HTR2C* -759C/T SNP on nearby DNA methylation rather than the distant CpG sites.

In contrast, the leptin -2548G/A polymorphism shows significant effects on the *HTR2C* promoter methylation at CpG-698 (SNP site-697G/C) of the *HTR2C* -697G/C region, and at the CpG1 and CpG4 of the Hs_*HTR2C*_01_PM region. This finding is interesting because the leptin -2548G/A polymorphism affects the extent of DNA methylation of

the *HTR2C* at the SNP site and at the CpGs near the transcription start site which normally is associated with lower methylation to initiate transcription. An effect on this region indicates the role of the leptin polymorphism on *HTR2C* methylation that might further influence the transcription and expression of the 5-HT2C receptors.

Although the *HTR2C* -759C/T and the leptin -2548G/A polymorphisms are not associated with BMI in this chronic cohort, the effect of these polymorphisms on DNA methylation of *HTR2C* suggests, at least, the interaction between leptin and *HTR2C* genes. However, the effect of these two polymorphisms on *HTR2C* DNA methylation may not directly link to body weight or BMI.

The results of the association of DNA methylation of the *HTR2C* and global *LINE-1* methylation with weight gain or BMI, and genetic polymorphism in first episode and chronic patients with schizophrenia are summarized in **table 6.3**.

Table 6.3: Summary of results of DNA methylation associated with weight gain or BMI and SNPs in each studied population groups

Patient group/	DNA methylation		
Factors	***LINE-1***	***HTR2C* -697G/C**	**Hs_*HTR2C*_01_PM**
Chronic patients			
- gender	- gender effect (female had lower methylation, p=0.016, CpG1).	- gender effect (female had higher methylation, p<0.05, all CpG).	- gender effect (female had higher methylation, p<0.05, all CpG).
- age	- age was not associated with methylation (all 3 CpGs).	- methylation increased with age (CpG-691, -670, -661, & -644).	- methylation increased with age (CpG1&CpG2).
- smoker	- smoker had higher methylation (p=0.019, CpG3; p=0.055, CpG2).	NA	NA
- Olanzapine/ clozapine	- olanzapine/clozapine trended lower methylation levels (p=0.059, CpG3).	NA	NA
- BMI obesity (>30 kg/m2)	NA	NA	NA
- central obesity	NA	NA	- associated with higher methylation (p=0.023, CpG1).
- metabolic syndrome	NA	NA	NA
- SNPs	- *FTO* rs9939609 AA genotype trended associated with higher methylation (p=0.055, CpG 3). - *MTHFR* 677C/T C allele associated with higher methylation (p=0.026, CpG3, correcting for smoking). - *MTHFR* 677C/T and smoking interaction (smoker with T allele had	- *HTR2C* -759C/T T allele associated with higher methylation (p=<0.05 at CpG-670 & CpG-661). - *MTHFR* 677C/T, *MTHFR* 1298A/C, and *FTO* rs9939609 had no association with methylation levels. - *LEP* -2548A/G GG genotype had higher methylation at CpG-698 (p=0.023).	- *HTR2C* -759C/T, *MTHFR* 677C/T, *MTHFR* 1298A/C, and *FTO* rs9939609 had no association with methylation levels. - *LEP* -2548A/G GG genotype had higher methylation at CpG1 (p=0.025) and CpG4 (p=0.009).

Patient group/	DNA methylation		
Factors	***LINE-1***	***HTR2C* -697G/C**	**Hs_*HTR2C*_01_PM**
	higher methylation, p=0.009, CpG3). - *MTHFR* 677C/T and gender interaction (female with T allele had higher methylation, p=0.003, correction for smoking). - *HTR2C* -759C/T and *MTHFR* 1298A/C were not associated with methylation levels.		
Chinese Han			
- gender	-	- gender effect (female had higher methylation, p<0.05, all CpG).	- gender effect (female had higher methylation, p<0.05, all CpG).
- age	-	NA	NA
- weight gain	-	- methylation did not associated with baseline weight, baseline BMI, change in weight, and change in BMI. - patients who had weight increased>7% had lower methylation (p=0.046, CpG-644).	- methylation did not associated with baseline weight, baseline BMI, change in weight, and change in BMI. - patients who had weight increased>7% had lower methylation (p=0.042, CpG3).
- SNPs	-	- *HTR2C* -759C/T T allele associated with higher methylation at CpG-670 (p=0.010) and CpG-644 (p=0.014). - *HTR2C* SNP effect on CpG-698 site (C allele of -759C/T (or G allele -697G/C) was associated with higher methylation, p=0.004). - a trend of T allele of *FTO* rs9939609	- *HTR2A* -1438G/A A allele was associated with lower methylation at CpG3 (p=0.030). - *HTR2C* -759C/T, *MTHFR* 677C/T, *MTHFR* 1298A/C, *FTO* re9939609, *BDNF* rs6265, and other SNPs were not associated with methylation levels.

Patient group/		DNA methylation	
Factors	***LINE-1***	***HTR2C* -697G/C**	**Hs_*HTR2C*_01_PM**
		associated with lower methylation at CpG-698 (p=0.052). -*BDNF* rs6265 G/A G allele was associated with lower methylation at CpG-698 (p=0.043) . - *MTHFR* 677C/T, *MTHFR* 1298A/C and other SNPs were not associated with methylation.	
Spanish			
- gender	-	- gender effect (female had higher methylation, p<0.05, all CpG).	- gender effect (female had higher methylation, p<0.05, all CpG).
- age	-	NA	NA
- weight gain	-	- methylation did not associated with baseline weight, baseline BMI, change in weight, change in BMI, and 7% weight increased groups.	- methylation did not associated with baseline weight, baseline BMI, change in weight, change in BMI, and 7% weight increased groups.
- SNPs	-	- *HTR2C* SNP effect on CpG-698 site (C allele of -759C/T (or G allele -697G/C) was associated with higher methylation, p=0.037). - *FTO* rs9939609 T allele was associated with lower methylation levels, particularly in male patients at all CpGs (p<0.05), except CpG-644 (p=0.054). - *MTHFR* 677C/T and *MTHFR* 1298A/C, were not associated with methylation levels.	- a trend of *FTO* rs9939609 T allele associated with lower methylation levels in male patients at CpG4 (p=0.060). - *HTR2C* -759C/T, *MTHFR* 677C/T and *MTHFR* 1298A/C, were not associated with methylation levels.

NA, no association

6.4 Effect of antipsychotic drugs on the *HTR2C* mRNA expression and DNA methylation of the *HTR2C* promoter sequences in SH-SY5Y cells

Methylation of the *HTR2C* promoter sequence near the -759C/T *HTR2C* SNP site as well as the mRNA expression of the 5-HT2C receptor were determined in a cell culture model to investigate the effect of antipsychotic drug treatment on the expression of the *HTR2C* mRNA and DNA methylation of the *HTR2C* promoter sequence. Non-differentiated and differentiated SH-SY5Y cells were used in this study to compare the effect of drugs on undifferentiated neuroblastoma cells and the more neuron like differentiated cells. Antipsychotic drugs used in this study consisted of clozapine and haloperidol in order to make a comparison between second-generation and first generation antipsychotic drugs. In addition, 5-aza-2'-deoxycytidine was also used in this study to investigate the effect of inhibiting DNA methylation on mRNA expression of the *HTR2C* gene.

Decreased DNA methylation of the *HTR2C* promoter sequence near the *HTR2C* -759C/T polymorphism and including the -697G/C polymorphism was observed on treatment with 5-aza-2'-deoxycytidine in both undifferentiated and differentiated SH-SY5Y cells. Treatment with this substance also showed an increase in the levels of the *HTR2C* mRNA expression. The changes in DNA methylation and mRNA expression of *HTR2C* in response to 5-aza-2'-deoxycytidine treatment suggest the suitability of the cells for studying the effect of antipsychotic drugs on DNA methylation and mRNA expression of *HTR2C*.

Clozapine and haloperidol did not change *HTR2C* promoter DNA methylation and mRNA expression in both undifferentiated and differentiated SH-SY5Y cells. A lack of significant changes of the *HTR2C* promoter DNA methylation and mRNA expression after treatment with clozapine may be due to the low concentration used (2 and 10 μM) or the very small effect of clozapine on the *HTR2C* promoter DNA methylation and mRNA expression. However, SH-SY5Y cells may respond to antipsychotic drugs in a different way from normal mature neurons because they are derived from neuroblastoma cells.

The lack of haloperidol influence on the *HTR2C* promoter DNA methylation and mRNA expression may be due to the low binding affinity of the haloperidol for the 5-HT2C receptors resulting in a lack of alteration in any cellular signalling including the DNA methylation of the *HTR2C* promoter and mRNA expression observed in the present study.

6.5 Effect of antipsychotic drugs on leptin secretion and the *Htr2c* mRNA expression in 3T3-L1 adipocytes

The present study is the first study to investigate the effect of antipsychotic drugs including clozapine, risperidone, haloperidol, and SB 242084 which is a selective 5-HT2C receptor antagonist, on leptin secretion from adipocytes, although there is one previous study that reported that clozapine treatment did not influence leptin secretion in human adipocytes (Hauner *et al.*, 2003). This study did not observe any change in leptin levels in the culture medium of the 3T3-L1 adipocytes exposed to antipsychotic drugs compared to control cells. The concentrations of the drugs used in this study were the therapeutic plasma concentrations and about a 10-fold higher concentration. The lack of an effect of antipsychotics on leptin secretion indicates that drugs may induce weight gain by acting at central pathways rather than peripheral action. Otherwise, the low concentrations of antipsychotic drugs used in this study may be the cause of no significant alteration, or the drugs may have effects on other receptors that might have antagonistic actions and result in unchanged leptin levels.

A previous study has reported an increased expression of the *Htr2c* mRNA in 3T3-L1 adipocytes during adipocyte differentiation and used these cells to investigate the role of this receptor in adipocyte differentiation (Kinoshita *et al.*, 2010). The measurable mRNA expression of the *Htr2c* in the differentiated 3T3-L1 adipocytes in this study may provide a cellular model for investigating the effect of antipsychotic drugs on its expression. This study did not find any change of the *Htr2c* mRNA expression in 3T3-L1 adipocytes after treatment with antipsychotic drugs compared to a control group. No influence of antipsychotic drugs on mRNA expression of the *Htr2c* in adipocytes supports the theory of central influence of antipsychotic drugs in inducing weight gain rather than the peripheral action. However, a change may be visible if cells are treated with very high concentrations of antipsychotic drugs.

6.6 Future studies

Further studies need to be carried out to confirm the findings of the present study and to establish the role of genetic and epigenetic factors in antipsychotic drug-induced weight gain in patients with schizophrenia. The novel genetic risk factors for antipsychotic drug-induced weight gain and replicates of the findings of the previously identified genetic risk factors still need further investigation.

The work in the area of DNA methylation in this study may extend into other genes relating to antipsychotic drug-induced weight gain; for example: the *MC4R*, *ADRA2A*, *HTR2A*, *MTHFR* and *FTO* polymorphisms which are the SNPs associated with antipsychotic drug-induced weight gain in the present work.

Further studies in cell culture could be carried out using SH-SY5Y human neuroblastoma cells to confirm the findings in this study and to investigate the role of antipsychotic treatment on DNA methylation and also the expression of the *HTR2C* gene or other genes.

The differentiated mouse 3T3-L1 adipocyte cells provide a good model for studying the leptin secretion and expression of the *Htr2c* transcripts. Further studies need to confirm the effect of antipsychotic drugs on leptin and the *Htr2c* mRNA expression by testing at the higher concentrations of antipsychotic drugs to point out the role of the 5-HT2C receptors in the regulation of leptin secretion.

6.7 General conclusions

The key novel findings of this study include:

- This study has identified a genetic risk factor for antipsychotic drug-induced weight gain in first episode schizophrenia. The T allele of the *MTHFR* 677C/T polymorphism was a protective allele for antipsychotic drug-induced weight gain. The *MC4R* rs489693 was also associated with weight gain in first episode Chinese Han schizophrenia patients.
- There are interactions of drug x genetic polymorphism of *HTR2A* and *ADRA2A* and these polymorphisms were associated with BMI gain in the risperidone subgroup of first episode Chinese Han schizophrenia patients.

- The *FTO* rs9939609 was previously reported to be associated with BMI in chronic (Belfast) schizophrenia patients in which the A allele carries had higher BMI than the TT genotype in this cohort.
- Global *LINE-1* methylation was not associated with BMI in chronic (Belfast) patients. In addition, the *FTO* rs9939609 and *HTR2C* -759C/T polymorphisms are not associated with global *LINE-1* DNA methylation. However, the *MTHFR* 677C/T polymorphism was associated with the global *LINE-1* DNA methylation (CpG3) in chronic Belfast schizophrenia patients.
- The *HTR2C* promoter DNA methylation showed a gender difference (females had higher methylation levels than males). The methylation of *HTR2C* also showed regional differences (the methylation levels of CpG sites located near the transcription start site have lower than those located far away from the transcription start site). The *HTR2C* SNP was more likely to have an effect on the extent of DNA methylation of the CpG sites that are located near to the SNP site than the CpG sites that are located at distance from the SNP site.
- The association of the T allele of the *HTR2C* -759C/T polymorphism and the higher levels of the *HTR2C* promoter DNA methylation in the first episode Chinese Han cohort and chronic (Belfast) patients may explain previous findings that the T allele has lower promoter activity (Hill and Reynolds, 2007). Therefore, this finding may add more evidence to the mechanistic link between the polymorphism and antipsychotic drug-induced weight gain.
- *FTO* rs9939609 was associated with *HTR2C* promoter methylation (-697G/C region), especially in first episode Spanish male patients with schizophrenia; however, no association between the *FTO* polymorphism and *HTR2C* promoter methylation was observed in chronic (Belfast) schizophrenia patients.
- The antipsychotic drugs, clozapine and haloperidol treatment did not have an effect on the *HTR2C* promoter DNA methylation and mRNA expression in human neuroblastoma SH-SY5Y cells. Whereas treatment with 5-aza-2'-deoxycytidine inhibited DNA methylation of the *HTR2C* promoter sequence and increased the *HTR2C* mRNA expression.
- The antipsychotic drugs including clozapine, risperidone, haloperidol treatment and the 5-HT2C receptor antagonist, SB 242084 treatment did not have any

effect on *Htr2c* mRNA expression and leptin secretion from mouse 3T3-L1 adipocyte cells.

The genetic factors and epigenetic factors implicated in antipsychotic drug-induced weight gain may provide genetic and epigenetic markers for weight gain following antipsychotic drug medication. This study provides more understanding of the mechanism underlying antipsychotic drug-induced weight gain. These genetic and epigenetic factors may be useful in the future for designing clinical medications such as antipsychotic drug treatment that is suitable for each individual (personalised medicine) in order to gain the most therapeutic efficacy and least adverse side effects.

References

Abdolmaleky , H. M., Shafa, R., Tsuang, M. T., & Thiagalingam, S. (2013). *Psychiatric epigenetics: A key to the molecular basis of and therapy for psychiatric disorders* (Peer ReviewedPsychiatric Times.

Abdolmaleky, H. M., Cheng, K. H., Faraone, S. V., Wilcox, M., Glatt, S. J., Gao, F., et al. (2006). Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. *Human Molecular Genetics, 15*(21), 3132-3145.

Abdolmaleky, H. M., Nohesara, S., Ghadirivasfi, M., Lambert, A. W., Ahmadkhaniha, H., Ozturk, S., et al. (2014). DNA hypermethylation of serotonin transporter gene promoter in drug naive patients with schizophrenia. *Schizophrenia Research, 152*(2-3), 373-380.

Abdolmaleky, H. M., Smith, C. L., Zhou, J. R., & Thiagalingam, S. (2008). Epigenetic alterations of the dopaminergic system in major psychiatric disorders. *Methods in Molecular Biology (Clifton, N.J.), 448*, 187-212.

Abdolmaleky, H. M., Thiagalingam, S., & Wilcox, M. (2005). Genetics and epigenetics in major psychiatric disorders: Dilemmas, achievements, applications, and future scope. *American Journal of Pharmacogenomics : Genomics-Related Research in Drug Development and Clinical Practice, 5*(3), 149-160.

Abdolmaleky, H. M., Yaqubi, S., Papageorgis, P., Lambert, A. W., Ozturk, S., Sivaraman, V., et al. (2011). Epigenetic dysregulation of HTR2A in the brain of patients with schizophrenia and bipolar disorder. *Schizophrenia Research, 129*(2-3), 183-190.

Abramowski, D., Rigo, M., Duc, D., Hoyer, D., & Staufenbiel, M. (1995). Localization of the 5-hydroxytryptamine2C receptor protein in human and rat brain using specific antisera. *Neuropharmacology, 34*(12), 1635-1645.

Adan, R. A., Tiesjema, B., Hillebrand, J. J., la Fleur, S. E., Kas, M. J., & de Krom, M. (2006). The MC4 receptor and control of appetite. *British Journal of Pharmacology, 149*(7), 815-827.

Ak, M., Sezlev, D., Sutcigil, L., Akarsu, S., Ozgen, F., & Yanik, T. (2013). The investigation of leptin and hypothalamic neuropeptides role in first attack psychotic male patients: Olanzapine monotherapy. *Psychoneuroendocrinology, 38*(3), 341-347.

Akbarian, S. (2010). The molecular pathology of schizophrenia--focus on histone and DNA modifications. *Brain Research Bulletin, 83*(3-4), 103-107.

Allison, D. B., & Heo, M. (1998). Meta-analysis of linkage data under worst-case conditions: A demonstration using the human OB region. *Genetics, 148*(2), 859-865.

Allison, D. B., Mackell, J. A., & McDonnell, D. D. (2003). The impact of weight gain on quality of life among persons with schizophrenia. *Psychiatric Services (Washington, D.C.), 54*(4), 565-567.

Allison, D. B., Mentore, J. L., Heo, M., Chandler, L. P., Cappelleri, J. C., Infante, M. C., et al. (1999). Antipsychotic-induced weight gain: A comprehensive research synthesis. *The American Journal of Psychiatry, 156*(11), 1686-1696.

Almen, M. S., Jacobsson, J. A., Moschonis, G., Benedict, C., Chrousos, G. P., Fredriksson, R., et al. (2012). Genome wide analysis reveals association of a FTO gene variant with epigenetic changes. *Genomics, 99*(3), 132-137.

Amano, T., Hosaka, S., Takami, H., Sugiyama, C., Oda, K., & Morikawa, R. (2012). The lipid metabolism abnormality in patients administered with olanzapine. *Nihon Shinkei Seishin Yakurigaku Zasshi = Japanese Journal of Psychopharmacology, 32*(5-6), 257-261.

American Psychiatric Association (Ed.). (1994). *Diagnostic and statistical manual of mental disorders* (4th ed.). Washington, DC: American Psychiatric Press.

Andrew, A., Knapp, M., McCrone, P., Parsonage, M., & Trachtenberg, M. (2012). Effective interventions in schizophrenia the economic case: *A report prepared for the schizophrenia commission*. London: Rethink Mental Illness.

Angelucci, F., Aloe, L., Iannitelli, A., Gruber, S. H., & Mathe, A. A. (2005). Effect of chronic olanzapine treatment on nerve growth factor and brain-derived neurotrophic factor in the rat brain. *European Neuropsychopharmacology : The Journal of the European College of Neuropsychopharmacology, 15*(3), 311-317.

Angelucci, F., Mathe, A. A., & Aloe, L. (2000). Brain-derived neurotrophic factor and tyrosine kinase receptor TrkB in rat brain are significantly altered after haloperidol and risperidone administration. *Journal of Neuroscience Research, 60*(6), 783-794.

Antequera, F. (2003). Structure, function and evolution of CpG island promoters. *Cellular and Molecular Life Sciences : CMLS, 60*(8), 1647-1658.

Anttila, S., Illi, A., Kampman, O., Mattila, K. M., Lehtimaki, T., & Leinonen, E. (2005). Lack of association between two polymorphisms of brain-derived neurotrophic factor and response to typical neuroleptics. *Journal of Neural Transmission (Vienna, Austria : 1996), 112*(7), 885-890.

Arner, P. (1992). Adrenergic receptor function in fat cells. *The American Journal of Clinical Nutrition, 55*(1 Suppl), 228S-236S.

Arooj, M., Rashid,F,A, & Gul, A. (2013). Role of epigenetic modifications in stem cell regulatory regions (Oct4, Sox2 and nanog) and cancer. *IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS), 5*(1), 76-81.

Arranz, M., Collier, D., Sodhi, M., Ball, D., Roberts, G., Price, J., et al. (1995). Association between clozapine response and allelic variation in 5-HT2A receptor gene. *Lancet, 346*(8970), 281-282.

Arranz, M. J., Munro, J., Owen, M. J., Spurlock, G., Sham, P. C., Zhao, J., et al. (1998). Evidence for association between polymorphisms in the promoter and coding regions

of the 5-HT2A receptor gene and response to clozapine. *Molecular Psychiatry, 3*(1), 61-66.

Atmaca, M., Kuloglu, M., Tezcan, E., & Ustundag, B. (2003). Serum leptin and triglyceride levels in patients on treatment with atypical antipsychotics. *The Journal of Clinical Psychiatry, 64*(5), 598-604.

Attwood, J. T., Yung, R. L., & Richardson, B. C. (2002). DNA methylation and the regulation of gene transcription. *Cellular and Molecular Life Sciences : CMLS, 59*(2), 241-257.

Aubert, R., Betoulle, D., Herbeth, B., Siest, G., & Fumeron, F. (2000). 5-HT2A receptor gene polymorphism is associated with food and alcohol intake in obese people. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity, 24*(7), 920-924.

Bachman, E. S., Dhillon, H., Zhang, C. Y., Cinti, S., Bianco, A. C., Kobilka, B. K., et al. (2002). betaAR signaling required for diet-induced thermogenesis and obesity resistance. *Science (New York, N.Y.), 297*(5582), 843-845.

Baker, R. A., Pikalov, A., Tran, Q. V., Kremenets, T., Arani, R. B., & Doraiswamy, P. M. (2009). Atypical antipsychotic drugs and diabetes mellitus in the US food and drug administration adverse event database: A systematic bayesian signal detection analysis. *Psychopharmacology Bulletin, 42*(1), 11-31.

Balt, S. L., Galloway, G. P., Baggott, M. J., Schwartz, Z., & Mendelson, J. (2011). Mechanisms and genetics of antipsychotic-associated weight gain. *Clinical Pharmacology and Therapeutics, 90*(1), 179-183.

Barlow, D., & Durand, V., (Eds.). (2005). *Abnormal psychology: An integrative approach* (4th [international student edition] ed.). USA: Wadsworth, a division of Thomson Learning, Inc.

Barnes, N. M., & Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology, 38*(8), 1083-1152.

Basile, V. S., Masellis, M., McIntyre, R. S., Meltzer, H. Y., Lieberman, J. A., & Kennedy, J. L. (2001). Genetic dissection of atypical antipsychotic-induced weight gain: Novel preliminary data on the pharmacogenetic puzzle. *The Journal of Clinical Psychiatry, 62 Suppl 23*, 45-66.

Baszczuk, A., Musialik, K., Kopczynski, J., Thielemann, A., Kopczynski, Z., Kesy, L., et al. (2014). Hyperhomocysteinemia, lipid and lipoprotein disturbances in patients with primary hypertension. *Advances in Medical Sciences, 59*(1), 68-73.

Bell, C. G., Finer, S., Lindgren, C. M., Wilson, G. A., Rakyan, V. K., Teschendorff, A. E., et al. (2010). Integrated genetic and epigenetic analysis identifies haplotype-specific methylation in the FTO type 2 diabetes and obesity susceptibility locus. *PloS One, 5*(11), e14040.

Bell, C. G., Walley, A. J., & Froguel, P. (2005). The genetics of human obesity. *Nature Reviews.Genetics, 6*(3), 221-234.

Bell, J. T., Pai, A. A., Pickrell, J. K., Gaffney, D. J., Pique-Regi, R., Degner, J. F., et al. (2011). DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. *Genome Biology, 12*(1), R10-2011-12-1-r10. Epub 2011 Jan 20.

Bell, J. T., Tsai, P. C., Yang, T. P., Pidsley, R., Nisbet, J., Glass, D., et al. (2012). Epigenome-wide scans identify differentially methylated regions for age and age-related phenotypes in a healthy ageing population. *PLoS Genetics, 8*(4), e1002629.

Benes, F. M. (2000). Emerging principles of altered neural circuitry in schizophrenia. *Brain Research.Brain Research Reviews, 31*(2-3), 251-269.

Berger, S. L., Kouzarides, T., Shiekhattar, R., & Shilatifard, A. (2009). An operational definition of epigenetics. *Genes & Development, 23*(7), 781-783.

Bernard, V., Le Moine, C., & Bloch, B. (1991). Striatal neurons express increased level of dopamine D2 receptor mRNA in response to haloperidol treatment: A quantitative in situ hybridization study. *Neuroscience, 45*(1), 117-126.

Berulava, T., & Horsthemke, B. (2010). The obesity-associated SNPs in intron 1 of the FTO gene affect primary transcript levels. *European Journal of Human Genetics : EJHG, 18*(9), 1054-1056.

Biebermann, H., Kuhnen, P., Kleinau, G., & Krude, H. (2012). The neuroendocrine circuitry controlled by POMC, MSH, and AGRP. *Handbook of Experimental Pharmacology,* (209), 47-75.

Biedler, J. L., Helson, L., & Spengler, B. A. (1973). Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Research, 33*(11), 2643-2652.

Biedler, J. L., Roffler-Tarlov, S., Schachner, M., & Freedman, L. S. (1978). Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Research, 38*(11 Pt 1), 3751-3757.

Bird, A. (2007). Perceptions of epigenetics *Nature, 447*(7143), 396-398.

Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation. *Nature, 321*(6067), 209-213.

Bishop, J. R., Ellingrod, V. L., Moline, J., & Miller, D. (2006). Pilot study of the G-protein beta3 subunit gene (C825T) polymorphism and clinical response to olanzapine or olanzapine-related weight gain in persons with schizophrenia. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research, 12*(2), BR47-50.

Bjornsson, H. T., Sigurdsson, M. I., Fallin, M. D., Irizarry, R. A., Aspelund, T., Cui, H., et al. (2008). Intra-individual change over time in DNA methylation with familial clustering. *Jama, 299*(24), 2877-2883.

Bjorntorp, P., & Rosmond, R. (2000). Obesity and cortisol. *Nutrition (Burbank, Los Angeles County, Calif.), 16*(10), 924-936.

Blundell, J. E., & Leshem, M. B. (1975). The effect of 5-hydroxytryptophan on food intake and on the anorexic action of amphetamine and fenfluramine. *The Journal of Pharmacy and Pharmacology, 27*(1), 31-37.

Bollati, V., Schwartz, J., Wright, R., Litonjua, A., Tarantini, L., Suh, H., et al. (2009). Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mechanisms of Ageing and Development, 130*(4), 234-239.

Bonasio, R., Tu, S., & Reinberg, D. (2010). Molecular signals of epigenetic states. *Science, 330*(6004), 612-616.

Bonhaus, D. W., Weinhardt, K. K., Taylor, M., DeSouza, A., McNeeley, P. M., Szczepanski, K., et al. (1997). RS-102221: A novel high affinity and selective, 5-HT2C receptor antagonist. *Neuropharmacology, 36*(4-5), 621-629.

Borcherding, D. C., Hugo, E. R., Idelman, G., De Silva, A., Richtand, N. W., Loftus, J., et al. (2011). Dopamine receptors in human adipocytes: Expression and functions. *PloS One, 6*(9), e25537.

Brawand, D., Soumillon, M., Necsulea, A., Julien, P., Csardi, G., Harrigan, P., et al. (2011). The evolution of gene expression levels in mammalian organs. *Nature, 478*(7369), 343-348.

Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., et al. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in drosophila. *Cell, 128*(6), 1089-1103.

Bromberg, A., Levine, J., Nemetz, B., Belmaker, R. H., & Agam, G. (2008). No association between global leukocyte DNA methylation and homocysteine levels in schizophrenia patients. *Schizophrenia Research, 101*(1-3), 50-57.

Bromel, T., Blum, W. F., Ziegler, A., Schulz, E., Bender, M., Fleischhaker, C., et al. (1998). Serum leptin levels increase rapidly after initiation of clozapine therapy. *Molecular Psychiatry, 3*(1), 76-80.

Buckland, P. R., D'Souza, U., Maher, N. A., & McGuffin, P. (1997). The effects of antipsychotic drugs on the mRNA levels of serotonin 5HT2A and 5HT2C receptors. *Brain Research.Molecular Brain Research, 48*(1), 45-52.

Buckland, P. R., Hoogendoorn, B., Guy, C. A., Smith, S. K., Coleman, S. L., & O'Donovan, M. C. (2005). Low gene expression conferred by association of an allele of the 5-HT2C receptor gene with antipsychotic-induced weight gain. *The American Journal of Psychiatry, 162*(3), 613-615.

Buckley, L. A., Maayan, N., Soares-Weiser, K., & Adams, C. E. (2015). Supportive therapy for schizophrenia. *The Cochrane Database of Systematic Reviews*, 4, CD004716.

Burghardt, K. J., Pilsner, J. R., Bly, M. J., & Ellingrod, V. L. (2012). DNA methylation in schizophrenia subjects: Gender and MTHFR 677C/T genotype differences. *Epigenomics, 4*(3), 261-268.

Burgio, E., Lopomo, A., & Migliore, L. (2015). Obesity and diabetes: From genetics to epigenetics. *Molecular Biology Reports, 42*(4), 799-818.

Burnet, P. W., Chen, C. P., McGowan, S., Franklin, M., & Harrison, P. J. (1996). The effects of clozapine and haloperidol on serotonin-1A, -2A and -2C receptor gene expression and serotonin metabolism in the rat forebrain. *Neuroscience, 73*(2), 531-540.

Burris, H. H., Rifas-Shiman, S. L., Baccarelli, A., Tarantini, L., Boeke, C. E., Kleinman, K., et al. (2012). Associations of LINE-1 DNA methylation with preterm birth in a prospective cohort study. *Journal of Developmental Origins of Health and Disease, 3*(3), 173-181.

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology, 25*(2), 169-193.

Calapai, G., Corica, F., Corsonello, A., Sautebin, L., Di Rosa, M., Campo, G. M., et al. (1999). Leptin increases serotonin turnover by inhibition of brain nitric oxide synthesis. *The Journal of Clinical Investigation, 104*(7), 975-982.

Cannon, M., Jones, P. B., & Murray, R. M. (2002). Obstetric complications and schizophrenia: Historical and meta-analytic review. *The American Journal of Psychiatry, 159*(7), 1080-1092.

Carlsson, A. (1988). The current status of the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 1*(3), 179-186.

Carlsson, A., & Lindqvist, M. (1963). Effect of chlorpromazine or haloperidol on formation of 3methoxytyramine and normetanephrine in mouse brain. *Acta Pharmacologica Et Toxicologica, 20*, 140-144.

Carpenter, W.,T, & Buchanan, R.,W. (2008). Lessons to take home from CATIE. *Psychiatr Serv*, 59(5), 523-525.

Carrard, A., Salzmann, A., Malafosse, A., & Karege, F. (2011). Increased DNA methylation status of the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder. *Journal of Affective Disorders, 132*(3), 450-453.

Carrel, L., & Willard, H. F. (2005). X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature, 434*(7031), 400-404.

Carthew, R. W., & Sontheimer, E. J. (2009). Origins and mechanisms of miRNAs and siRNAs. *Cell, 136*(4), 642-655.

Casey, D. E., Haupt, D. W., Newcomer, J. W., Henderson, D. C., Sernyak, M. J., Davidson, M., et al. (2004). Antipsychotic-induced weight gain and metabolic abnormalities: Implications for increased mortality in patients with schizophrenia. *The Journal of Clinical Psychiatry, 65 Suppl 7*, 4-18; quiz 19-20.

Casey, D. E., & Zorn, S. H. (2001). The pharmacology of weight gain with antipsychotics. *The Journal of Clinical Psychiatry, 62 Suppl 7*, 4-10.

Cavarec, L., Vincent, L., Le Borgne, C., Plusquellec, C., Ollivier, N., Normandie-Levi, P., et al. (2013). In vitro screening for drug-induced depression and/or suicidal adverse effects: A new toxicogenomic assay based on CE-SSCP analysis of HTR2C mRNA editing in SH-SY5Y cells. *Neurotoxicity Research, 23*(1), 49-62.

Cernaianu, G., Brandmaier, P., Scholz, G., Ackermann, O. P., Alt, R., Rothe, K., et al. (2008). All-trans retinoic acid arrests neuroblastoma cells in a dormant state. subsequent nerve growth factor/brain-derived neurotrophic factor treatment adds modest benefit. *Journal of Pediatric Surgery, 43*(7), 1284-1294.

Chaudhry, M. A., & Omaruddin, R. A. (2012). Differential DNA methylation alterations in radiation-sensitive and -resistant cells. *DNA and Cell Biology, 31*(6), 908-916.

Chen, A. R., Zhang, H. G., Wang, Z. P., Fu, S. J., Yang, P. Q., Ren, J. G., et al. (2010). C-reactive protein, vitamin B12 and C677T polymorphism of N-5,10-methylenetetrahydrofolate reductase gene are related to insulin resistance and risk factors for metabolic syndrome in chinese population. *Clinical and Investigative Medicine.Medecine Clinique Et Experimentale, 33*(5), E290-7.

Chen, X., Margolis, K. J., Gershon, M. D., Schwartz, G. J., & Sze, J. Y. (2012). Reduced serotonin reuptake transporter (SERT) function causes insulin resistance and hepatic steatosis independent of food intake. *PloS One, 7*(3), e32511.

Chen, Z. Y., Patel, P. D., Sant, G., Meng, C. X., Teng, K. K., Hempstead, B. L., et al. (2004). Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 24*(18), 4401-4411.

Chenais, B. (2015). Transposable elements in cancer and other human diseases. *Current Cancer Drug Targets,*

Cheng, Y. Y., Jin, H., Liu, X., Siu, J. M., Wong, Y. P., Ng, E. K., et al. (2008). Fibulin 1 is downregulated through promoter hypermethylation in gastric cancer. *British Journal of Cancer, 99*(12), 2083-2087.

Cheng, Z., Jiang, X., Kruger, W. D., Pratico, D., Gupta, S., Mallilankaraman, K., et al. (2011). Hyperhomocysteinemia impairs endothelium-derived hyperpolarizing factor-mediated vasorelaxation in transgenic cystathionine beta synthase-deficient mice. *Blood, 118*(7), 1998-2006.

Cheon, K. A., Cho, D. Y., Koo, M. S., Song, D. H., & Namkoong, K. (2009). Association between homozygosity of a G allele of the alpha-2a-adrenergic receptor gene and

methylphenidate response in Korean children and adolescents with attention-deficit/hyperactivity disorder. *Biological Psychiatry,* 65(7), 564-570.

Cheung, M. K., Gulati, P., O'Rahilly, S., & Yeo, G. S. (2013). FTO expression is regulated by availability of essential amino acids. *International Journal of Obesity (2005), 37*(5), 744-747.

Cheung, M. K., & Yeo, G. S. (2011). FTO biology and obesity: Why do a billion of us weigh 3 kg more? *Frontiers in Endocrinology, 2*, 4.

Cheung, Y. T., Lau, W. K., Yu, M. S., Lai, C. S., Yeung, S. C., So, K. F., et al. (2009). Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology, 30*(1), 127-135.

Chiaruttini, C., Vicario, A., Li, Z., Baj, G., Braiuca, P., Wu, Y., et al. (2009). Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation. *Proceedings of the National Academy of Sciences of the United States of America, 106*(38), 16481-16486.

Chowdhury, N. I., Tiwari, A. K., Souza, R. P., Zai, C. C., Shaikh, S. A., Chen, S., et al. (2013). Genetic association study between antipsychotic-induced weight gain and the melanocortin-4 receptor gene. *The Pharmacogenomics Journal, 13*(3), 272-279.

Christensen, B. C., Houseman, E. A., Marsit, C. J., Zheng, S., Wrensch, M. R., Wiemels, J. L., et al. (2009). Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. *PLoS Genetics, 5*(8), e1000602.

Christin, L., O'Connell, M., Bogardus, C., Danforth, E.,Jr, & Ravussin, E. (1993). Norepinephrine turnover and energy expenditure in pima indian and white men. *Metabolism: Clinical and Experimental, 42*(6), 723-729.

Church, C., Lee, S., Bagg, E. A., McTaggart, J. S., Deacon, R., Gerken, T., et al. (2009). A mouse model for the metabolic effects of the human fat mass and obesity associated FTO gene. *PLoS Genetics, 5*(8), e1000599.

Church, C., Moir, L., McMurray, F., Girard, C., Banks, G. T., Teboul, L., et al. (2010). Overexpression of fto leads to increased food intake and results in obesity. *Nature Genetics, 42*(12), 1086-1092.

Ciccarone, V., Spengler, B. A., Meyers, M. B., Biedler, J. L., & Ross, R. A. (1989). Phenotypic diversification in human neuroblastoma cells: Expression of distinct neural crest lineages. *Cancer Research, 49*(1), 219-225.

Clemett, D. A., Punhani, T., Duxon, M. S., Blackburn, T. P., & Fone, K. C. (2000). Immunohistochemical localisation of the 5-HT2C receptor protein in the rat CNS. *Neuropharmacology, 39*(1), 123-132.

Clifton, P. G., Lee, M. D., & Dourish, C. T. (2000). Similarities in the action of ro 60-0175, a 5-HT2C receptor agonist and d-fenfluramine on feeding patterns in the rat. *Psychopharmacology, 152*(3), 256-267.

Clifton, P. G., Rusk, I. N., & Cooper, S. J. (1991). Effects of dopamine D1 and dopamine D2 antagonists on the free feeding and drinking patterns of rats. *Behavioral Neuroscience, 105*(2), 272-281.

Comer, R., (Ed.). (2004). *Abnormal psychology* (5th ed.). New York: Worth Publishers and W.H. Freeman and Company.

Comuzzie, A. G., & Allison, D. B. (1998). The search for human obesity genes. *Science (New York, N.Y.), 280*(5368), 1374-1377.

Cone, R. D. (2006). Studies on the physiological functions of the melanocortin system. *Endocrine Reviews, 27*(7), 736-749.

Copois, V., Bibeau, F., Bascoul-Mollevi, C., Salvetat, N., Chalbos, P., Bareil, C., et al. (2007). Impact of RNA degradation on gene expression profiles: Assessment of different methods to reliably determine RNA quality. *Journal of Biotechnology, 127*(4), 549-559.

Correll, C. U. (2008). Antipsychotic use in children and adolescents: Minimizing adverse effects to maximize outcomes. *Journal of the American Academy of Child and Adolescent Psychiatry, 47*(1), 9-20.

Coryell, W., Miller, D. D., & Perry, P. J. (1998). Haloperidol plasma levels and dose optimization. *The American Journal of Psychiatry, 155*(1), 48-53.

Costa, E., Dong, E., Grayson, D. R., Guidotti, A., Ruzicka, W., & Veldic, M. (2007). Reviewing the role of DNA (cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. *Epigenetics : Official Journal of the DNA Methylation Society, 2*(1), 29-36.

Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdan, M. G., Diano, S., Horvath, T. L., et al. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature, 411*(6836), 480-484.

Creed-Carson, M., Oraha, A., & Nobrega, J. N. (2011). Effects of 5-HT(2A) and 5-HT(2C) receptor antagonists on acute and chronic dyskinetic effects induced by haloperidol in rats. *Behavioural Brain Research, 219*(2), 273-279.

Creese, I., Burt, D. R., & Snyder, S. H. (1976). Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. *Science (New York, N.Y.), 192*(4238), 481-483.

Crossley, N. A., Constante, M., McGuire, P., & Power, P. (2010). Efficacy of atypical v. typical antipsychotics in the treatment of early psychosis: Meta-analysis. *The British Journal of Psychiatry : The Journal of Mental Science*, 196(6), 434-439.

Crow, T. J., & Done, D. J. (1992). Prenatal exposure to influenza does not cause schizophrenia. *The British Journal of Psychiatry : The Journal of Mental Science, 161*, 390-393.

Crujeiras, A. B., Campion, J., Diaz-Lagares, A., Milagro, F. I., Goyenechea, E., Abete, I., et al. (2013). Association of weight regain with specific methylation levels in the NPY and POMC promoters in leukocytes of obese men: A translational study. *Regulatory Peptides, 186*, 1-6.

Currie, P. J., & Coscina, D. V. (1998). 5-hydroxytryptaminergic receptor agonists: Effects on neuropeptide Y potentiation of feeding and respiratory quotient. *Brain Research, 803*(1-2), 212-217.

Czerwensky, F., Leucht, S., & Steimer, W. (2013a). Association of the common MC4R rs17782313 polymorphism with antipsychotic-related weight gain. *Journal of Clinical Psychopharmacology, 33*(1), 74-79.

Czerwensky, F., Leucht, S., & Steimer, W. (2013b). MC4R rs489693: A clinical risk factor for second generation antipsychotic-related weight gain? *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP), 16*(9), 2103-2109.

Datki, Z., Juhasz, A., Galfi, M., Soos, K., Papp, R., Zadori, D., et al. (2003). Method for measuring neurotoxicity of aggregating polypeptides with the MTT assay on differentiated neuroblastoma cells. *Brain Research Bulletin, 62*(3), 223-229.

Davis, K. L., Kahn, R. S., Ko, G., & Davidson, M. (1991). Dopamine in schizophrenia: A review and reconceptualization. *The American Journal of Psychiatry, 148*(11), 1474-1486.

Davoodi, N., Kalinichev, M., & Clifton, P. G. (2008). Comparative effects of olanzapine and ziprasidone on hypophagia induced by enhanced histamine neurotransmission in the rat. *Behavioural Pharmacology, 19*(2), 121-128.

de Arruda, I. T., Persuhn, D. C., & de Oliveira, N. F. (2013). The MTHFR C677T polymorphism and global DNA methylation in oral epithelial cells. *Genetics and Molecular Biology, 36*(4), 490-493.

DE Hert, M., Schreurs, V., Vancampfort, D., & VAN Winkel, R. (2009). Metabolic syndrome in people with schizophrenia: A review. *World Psychiatry : Official Journal of the World Psychiatric Association (WPA), 8*(1), 15-22.

De Hert, M. A., van Winkel, R., Van Eyck, D., Hanssens, L., Wampers, M., Scheen, A., et al. (2006). Prevalence of the metabolic syndrome in patients with schizophrenia treated with antipsychotic medication. *Schizophrenia Research, 83*(1), 87-93.

De Luca, V., Souza, R. P., Viggiano, E., Sickert, L., Teo, C., Zai, C., et al. (2011). Genetic interactions in the adrenergic system genes: Analysis of antipsychotic-induced weight gain. *Human Psychopharmacology, 26*(6), 386-391.

Delitala, G., Trainer, P. J., Oliva, O., Fanciulli, G., & Grossman, A. B. (1994). Opioid peptide and alpha-adrenoceptor pathways in the regulation of the pituitary-adrenal axis in man. *The Journal of Endocrinology, 141*(1), 163-168.

den Hoed, M., Westerterp-Plantenga, M. S., Bouwman, F. G., Mariman, E. C., & Westerterp, K. R. (2009). Postprandial responses in hunger and satiety are associated with the rs9939609 single nucleotide polymorphism in FTO. *The American Journal of Clinical Nutrition, 90*(5), 1426-1432.

Deupree, J. D., Smith, S. D., Kratochvil, C. J., Bohac, D., Ellis, C. R., Polaha, J., *et al.* (2006). Possible involvement of alpha-2A adrenergic receptors in attention deficit hyperactivity disorder: Radioligand binding and polymorphism studies. *Am J Med Genet B Neuropsychiatr Genet.* 141B(8):877-884.

Devedjian, J. C., Pujol, A., Cayla, C., George, M., Casellas, A., Paris, H., *et al.* (2000). Transgenic mice overexpressing alpha2A-adrenoceptors in pancreatic beta-cells show altered regulation of glucose homeostasis. *Diabetologia, 43*(7), 899-906.

Dheda, K., Huggett, J. F., Bustin, S. A., Johnson, M. A., Rook, G., & Zumla, A. (2004). Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques, 37*(1), 112-4, 116, 118-9.

Di Narzo, A. F., Kozlenkov, A., Roussos, P., Hao, K., Hurd, Y., Lewis, D. A., *et al.* (2014). A unique gene expression signature associated with serotonin 2C receptor RNA editing in the prefrontal cortex and altered in suicide. *Human Molecular Genetics, 23*(18), 4801-4813.

Dickerson, F. B., Brown, C. H., Kreyenbuhl, J. A., Fang, L., Goldberg, R. W., Wohlheiter, K., et al. (2006). Obesity among individuals with serious mental illness. *Acta Psychiatrica Scandinavica, 113*(4), 306-313.

Dinan, T. G. (1996). Serotonin and the regulation of hypothalamic-pituitary-adrenal axis function. *Life Sciences, 58*(20), 1683-1694.

Dolinoy, D. C., Weidman, J. R., & Jirtle, R. L. (2007). Epigenetic gene regulation: Linking early developmental environment to adult disease. *Reproductive Toxicology (Elmsford, N.Y.), 23*(3), 297-307.

Dong, E., Nelson, M., Grayson, D. R., Costa, E., & Guidotti, A. (2008). Clozapine and sulpiride but not haloperidol or olanzapine activate brain DNA demethylation. *Proceedings of the National Academy of Sciences of the United States of America, 105*(36), 13614-13619.

Dourish, C. T., Hutson, P. H., & Curzon, G. (1985). Low doses of the putative serotonin agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) elicit feeding in the rat. *Psychopharmacology, 86*(1-2), 197-204.

Dryden, S., Wang, Q., Frankish, H. M., & Williams, G. (1996). Differential effects of the 5-HT 1B/2C receptor agonist mCPP and the 5-HT1A agonist flesinoxan on hypothalamic neuropeptide Y in the rat: Evidence that NPY may mediate serotonin's effects on food intake. *Peptides, 17*(6), 943-949.

Duggan, C., Xiao, L., Terry, M. B., & McTiernan, A. (2014). No effect of weight loss on LINE-1 methylation levels in peripheral blood leukocytes from postmenopausal overweight women. *Obesity (Silver Spring, Md.), 22*(9), 2091-2096.

Duncan, E. J., Woolson, S. L., Hamer, R. M., & Dunlop, B. W. (2009). Risk of lipid abnormality with haloperidol, olanzapine, quetiapine, and risperidone in a veterans affairs population. *International Clinical Psychopharmacology, 24*(4), 204-213.

Duncan, G. E., Sheitman, B. B., & Lieberman, J. A. (1999). An integrated view of pathophysiological models of schizophrenia. *Brain Research.Brain Research Reviews, 29*(2-3), 250-264.

Eden, S., Constancia, M., Hashimshony, T., Dean, W., Goldstein, B., Johnson, A. C., et al. (2001). An upstream repressor element plays a role in Igf2 imprinting. *The EMBO Journal, 20*(13), 3518-3525.

Egan, M. F., Kojima, M., Callicott, J. H., Goldberg, T. E., Kolachana, B. S., Bertolino, A., et al. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell, 112*(2), 257-269.

Ehrlich, M., Gama-Sosa, M. A., Huang, L. H., Midgett, R. M., Kuo, K. C., McCune, R. A., et al. (1982). Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Research, 10*(8), 2709-2721.

Ellingrod, V. L., Miller, D. D., Taylor, S. F., Moline, J., Holman, T., & Kerr, J. (2008). Metabolic syndrome and insulin resistance in schizophrenia patients receiving antipsychotics genotyped for the methylenetetrahydrofolate reductase (MTHFR) 677C/T and 1298A/C variants. *Schizophrenia Research, 98*(1-3), 47-54.

Ellingrod, V. L., Perry, P. J., Ringold, J. C., Lund, B. C., Bever-Stille, K., Fleming, F., et al. (2005). Weight gain associated with the -759C/T polymorphism of the 5HT2C receptor and olanzapine. *American Journal of Medical Genetics.Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics, 134B*(1), 76-78.

Ellingrod, V. L., Taylor, S. F., Dalack, G., Grove, T. B., Bly, M. J., Brook, R. D., et al. (2012). Risk factors associated with metabolic syndrome in bipolar and schizophrenia subjects treated with antipsychotics: The role of folate pharmacogenetics. *Journal of Clinical Psychopharmacology, 32*(2), 261-265.

El-Maarri, O., Walier, M., Behne, F., van Uum, J., Singer, H., Diaz-Lacava, A., et al. (2011). Methylation at global LINE-1 repeats in human blood are affected by gender but not by age or natural hormone cycles. *PloS One, 6*(1), e16252.

Emery, R., & Oltmanns, T., (Eds.). (2000). *Essentials of abnormal psychology*. New Jersey: Prentice-Hall.

Eseberri, I., Lasa, A., Churruca, I., & Portillo, M. P. (2013). Resveratrol metabolites modify adipokine expression and secretion in 3T3-L1 pre-adipocytes and mature adipocytes. *PloS One, 8*(5), e63918.

Espinoza, C. A., & Ren, B. (2011). Mapping higher order structure of chromatin domains. *Nature Genetics, 43*(7), 615-616.

Estey, E. H. (2013). Epigenetics in clinical practice: The examples of azacitidine and decitabine in myelodysplasia and acute myeloid leukemia. *Leukemia, 27*(9), 1803-1812.

Fagerstrom, S., Pahlman, S., Gestblom, C., & Nanberg, E. (1996). Protein kinase C-epsilon is implicated in neurite outgrowth in differentiating human neuroblastoma cells. *Cell Growth & Differentiation : The Molecular Biology Journal of the American Association for Cancer Research, 7*(6), 775-785.

Fain, J. N., & Garcija-Sainz, J. A. (1983). Adrenergic regulation of adipocyte metabolism. *Journal of Lipid Research, 24*(8), 945-966.

Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., & Cone, R. D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature, 385*(6612), 165-168.

Fan, W., Ellacott, K. L., Halatchev, I. G., Takahashi, K., Yu, P., & Cone, R. D. (2004). Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nature Neuroscience, 7*(4), 335-336.

Fawcett, K. A., & Barroso, I. (2010). The genetics of obesity: FTO leads the way. *Trends in Genetics : TIG, 26*(6), 266-274.

Feng, Q., & Zhang, Y. (2001). The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes. *Genes & Development, 15*(7), 827-832.

Fernandez-Galaz, M. C., Fernandez-Agullo, T., Carrascosa, J. M., Ros, M., & Garcia-Segura, L. M. (2010). Leptin accumulation in hypothalamic and dorsal raphe neurons is inversely correlated with brain serotonin content. *Brain Research, 1329*, 194-202.

Fischer, J., Koch, L., Emmerling, C., Vierkotten, J., Peters, T., Bruning, J. C., et al. (2009). Inactivation of the fto gene protects from obesity. *Nature, 458*(7240), 894-898.

Flom, J. D., Ferris, J. S., Liao, Y., Tehranifar, P., Richards, C. B., Cho, Y. H., et al. (2011). Prenatal smoke exposure and genomic DNA methylation in a multiethnic birth cohort. *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 20*(12), 2518-2523.

Flomen, R., Knight, J., Sham, P., Kerwin, R., & Makoff, A. (2004). Evidence that RNA editing modulates splice site selection in the 5-HT2C receptor gene. *Nucleic Acids Research, 32*(7), 2113-2122.

Fox, J. T., & Stover, P. J. (2008). Folate-mediated one-carbon metabolism. *Vitamins and Hormones, 79*, 1-44.

Frayling, T. M., Timpson, N. J., Weedon, M. N., Zeggini, E., Freathy, R. M., Lindgren, C. M., et al. (2007). A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science (New York, N.Y.), 316*(5826), 889-894.

Fries, P. (2009). Neuronal gamma-band synchronization as a fundamental process in cortical computation. *Annual Review of Neuroscience,* 32, 209-224.

Friso, S., & Choi, S. W. (2002). Gene-nutrient interactions and DNA methylation. *The Journal of Nutrition, 132*(8 Suppl), 2382S-2387S.

Friso, S., Choi, S. W., Girelli, D., Mason, J. B., Dolnikowski, G. G., Bagley, P. J., et al. (2002). A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proceedings of the National Academy of Sciences of the United States of America, 99*(8), 5606-5611.

Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., . . . van den Heuvel, L. P. (1995). A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. *Nature Genetics,* 10(1), 111-113.

Fu, Y., Luo, N., Klein, R. L., & Garvey, W. T. (2005). Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. *Journal of Lipid Research, 46*(7), 1369-1379.

Fujisawa, T., Ikegami, H., Kawaguchi, Y., & Ogihara, T. (1998). Meta-analysis of the association of Trp64Arg polymorphism of beta 3-adrenergic receptor gene with body mass index. *The Journal of Clinical Endocrinology and Metabolism, 83*(7), 2441-2444.

Galgani, J., & Ravussin, E. (2008). Energy metabolism, fuel selection and body weight regulation. *International Journal of Obesity (2005), 32 Suppl 7*, S109-19.

Garenc, C., Perusse, L., Chagnon, Y. C., Rankinen, T., Gagnon, J., Borecki, I. B., et al. (2002). The alpha 2-adrenergic receptor gene and body fat content and distribution: The HERITAGE family study. *Molecular Medicine (Cambridge, Mass.), 8*(2), 88-94.

Gautam, S., & Meena, P. S. (2011). Drug-emergent metabolic syndrome in patients with schizophrenia receiving atypical (second-generation) antipsychotics. *Indian Journal of Psychiatry, 53*(2), 128-133.

Gautron, L., Lee, C., Funahashi, H., Friedman, J., Lee, S., & Elmquist, J. (2010). Melanocortin-4 receptor expression in a vago-vagal circuitry involved in postprandial functions. *The Journal of Comparative Neurology, 518*(1), 6-24.

Geddes, J. R., Verdoux, H., Takei, N., Lawrie, S. M., Bovet, P., Eagles, J. M., et al. (1999). Schizophrenia and complications of pregnancy and labor: An individual patient data meta-analysis. *Schizophrenia Bulletin, 25*(3), 413-423.

Gerken, T., Girard, C. A., Tung, Y. C., Webby, C. J., Saudek, V., Hewitson, K. S., et al. (2007). The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. *Science (New York, N.Y.), 318*(5855), 1469-1472.

Ghadirivasfi, M., Nohesara, S., Ahmadkhaniha, H. R., Eskandari, M. R., Mostafavi, S., Thiagalingam, S., et al. (2011). Hypomethylation of the serotonin receptor type-2A gene (HTR2A) at T102C polymorphic site in DNA derived from the saliva of patients

with schizophrenia and bipolar disorder. *American Journal of Medical Genetics.Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics, 156B*(5), 536-545.

Gilbert, D. B., & Cooper, S. J. (1985). Analysis of dopamine D1 and D2 receptor involvement in d- and l-amphetamine-induced anorexia in rats. *Brain Research Bulletin, 15*(4), 385-389.

Godlewska, B. R., Olajossy-Hilkesberger, L., Ciwoniuk, M., Olajossy, M., Marmurowska-Michalowska, H., Limon, J., et al. (2009). Olanzapine-induced weight gain is associated with the -759C/T and -697G/C polymorphisms of the HTR2C gene. *The Pharmacogenomics Journal, 9*(4), 234-241.

Goeb, J. L., Marco, S., Duhamel, A., Kechid, G., Bordet, R., Thomas, P., et al. (2010). Metabolic side effects of risperidone in early onset schizophrenia. [Effets secondaires metaboliques de la risperidone dans les schizophrenies a debut precoce] *L'Encephale, 36*(3), 242-252.

Goldman, C. K., Marino, L., & Leibowitz, S. F. (1985). Postsynaptic alpha 2-noradrenergic receptors mediate feeding induced by paraventricular nucleus injection of norepinephrine and clonidine. *European Journal of Pharmacology, 115*(1), 11-19.

Gonzalez-Burgos, G., Fish, K. N., & Lewis, D. A. (2011). GABA neuron alterations, cortical circuit dysfunction and cognitive deficits in schizophrenia. *Neural Plasticity*, 2011, 723184.

Goto, T., & Monk, M. (1998). Regulation of X-chromosome inactivation in development in mice and humans. *Microbiology and Molecular Biology Reviews : MMBR, 62*(2), 362-378.

Gottesman, I,I, (Ed.). (1991). *Schizophrenia genesis: The origin of madness*. New York: Freeman.

Goudie, A. J., Cooper, G. D., & Halford, J. C. (2005). Antipsychotic-induced weight gain. *Diabetes, Obesity & Metabolism, 7*(5), 478-487.

Gratacos, M., Gonzalez, J. R., Mercader, J. M., de Cid, R., Urretavizcaya, M., & Estivill, X. (2007). Brain-derived neurotrophic factor Val66Met and psychiatric disorders: Meta-analysis of case-control studies confirm association to substance-related disorders, eating disorders, and schizophrenia. *Biological Psychiatry, 61*(7), 911-922.

Grayson, D. R., Jia, X., Chen, Y., Sharma, R. P., Mitchell, C. P., Guidotti, A., et al. (2005). Reelin promoter hypermethylation in schizophrenia. *Proceedings of the National Academy of Sciences of the United States of America, 102*(26), 9341-9346.

Gregoor, J. G., Mulder, H., Cohen, D., van Megen, H. J., Egberts, T. C., Heerdink, E. R., et al. (2010). Combined HTR2C-LEP genotype as a determinant of obesity in patients using antipsychotic medication. *Journal of Clinical Psychopharmacology, 30*(6), 702-705.

Gregoor, J. G., van der Weide, J., Loovers, H. M., van Megen, H. J., Egberts, T. C., & Heerdink, E. R. (2011). Polymorphisms of the LEP, LEPR and HTR2C gene: Obesity and

BMI change in patients using antipsychotic medication in a naturalistic setting. *Pharmacogenomics, 12*(6), 919-923.

Gregoor, J. G., van der Weide, J., Mulder, H., Cohen, D., van Megen, H. J., Egberts, A. C., et al. (2009). Polymorphisms of the LEP- and LEPR gene and obesity in patients using antipsychotic medication. *Journal of Clinical Psychopharmacology, 29*(1), 21-25.

Grewal, S. I. (2010). RNAi-dependent formation of heterochromatin and its diverse functions. *Current Opinion in Genetics & Development, 20*(2), 134-141.

Gronniger, E., Weber, B., Heil, O., Peters, N., Stab, F., Wenck, H., et al. (2010). Aging and chronic sun exposure cause distinct epigenetic changes in human skin. *PLoS Genetics, 6*(5), e1000971.

Guan, X., Shi, X., Li, X., Chang, B., Wang, Y., Li, D., et al. (2012). GLP-2 receptor in POMC neurons suppresses feeding behavior and gastric motility. *American Journal of Physiology.Endocrinology and Metabolism, 303*(7), E853-64.

Guarnieri, S., Pilla, R., Morabito, C., Sacchetti, S., Mancinelli, R., Fano, G., et al. (2009). Extracellular guanosine and GTP promote expression of differentiation markers and induce S-phase cell-cycle arrest in human SH-SY5Y neuroblastoma cells. *International Journal of Developmental Neuroscience : The Official Journal of the International Society for Developmental Neuroscience, 27*(2), 135-147.

Guidotti, A., Auta, J., Davis, J. M., Dong, E., Grayson, D. R., Veldic, M., et al. (2005). GABAergic dysfunction in schizophrenia: New treatment strategies on the horizon. *Psychopharmacology, 180*(2), 191-205.

Guidotti, A., Dong, E., Kundakovic, M., Satta, R., Grayson, D. R., & Costa, E. (2009). Characterization of the action of antipsychotic subtypes on valproate-induced chromatin remodeling. *Trends in Pharmacological Sciences, 30*(2), 55-60.

Guidotti, A., & Grayson, D. R. (2014). DNA methylation and demethylation as targets for antipsychotic therapy. *Dialogues in Clinical Neuroscience, 16*(3), 419-429.

Guilland, J. C., Favier, A., Potier de Courcy, G., Galan, P., & Hercberg, S. (2003). Hyperhomocysteinemia: An independent risk factor or a simple marker of vascular disease?. 1. basic data. [L'hyperhomocysteinemie: facteur de risque cardiovasculaire ou simple marqueur ? 1. Donnees fondamentales] *Pathologie-Biologie, 51*(2), 101-110.

Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., et al. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5' end formation in drosophila. *Science (New York, N.Y.), 315*(5818), 1587-1590.

Gunes, A., Melkersson, K. I., Scordo, M. G., & Dahl, M. L. (2009). Association between HTR2C and HTR2A polymorphisms and metabolic abnormalities in patients treated with olanzapine or clozapine. *Journal of Clinical Psychopharmacology, 29*(1), 65-68.

Guo, H., Ingolia, N. T., Weissman, J. S., & Bartel, D. P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature, 466*(7308), 835-840.

Gupta, S., & Kulhara, P. (2010). What is schizophrenia: A neurodevelopmental or neurodegenerative disorder or a combination of both? A critical analysis. *Indian Journal of Psychiatry, 52*(1), 21-27.

Gurevich, E. V., & Joyce, J. N. (1997). Alterations in the cortical serotonergic system in schizophrenia: A postmortem study. *Biological Psychiatry, 42*(7), 529-545.

Hajnal, A., Mark, G. P., Rada, P. V., Lenard, L., & Hoebel, B. G. (1997). Norepinephrine microinjections in the hypothalamic paraventricular nucleus increase extracellular dopamine and decrease acetylcholine in the nucleus accumbens: Relevance to feeding reinforcement. *Journal of Neurochemistry, 68*(2), 667-674.

Halford, J. C., & Blundell, J. E. (2000). Separate systems for serotonin and leptin in appetite control. *Annals of Medicine, 32*(3), 222-232.

Hamilton, B. S., Paglia, D., Kwan, A. Y., & Deitel, M. (1995). Increased obese mRNA expression in omental fat cells from massively obese humans. *Nature Medicine, 1*(9), 953-956.

Han, M., Deng, C., Burne, T. H., Newell, K. A., & Huang, X. F. (2008). Short- and long-term effects of antipsychotic drug treatment on weight gain and H1 receptor expression. *Psychoneuroendocrinology, 33*(5), 569-580.

Han, Z., Niu, T., Chang, J., Lei, X., Zhao, M., Wang, Q., et al. (2010). Crystal structure of the FTO protein reveals basis for its substrate specificity. *Nature, 464*(7292), 1205-1209.

Harker, L. A., Harlan, J. M., & Ross, R. (1983). Effect of sulfinpyrazone on homocysteine-induced endothelial injury and arteriosclerosis in baboons. *Circulation Research, 53*(6), 731-739.

Hashimoto, T., Nishino, N., Nakai, H., & Tanaka, C. (1991). Increase in serotonin 5-HT1A receptors in prefrontal and temporal cortices of brains from patients with chronic schizophrenia. *Life Sciences, 48*(4), 355-363.

Hasnain, M., Fredrickson, S. K., Vieweg, W. V., & Pandurangi, A. K. (2010). Metabolic syndrome associated with schizophrenia and atypical antipsychotics. *Current Diabetes Reports, 10*(3), 209-216.

Hasnain, M., Vieweg, W. V., Fredrickson, S. K., Beatty-Brooks, M., Fernandez, A., & Pandurangi, A. K. (2009). Clinical monitoring and management of the metabolic syndrome in patients receiving atypical antipsychotic medications. *Primary Care Diabetes, 3*(1), 5-15.

Hauner, H., Rohrig, K., Hebebrand, J., & Skurk, T. (2003). No evidence for a direct effect of clozapine on fat-cell formation and production of leptin and other fat-cell-derived factors. *Molecular Psychiatry, 8*(3), 258-259.

Haupt, D. W., Luber, A., Maeda, J., Melson, A. K., Schweiger, J. A., & Newcomer, J. W. (2005). Plasma leptin and adiposity during antipsychotic treatment of schizophrenia.

Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 30(1), 184-191.

Hayashi, A., Suzuki, M., Sasamata, M., & Miyata, K. (2005). Agonist diversity in 5-HT(2C) receptor-mediated weight control in rats. *Psychopharmacology, 178*(2-3), 241-249.

Hay-Schmidt, A., Helboe, L., & Larsen, P. J. (2001). Leptin receptor immunoreactivity is present in ascending serotonergic and catecholaminergic neurons of the rat. *Neuroendocrinology, 73*(4), 215-226.

He, J., Xi, B., Ruiter, R., Shi, T. Y., Zhu, M. L., Wang, M. Y., et al. (2013). Association of LEP G2548A and LEPR Q223R polymorphisms with cancer susceptibility: Evidence from a meta-analysis. *PloS One, 8*(10), e75135.

Heijmans, B. T., Tobi, E. W., Stein, A. D., Putter, H., Blauw, G. J., Susser, E. S., et al. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences of the United States of America, 105*(44), 17046-17049.

Hein, L., Altman, J. D., & Kobilka, B. K. (1999). Two functionally distinct alpha2-adrenergic receptors regulate sympathetic neurotransmission. *Nature, 402*(6758), 181-184.

Heisler, L. K., Jobst, E. E., Sutton, G. M., Zhou, L., Borok, E., Thornton-Jones, Z., et al. (2006). Serotonin reciprocally regulates melanocortin neurons to modulate food intake. *Neuron, 51*(2), 239-249.

Hemmrich, K., Gummersbach, C., Pallua, N., Luckhaus, C., & Fehsel, K. (2006). Clozapine enhances differentiation of adipocyte progenitor cells. *Molecular Psychiatry, 11*(11), 980-981.

Henderson, D. C., Cagliero, E., Gray, C., Nasrallah, R. A., Hayden, D. L., Schoenfeld, D. A., et al. (2000). Clozapine, diabetes mellitus, weight gain, and lipid abnormalities: A five-year naturalistic study. *The American Journal of Psychiatry, 157*(6), 975-981.

Henderson, J. A., & Bethea, C. L. (2008). Differential effects of ovarian steroids and raloxifene on serotonin 1A and 2C receptor protein expression in macaques. *Endocrine, 33*(3), 285-293.

Herken, H., Erdal, M., Aydin, N., Sengul, C., Karadag, F., Barlas, O., et al. (2009). The association of olanzapine-induced weight gain with peroxisome proliferator-activated receptor-gamma2 Pro12Ala polymorphism in patients with schizophrenia. *DNA and Cell Biology, 28*(10), 515-519.

Hernando-Herraez, I., Prado-Martinez, J., Garg, P., Fernandez-Callejo, M., Heyn, H., Hvilsom, C., et al. (2013). Dynamics of DNA methylation in recent human and great ape evolution. *PLoS Genetics, 9*(9), e1003763.

Hess, M. E., Hess, S., Meyer, K. D., Verhagen, L. A., Koch, L., Bronneke, H. S., et al. (2013). The fat mass and obesity associated gene (fto) regulates activity of the dopaminergic midbrain circuitry. *Nature Neuroscience, 16*(8), 1042-1048.

Hetherington,A,W, & Ranson,S,W,. (1940). Hypothalamic lesions and adiposity in the rat. *The Anatomical Record, 78*, 148-172.

Hiemke, C., Baumann, P., Bergemann, N., Conca, A., Dietmaier, O., Egberts, K., et al. (2011). AGNP consensus guidelines for therapeutic drug monitoring in psychiatry: Update 2011. *Pharmacopsychiatry, 44*(6), 195-235.

Hill, M. J., & Reynolds, G. P. (2007). 5-HT2C receptor gene polymorphisms associated with antipsychotic drug action alter promoter activity. *Brain Research, 1149*, 14-17.

Hill, M. J., & Reynolds, G. P. (2011). Functional consequences of two HTR2C polymorphisms associated with antipsychotic-induced weight gain. *Pharmacogenomics, 12*(5), 727-734.

Hinney, A., Volckmar, A. L., & Knoll, N. (2013). Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis. *Progress in Molecular Biology and Translational Science, 114*, 147-191.

Hinney, A., Ziegler, A., Nothen, M. M., Remschmidt, H., & Hebebrand, J. (1997). 5-HT2A receptor gene polymorphisms, anorexia nervosa, and obesity. *Lancet, 350*(9087), 1324-1325.

Hinuy, H. M., Hirata, M. H., Forti, N., Diament, J., Sampaio, M. F., Armaganijan, D., et al. (2008). Leptin G-2548A promoter polymorphism is associated with increased plasma leptin and BMI in brazilian women. *Arquivos Brasileiros De Endocrinologia e Metabologia, 52*(4), 611-616.

Hoffstedt, J., Eriksson, P., Mottagui-Tabar, S., & Arner, P. (2002). A polymorphism in the leptin promoter region (-2548 G/A) influences gene expression and adipose tissue secretion of leptin. *Hormone and Metabolic Research = Hormon- Und Stoffwechselforschung = Hormones Et Metabolisme, 34*(7), 355-359.

Hoffstedt, J., Poirier, O., Thorne, A., Lonnqvist, F., Herrmann, S. M., Cambien, F., et al. (1999). Polymorphism of the human beta3-adrenoceptor gene forms a well-conserved haplotype that is associated with moderate obesity and altered receptor function. *Diabetes, 48*(1), 203-205.

Holliday, R. (1994). Epigenetics: An overview. *Developmental Genetics, 15*(6), 453-457.

Hong, C. J., Liou, Y. J., Bai, Y. M., Chen, T. T., Wang, Y. C., & Tsai, S. J. (2010). Dopamine receptor D2 gene is associated with weight gain in schizophrenic patients under long-term atypical antipsychotic treatment. *Pharmacogenetics and Genomics, 20*(6), 359-366.

Hong, C. J., Yu, Y. W., Lin, C. H., & Tsai, S. J. (2003). An association study of a brain-derived neurotrophic factor Val66Met polymorphism and clozapine response of schizophrenic patients. *Neuroscience Letters, 349*(3), 206-208.

Horstmann, A., Kovacs, P., Kabisch, S., Boettcher, Y., Schloegl, H., Tonjes, A., et al. (2013). Common genetic variation near MC4R has a sex-specific impact on human brain structure and eating behavior. *PloS One, 8*(9), e74362.

Horvath, S., Zhang, Y., Langfelder, P., Kahn, R. S., Boks, M. P., van Eijk, K., et al. (2012). Aging effects on DNA methylation modules in human brain and blood tissue. *Genome Biology, 13*(10), R97-2012-13-10-r97.

Hsiung, D. T., Marsit, C. J., Houseman, E. A., Eddy, K., Furniss, C. S., McClean, M. D., et al. (2007). Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 16*(1), 108-114.

Hu, Y., Kutscher, E., & Davies, G. E. (2010). Berberine inhibits SREBP-1-related clozapine and risperidone induced adipogenesis in 3T3-L1 cells. *Phytotherapy Research : PTR, 24*(12), 1831-1838.

Huang, X. F., Tan, Y. Y., Huang, X., & Wang, Q. (2007). Effect of chronic treatment with clozapine and haloperidol on 5-HT(2A and 2C) receptor mRNA expression in the rat brain. *Neuroscience Research, 59*(3), 314-321.

Huen, K., Yousefi, P., Bradman, A., Yan, L., Harley, K. G., Kogut, K., et al. (2014). Effects of age, sex, and persistent organic pollutants on DNA methylation in children. *Environmental and Molecular Mutagenesis, 55*(3), 209-222.

Jablensky, A., Sartorius, N., Ernberg, G., Anker, M., Korten, A., Cooper, J. E., et al. (1992). Schizophrenia: Manifestations, incidence and course in different cultures. A world health organization ten-country study. *Psychological Medicine.Monograph Supplement, 20*, 1-97.

Jeon, Y., & Lee, J. T. (2011). YY1 tethers xist RNA to the inactive X nucleation center. *Cell, 146*(1), 119-133.

Jia, G., Yang, C. G., Yang, S., Jian, X., Yi, C., Zhou, Z., et al. (2008). Oxidative demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA by mouse and human FTO. *FEBS Letters, 582*(23-24), 3313-3319.

Jiang, Y., Langley, B., Lubin, F. D., Renthal, W., Wood, M. A., Yasui, D. H., et al. (2008). Epigenetics in the nervous system. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 28*(46), 11753-11759.

Jin, H., Meyer, J. M., Mudaliar, S., & Jeste, D. V. (2008). Impact of atypical antipsychotic therapy on leptin, ghrelin, and adiponectin. *Schizophrenia Research, 100*(1-3), 70-85.

Jintaridth, P., & Mutirangura, A. (2010). Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. *Physiological Genomics, 41*(2), 194-200.

Jirtle, R. L., & Skinner, M. K. (2007). Environmental epigenomics and disease susceptibility. *Nature Reviews.Genetics, 8*(4), 253-262.

Johnson, W., Spychala, J., Stenroos, E., Scholl, T., & Schroeder, C. (2001). Smoking behavior and the C677T allele of the methylenetetrahydrofolate reductase (MTHFR) gene. *American Journal of Medical Genetics, 98*(4), 361-362.

Johnston, C. M., Lovell, F. L., Leongamornlert, D. A., Stranger, B. E., Dermitzakis, E. T., & Ross, M. T. (2008). Large-scale population study of human cell lines indicates that dosage compensation is virtually complete. *PLoS Genetics, 4*(1), e9.

Jones, P. A. (1999). The DNA methylation paradox. *Trends in Genetics : TIG, 15*(1), 34-37.

Jones, P. A. (2012). Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature Reviews.Genetics, 13*(7), 484-492.

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., et al. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature Genetics, 19*(2), 187-191.

Joshi, S., Guleria, R., Pan, J., DiPette, D., & Singh, U. S. (2006). Retinoic acid receptors and tissue-transglutaminase mediate short-term effect of retinoic acid on migration and invasion of neuroblastoma SH-SY5Y cells. *Oncogene, 25*(2), 240-247.

Kaikkonen, M. U., Lam, M. T., & Glass, C. K. (2011). Non-coding RNAs as regulators of gene expression and epigenetics. *Cardiovascular Research, 90*(3), 430-440.

Kane, J. M. (1994). Risperidone: New horizons for the schizophrenic patient. 9th world congress of psychiatry of the world psychiatric association, rio de janeiro, june 1993. *The Journal of Clinical Psychiatry, 55 Suppl*, 3-4.

Kaplan,H,I, & Sadock,B,J, (Eds.). (1995). *Comprehensive textbook of psychiatry*. Baltimore, MD: Williams & Wilkins.

Kapur, S., Zipursky, R., Jones, C., Remington, G., & Houle, S. (2000). Relationship between dopamine D(2) occupancy, clinical response, and side effects: A double-blind PET study of first-episode schizophrenia. *The American Journal of Psychiatry, 157*(4), 514-520.

Karpf, A. R., & Matsui, S. (2005). Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. *Cancer Research, 65*(19), 8635-8639.

Kawazoe, Y., Tanaka, S., & Uesugi, M. (2004). Chemical genetic identification of the histamine H1 receptor as a stimulator of insulin-induced adipogenesis. *Chemistry & Biology, 11*(7), 907-913.

Kendell, R. E., & Kemp, I. W. (1989). Maternal influenza in the etiology of schizophrenia. *Archives of General Psychiatry, 46*(10), 878-882.

Kennett, G. A., Wood, M. D., Bright, F., Trail, B., Riley, G., Holland, V., et al. (1997). SB 242084, a selective and brain penetrant 5-HT2C receptor antagonist. *Neuropharmacology, 36*(4-5), 609-620.

Kiani, J. G., Saeed, M., Parvez, S. H., & Frossard, P. M. (2005). Association of G-protein beta-3 subunit gene (GNB3) T825 allele with type II diabetes. *Neuro Endocrinology Letters, 26*(2), 87-88.

Kim, S. F., Huang, A. S., Snowman, A. M., Teuscher, C., & Snyder, S. H. (2007). From the cover: Antipsychotic drug-induced weight gain mediated by histamine H1 receptor-linked activation of hypothalamic AMP-kinase. *Proceedings of the National Academy of Sciences of the United States of America, 104*(9), 3456-3459.

Kim, T. K., Sul, J. Y., Peternko, N. B., Lee, J. H., Lee, M., Patel, V. V., . . . Eberwine, J. H. (2011). Transcriptome transfer provides a model for understanding the phenotype of cardiomyocytes. *Proceedings of the National Academy of Sciences of the United States of America,* 108(29), 11918-11923.

Kinoshita, M., Ono, K., Horie, T., Nagao, K., Nishi, H., Kuwabara, Y., et al. (2010). Regulation of adipocyte differentiation by activation of serotonin (5-HT) receptors 5-HT2AR and 5-HT2CR and involvement of microRNA-448-mediated repression of KLF5. *Molecular Endocrinology (Baltimore, Md.), 24*(10), 1978-1987.

Kirk, S. L., Cahir, M., & Reynolds, G. P. (2006). Clozapine, but not haloperidol, increases neuropeptide Y neuronal expression in the rat hypothalamus. *Journal of Psychopharmacology (Oxford, England), 20*(4), 577-579.

Klein, S., Coppack, S. W., Mohamed-Ali, V., & Landt, M. (1996). Adipose tissue leptin production and plasma leptin kinetics in humans. *Diabetes, 45*(7), 984-987.

Klenke, S., Kussmann, M., & Siffert, W. (2011). The GNB3 C825T polymorphism as a pharmacogenetic marker in the treatment of hypertension, obesity, and depression. *Pharmacogenetics and Genomics, 21*(9), 594-606.

Kluge, M., Schuld, A., Schacht, A., Himmerich, H., Dalal, M. A., Wehmeier, P. M., et al. (2009). Effects of clozapine and olanzapine on cytokine systems are closely linked to weight gain and drug-induced fever. *Psychoneuroendocrinology, 34*(1), 118-128.

Kohlrausch, F. B., Salatino-Oliveira, A., Gama, C. S., Lobato, M. I., Belmonte-de-Abreu, P., & Hutz, M. H. (2008). G-protein gene 825C>T polymorphism is associated with response to clozapine in brazilian schizophrenics. *Pharmacogenomics, 9*(10), 1429-1436.

Konopaske, G. T., & Coyle, J. T. (2015). Chapter 39 – Schizophrenia. In M. J. Zigmond, L. P. Rowland & J. T. Coyle (Eds.), *Neurobiology of brain disorders: Biological basis of neurological and psychiatric disorders* (pp. 639-653). London, UK: Elsevier Inc.

Korecka, J. A., van Kesteren, R. E., Blaas, E., Spitzer, S. O., Kamstra, J. H., Smit, A. B., et al. (2013). Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling. *PloS One, 8*(5), e63862.

Kovalevich, J., & Langford, D. (2013). Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods in Molecular Biology (Clifton, N.J.), 1078*, 9-21.

Kraus, T., Haack, M., Schuld, A., Hinze-Selch, D., Kuhn, M., Uhr, M., et al. (1999). Body weight and leptin plasma levels during treatment with antipsychotic drugs. *The American Journal of Psychiatry, 156*(2), 312-314.

Krol, J., Loedige, I., & Filipowicz, W. (2010). The widespread regulation of microRNA biogenesis, function and decay. *Nature Reviews.Genetics, 11*(9), 597-610.

Krystal, J. H., Karper, L. P., Seibyl, J. P., Freeman, G. K., Delaney, R., Bremner, J. D., et al. (1994). Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans. psychotomimetic, perceptual, cognitive, and neuroendocrine responses. *Archives of General Psychiatry, 51*(3), 199-214.

Kume, T., Kawato, Y., Osakada, F., Izumi, Y., Katsuki, H., Nakagawa, T., et al. (2008). Dibutyryl cyclic AMP induces differentiation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype. *Neuroscience Letters, 443*(3), 199-203.

Kunugi, H., Nanko, S., Takei, N., Saito, K., Hayashi, N., & Kazamatsuri, H. (1995). Schizophrenia following in utero exposure to the 1957 influenza epidemics in japan. *The American Journal of Psychiatry, 152*(3), 450-452.

Kuo, P. H., Kao, C. F., Chen, P. Y., Chen, C. H., Tsai, Y. S., Lu, M. L., et al. (2011). Polymorphisms of INSIG2, MC4R, and LEP are associated with obesity- and metabolic-related traits in schizophrenic patients. *Journal of Clinical Psychopharmacology, 31*(6), 705-711.

Kuoppamaki, M., Palvimaki, E. P., Hietala, J., & Syvalahti, E. (1995). Differential regulation of rat 5-HT2A and 5-HT2C receptors after chronic treatment with clozapine, chlorpromazine and three putative atypical antipsychotic drugs. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 13*(2), 139-150.

Kuoppamaki, M., Seppala, T., Syvalahti, E., & Hietala, J. (1993). Chronic clozapine treatment decreases 5-hydroxytryptamine1C receptor density in the rat choroid plexus: Comparison with haloperidol. *The Journal of Pharmacology and Experimental Therapeutics, 264*(3), 1262-1267.

Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Totoki, Y., Toyoda, A., Ikawa, M., et al. (2008). DNA methylation of retrotransposon genes is regulated by piwi family members MILI and MIWI2 in murine fetal testes. *Genes & Development, 22*(7), 908-917.

Kursungoz, C., Ak, M., & Yanik, T. (2015). Effects of risperidone treatment on the expression of hypothalamic neuropeptide in appetite regulation in wistar rats. *Brain Research, 1596*, 146-155.

Kuzman, M. R., & Muller, D. J. (2012). Association of the MTHFR gene with antipsychotic-induced metabolic abnormalities in patients with schizophrenia. *Pharmacogenomics, 13*(8), 843-846.

Lam, D. D., Leinninger, G. M., Louis, G. W., Garfield, A. S., Marston, O. J., Leshan, R. L., et al. (2011). Leptin does not directly affect CNS serotonin neurons to influence appetite. *Cell Metabolism, 13*(5), 584-591.

Lambert, M., Haro, J. M., Novick, D., Edgell, E. T., Kennedy, L., Ratcliffe, M., et al. (2005). Olanzapine vs. other antipsychotics in actual out-patient settings: Six months

tolerability results from the european schizophrenia out-patient health outcomes study. *Acta Psychiatrica Scandinavica, 111*(3), 232-243.

Lambert, M. T., Copeland, L. A., Sampson, N., & Duffy, S. A. (2006). New-onset type-2 diabetes associated with atypical antipsychotic medications. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 30*(5), 919-923.

Langer, S. Z. (1997). 25 years since the discovery of presynaptic receptors: Present knowledge and future perspectives. *Trends in Pharmacological Sciences, 18*(3), 95-99.

Larsen, F., Gundersen, G., Lopez, R., & Prydz, H. (1992). CpG islands as gene markers in the human genome. *Genomics, 13*(4), 1095-1107.

Laruelle, M., Abi-Dargham, A., Casanova, M. F., Toti, R., Weinberger, D. R., & Kleinman, J. E. (1993). Selective abnormalities of prefrontal serotonergic receptors in schizophrenia. A postmortem study. *Archives of General Psychiatry, 50*(10), 810-818.

Le Hellard, S., Theisen, F. M., Haberhausen, M., Raeder, M. B., Ferno, J., Gebhardt, S., et al. (2009). Association between the insulin-induced gene 2 (INSIG2) and weight gain in a german sample of antipsychotic-treated schizophrenic patients: Perturbation of SREBP-controlled lipogenesis in drug-related metabolic adverse effects? *Molecular Psychiatry, 14*(3), 308-317.

Le Marchand, L., Donlon, T., Hankin, J. H., Kolonel, L. N., Wilkens, L. R., & Seifried, A. (2002). B-vitamin intake, metabolic genes, and colorectal cancer risk (united states). *Cancer Causes & Control : CCC,* 13(3), 239-248.

Lebrun, B., Bariohay, B., Moyse, E., & Jean, A. (2006). Brain-derived neurotrophic factor (BDNF) and food intake regulation: A minireview. *Autonomic Neuroscience : Basic & Clinical, 126-127*, 30-38.

Leibowitz, S. F., Roossin, P., & Rosenn, M. (1984). Chronic norepinephrine injection into the hypothalamic paraventricular nucleus produces hyperphagia and increased body weight in the rat. *Pharmacology, Biochemistry, and Behavior, 21*(5), 801-808.

Lembreghts, M., & Ansseau, M. (1993). Biological markers in schizophrenia. [Les marqueurs biologiques dans les schizophrenies] *L'Encephale, 19*(5), 501-523.

Lencz, T., Robinson, D. G., Napolitano, B., Sevy, S., Kane, J. M., Goldman, D., et al. (2010). DRD2 promoter region variation predicts antipsychotic-induced weight gain in first episode schizophrenia. *Pharmacogenetics and Genomics, 20*(9), 569-572.

Lett, T. A., Wallace, T. J., Chowdhury, N. I., Tiwari, A. K., Kennedy, J. L., & Muller, D. J. (2012). Pharmacogenetics of antipsychotic-induced weight gain: Review and clinical implications. *Molecular Psychiatry, 17*(3), 242-266.

Levin, B. E., & Planas, B. (1993). Defective glucoregulation of brain alpha 2-adrenoceptors in obesity-prone rats. *The American Journal of Physiology, 264*(2 Pt 2), R305-11.

Levine, J. A., Eberhardt, N. L., & Jensen, M. D. (1999). Role of nonexercise activity thermogenesis in resistance to fat gain in humans. *Science (New York, N.Y.), 283*(5399), 212-214.

Lewis, D. A., & Lieberman, J. A. (2000). Catching up on schizophrenia: Natural history and neurobiology. *Neuron, 28*(2), 325-334.

Lewis, S. J., Lawlor, D. A., Davey Smith, G., Araya, R., Timpson, N., Day, I. N., et al. (2006). The thermolabile variant of MTHFR is associated with depression in the british women's heart and health study and a meta-analysis. *Molecular Psychiatry, 11*(4), 352-360.

Leysen, J. E., Janssen, P. M., Schotte, A., Luyten, W. H., & Megens, A. A. (1993). Interaction of antipsychotic drugs with neurotransmitter receptor sites in vitro and in vivo in relation to pharmacological and clinical effects: Role of 5HT2 receptors. *Psychopharmacology, 112*(1 Suppl), S40-54.

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Reviews.Genetics, 3*(9), 662-673.

Li, E., Beard, C., & Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. *Nature, 366*(6453), 362-365.

Li, E., & Zhang, Y. (2014). DNA methylation in mammals. *Cold Spring Harbor Perspectives in Biology, 6*(5), a019133.

Licinio, J., Dong, C., & Wong, M. L. (2009). Novel sequence variations in the brain-derived neurotrophic factor gene and association with major depression and antidepressant treatment response. *Archives of General Psychiatry, 66*(5), 488-497.

Lieberman, J. A., Kane, J. M., & Alvir, J. (1987). Provocative tests with psychostimulant drugs in schizophrenia. *Psychopharmacology, 91*(4), 415-433.

Lieberman, J. A., Stroup, T. S., McEvoy, J. P., Swartz, M. S., Rosenheck, R. A., Perkins, D. O., Keefe, R. S., Davis, S. M., Davis, C. E., Lebowitz, B. D.;, Severe, J., Hsiao, J. K., Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) Investigators. (2005). Effectiveness of antipsychotic drugs in patients with chronic schizophrenia. *The New England Journal of Medicine*, 353(12), 1209-1223.

Lim, U., & Cassano, P. A. (2002). Homocysteine and blood pressure in the third national health and nutrition examination survey, 1988-1994. *American Journal of Epidemiology, 156*(12), 1105-1113.

Lin, R. K., Hsu, H. S., Chang, J. W., Chen, C. Y., Chen, J. T., & Wang, Y. C. (2007). Alteration of DNA methyltransferases contributes to 5'CpG methylation and poor prognosis in lung cancer. *Lung Cancer (Amsterdam, Netherlands), 55*(2), 205-213.

Lindenmayer, J. P., & Khan, A. (2006). Psychopathology. In J. A. Lieberman, T. S. Stroup & D. O. Perkins (Eds.), *The american psychiatric publishing textbook of schizophrenia* (pp. 187-221). Washington, DC: American Psychiatric Publishing.

Linnebank, M., Moskau, S., Semmler, A., Hoefgen, B., Bopp, G., Kallweit, U., et al. (2012). A possible genetic link between MTHFR genotype and smoking behavior. *PloS One, 7*(12), e53322.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods (San Diego, Calif.), 25*(4), 402-408.

Lochman, J., Balcar, V. J., Stastny, F., & Sery, O. (2013). Preliminary evidence for association between schizophrenia and polymorphisms in the regulatory regions of the ADRA2A, DRD3 and SNAP-25 genes. *Psychiatry Research*, 205(1-2), 7-12.

Lokk, K., Modhukur, V., Rajashekar, B., Martens, K., Magi, R., Kolde, R., et al. (2014). DNA methylome profiling of human tissues identifies global and tissue-specific methylation patterns. *Genome Biology, 15*(4), r54-2014-15-4-r54.

Loos, R. J. (2011). The genetic epidemiology of melanocortin 4 receptor variants. *European Journal of Pharmacology, 660*(1), 156-164.

Loos, R. J., Lindgren, C. M., Li, S., Wheeler, E., Zhao, J. H., Prokopenko, I., et al. (2008). Common variants near MC4R are associated with fat mass, weight and risk of obesity. *Nature Genetics, 40*(6), 768-775.

Loos, R. J., & Yeo, G. S. (2014). The bigger picture of FTO: The first GWAS-identified obesity gene. *Nature Reviews.Endocrinology, 10*(1), 51-61.

Lott, S. A., Burghardt, P. R., Burghardt, K. J., Bly, M. J., Grove, T. B., & Ellingrod, V. L. (2013). The influence of metabolic syndrome, physical activity and genotype on catechol-O-methyl transferase promoter-region methylation in schizophrenia. *The Pharmacogenomics Journal, 13*(3), 264-271.

Lu, Q., Qiu, X., Hu, N., Wen, H., Su, Y., & Richardson, B. C. (2006). Epigenetics, disease, and therapeutic interventions. *Ageing Research Reviews, 5*(4), 449-467.

Lucock, M. (2000). Folic acid: Nutritional biochemistry, molecular biology, and role in disease processes. *Molecular Genetics and Metabolism, 71*(1-2), 121-138.

Lunegova, O. S., Kerimkulova, A. S., Turdakmatov, N. B., Sovkhozova, N. A., Nabiev, M. P., Isakova, Z., et al. (2011). Association of C677T gene polymorphism of methylenetetrahydrofolate reductase with insulin resistance among kirghizes. *Kardiologiia, 51*(3), 58-62.

Lv, J., Liu, H., Su, J., Wu, X., Liu, H., Li, B., et al. (2012). DiseaseMeth: A human disease methylation database. *Nucleic Acids Research, 40*(Database issue), D1030-5.

Lyons, W. E., Mamounas, L. A., Ricaurte, G. A., Coppola, V., Reid, S. W., Bora, S. H., et al. (1999). Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proceedings of the National Academy of Sciences of the United States of America, 96*(26), 15239-15244.

Ma, X., Bruning, J., & Ashcroft, F. M. (2007). Glucagon-like peptide 1 stimulates hypothalamic proopiomelanocortin neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 27*(27), 7125-7129.

Machinal, F., Dieudonne, M. N., Leneveu, M. C., Pecquery, R., & Giudicelli, Y. (1999). In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: Evidence for a regional specific regulation by sex steroid hormones. *Endocrinology, 140*(4), 1567-1574.

Machinal-Quelin, F., Dieudonne, M. N., Pecquery, R., Leneveu, M. C., & Giudicelli, Y. (2002). Direct in vitro effects of androgens and estrogens on ob gene expression and leptin secretion in human adipose tissue. *Endocrine, 18*(2), 179-184.

Malbon, C. C. (1997). Heterotrimeric G-proteins and development. *Biochemical Pharmacology, 53*(1), 1-4.

Malhotra, A. K., Correll, C. U., Chowdhury, N. I., Muller, D. J., Gregersen, P. K., Lee, A. T., et al. (2012). Association between common variants near the melanocortin 4 receptor gene and severe antipsychotic drug-induced weight gain. *Archives of General Psychiatry, 69*(9), 904-912.

Malla, A. K., Takhar, J. J., Norman, R. M., Manchanda, R., Cortese, L., Haricharan, R., et al. (2002). Negative symptoms in first episode non-affective psychosis. *Acta Psychiatrica Scandinavica, 105*(6), 431-439.

Malone, C. D., Brennecke, J., Dus, M., Stark, A., McCombie, W. R., Sachidanandam, R., et al. (2009). Specialized piRNA pathways act in germline and somatic tissues of the drosophila ovary. *Cell, 137*(3), 522-535.

Mammes, O., Betoulle, D., Aubert, R., Herbeth, B., Siest, G., & Fumeron, F. (2000). Association of the G-2548A polymorphism in the 5' region of the LEP gene with overweight. *Annals of Human Genetics, 64*(Pt 5), 391-394.

Mann, J. R. (2014). Epigenetics and memigenetics. *Cellular and Molecular Life Sciences : CMLS, 71*(7), 1117-1122.

Martienssen, R. (1998). Transposons, DNA methylation and gene control. *Trends in Genetics : TIG, 14*(7), 263-264.

Martin-Nunez, G. M., Cabrera-Mulero, R., Rubio-Martin, E., Rojo-Martinez, G., Olveira, G., Valdes, S., et al. (2014). Methylation levels of the SCD1 gene promoter and LINE-1 repeat region are associated with weight change: An intervention study. *Molecular Nutrition & Food Research, 58*(7), 1528-1536.

Masaki, T., Chiba, S., Yasuda, T., Noguchi, H., Kakuma, T., Watanabe, T., et al. (2004). Involvement of hypothalamic histamine H1 receptor in the regulation of feeding rhythm and obesity. *Diabetes, 53*(9), 2250-2260.

Masaki, T., Yoshimatsu, H., Chiba, S., Watanabe, T., & Sakata, T. (2001). Targeted disruption of histamine H1-receptor attenuates regulatory effects of leptin on feeding, adiposity, and UCP family in mice. *Diabetes, 50*(2), 385-391.

Mattick, J. S., & Makunin, I. V. (2006). Non-coding RNA. *Human Molecular Genetics*, 15 Spec No 1, R17-29.

McCabe, J. T., DeBellis, M., & Leibowitz, S. F. (1984). Clonidine-induced feeding: Analysis of central sites of action and fiber projections mediating this response. *Brain Research, 309*(1), 85-104.

McCarthy, S., Mottagui-Tabar, S., Mizuno, Y., Sennblad, B., Hoffstedt, J., Arner, P., et al. (2005). Complex HTR2C linkage disequilibrium and promoter associations with body mass index and serum leptin. *Human Genetics, 117*(6), 545-557.

McKay, J. A., Groom, A., Potter, C., Coneyworth, L. J., Ford, D., Mathers, J. C., et al. (2012). Genetic and non-genetic influences during pregnancy on infant global and site specific DNA methylation: Role for folate gene variants and vitamin B12. *PloS One, 7*(3), e33290.

McMahon, F. J., Buervenich, S., Charney, D., Lipsky, R., Rush, A. J., Wilson, A. F., et al. (2006). Variation in the gene encoding the serotonin 2A receptor is associated with outcome of antidepressant treatment. *American Journal of Human Genetics, 78*(5), 804-814.

McNeil, T. F., Cantor-Graae, E., & Sjostrom, K. (1994). Obstetric complications as antecedents of schizophrenia: Empirical effects of using different obstetric complication scales. *Journal of Psychiatric Research, 28*(6), 519-530.

Meaney, M. J., & Ferguson-Smith, A. C. (2010). Epigenetic regulation of the neural transcriptome: The meaning of the marks. *Nature Neuroscience, 13*(11), 1313-1318.

Mednick, S. A., Machon, R. A., Huttunen, M. O., & Bonett, D. (1988). Adult schizophrenia following prenatal exposure to an influenza epidemic. *Archives of General Psychiatry, 45*(2), 189-192.

Meguid, M. M., Fetissov, S. O., Varma, M., Sato, T., Zhang, L., Laviano, A., et al. (2000). Hypothalamic dopamine and serotonin in the regulation of food intake. *Nutrition (Burbank, Los Angeles County, Calif.), 16*(10), 843-857.

Meier, U., & Gressner, A. M. (2004). Endocrine regulation of energy metabolism: Review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clinical Chemistry, 50*(9), 1511-1525.

Meissner, A. (2010). Epigenetic modifications in pluripotent and differentiated cells. *Nature Biotechnology, 28*(10), 1079-1088.

Melas, P. A., Rogdaki, M., Osby, U., Schalling, M., Lavebratt, C., & Ekstrom, T. J. (2012). Epigenetic aberrations in leukocytes of patients with schizophrenia: Association of global DNA methylation with antipsychotic drug treatment and disease onset. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 26*(6), 2712-2718.

Melka, M. G., Castellani, C. A., Laufer, B. I., Rajakumar, R. N., O'Reilly, R., & Singh, S. M. (2013). Olanzapine induced DNA methylation changes support the dopamine

hypothesis of psychosis. *Journal of Molecular Psychiatry, 1*(1), 19-9256-1-19. eCollection 2013.

Melka, M. G., Laufer, B. I., McDonald, P., Castellani, C. A., Rajakumar, N., O'Reilly, R., et al. (2014). The effects of olanzapine on genome-wide DNA methylation in the hippocampus and cerebellum. *Clinical Epigenetics, 6*(1), 1-7083-6-1.

Melkersson, K. I., & Hulting, A. L. (2001). Insulin and leptin levels in patients with schizophrenia or related psychoses--a comparison between different antipsychotic agents. *Psychopharmacology, 154*(2), 205-212.

Melkersson, K. I., Hulting, A. L., & Brismar, K. E. (2000). Elevated levels of insulin, leptin, and blood lipids in olanzapine-treated patients with schizophrenia or related psychoses. *The Journal of Clinical Psychiatry, 61*(10), 742-749.

Melnik, T., Soares, B. G., Puga, M. E., & Atallah, A. N. (2010). Efficacy and safety of atypical antipsychotic drugs (quetiapine, risperidone, aripiprazole and paliperidone) compared with placebo or typical antipsychotic drugs for treating refractory schizophrenia: Overview of systematic reviews. *Sao Paulo Medical Journal = Revista Paulista De Medicina*, 128(3), 141-166.

Meltzer, H. Y., & Sumiyoshi, T. (2003). Atypical antipsychotic drugs improve cognition in schizophrenia. *Biological Psychiatry, 53*(3), 265-7; author reply 267-8.

Milatovich, A., Hsieh, C. L., Bonaminio, G., Tecott, L., Julius, D., & Francke, U. (1992). Serotonin receptor 1c gene assigned to X chromosome in human (band q24) and mouse (bands D-F4). *Human Molecular Genetics, 1*(9), 681-684.

Mill, J., & Petronis, A. (2007). Molecular studies of major depressive disorder: The epigenetic perspective. *Molecular Psychiatry, 12*(9), 799-814.

Millan, M. J. (2000). Improving the treatment of schizophrenia: Focus on serotonin (5-HT)(1A) receptors. *The Journal of Pharmacology and Experimental Therapeutics, 295*(3), 853-861.

Miller, D. D., Ellingrod, V. L., Holman, T. L., Buckley, P. F., & Arndt, S. (2005). Clozapine-induced weight gain associated with the 5HT2C receptor -759C/T polymorphism. *American Journal of Medical Genetics.Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics, 133B*(1), 97-100.

Minet-Ringuet, J., Even, P. C., Valet, P., Carpene, C., Visentin, V., Prevot, D., et al. (2007). Alterations of lipid metabolism and gene expression in rat adipocytes during chronic olanzapine treatment. *Molecular Psychiatry, 12*(6), 562-571.

Mita, T., Hanada, S., Nishino, N., Kuno, T., Nakai, H., Yamadori, T., et al. (1986). Decreased serotonin S2 and increased dopamine D2 receptors in chronic schizophrenics. *Biological Psychiatry, 21*(14), 1407-1414.

Miura, A., Yonebayashi, S., Watanabe, K., Toyama, T., Shimada, H., & Kakutani, T. (2001). Mobilization of transposons by a mutation abolishing full DNA methylation in arabidopsis. *Nature, 411*(6834), 212-214.

Moller, C. L., Raun, K., Jacobsen, M. L., Pedersen, T. A., Holst, B., Conde-Frieboes, K. W., et al. (2011). Characterization of murine melanocortin receptors mediating adipocyte lipolysis and examination of signalling pathways involved. *Molecular and Cellular Endocrinology, 341*(1-2), 9-17.

Mollereau, C., Zajac, J. M., & Roumy, M. (2007). Staurosporine differentiation of NPFF2 receptor-transfected SH-SY5Y neuroblastoma cells induces selectivity of NPFF activity towards opioid receptors. *Peptides, 28*(5), 1125-1128.

Monteleone, P., Fabrazzo, M., Tortorella, A., La Pia, S., & Maj, M. (2002). Pronounced early increase in circulating leptin predicts a lower weight gain during clozapine treatment. *Journal of Clinical Psychopharmacology, 22*(4), 424-426.

Morrow, J. K., Tian, L., & Zhang, S. (2010). Molecular networks in drug discovery. *Critical Reviews in Biomedical Engineering, 38*(2), 143-156.

Mottillo, E. P., Shen, X. J., & Granneman, J. G. (2010). beta3-adrenergic receptor induction of adipocyte inflammation requires lipolytic activation of stress kinases p38 and JNK. *Biochimica Et Biophysica Acta, 1801*(9), 1048-1055.

Mou, X. D., Zhang, Z. J., Yao, Z. J., Liu, W., Zhang, X. R., Shi, J. B., et al. (2005). No association of -1438G/A polymorphism in promoter region of 5-HT2A receptor gene with antipsychotic agent-induced weight gain. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi = Zhonghua Yixue Yichuanxue Zazhi = Chinese Journal of Medical Genetics, 22*(5), 575-576.

Mujumdar, V. S., Tummalapalli, C. M., Aru, G. M., & Tyagi, S. C. (2002). Mechanism of constrictive vascular remodeling by homocysteine: Role of PPAR. *American Journal of Physiology.Cell Physiology, 282*(5), C1009-15.

Mukherjee, R., & Yun, J. W. (2013). Bromocriptine inhibits adipogenesis and lipogenesis by agonistic action on alpha2-adrenergic receptor in 3T3-L1 adipocyte cells. *Molecular Biology Reports, 40*(5), 3783-3792.

Muller, D. J., De Luca, V., Sicard, T., King, N., Hwang, R., Volavka, J., et al. (2005). Suggestive association between the C825T polymorphism of the G-protein beta3 subunit gene (GNB3) and clinical improvement with antipsychotics in schizophrenia. *European Neuropsychopharmacology : The Journal of the European College of Neuropsychopharmacology, 15*(5), 525-531.

Muller, D. J., Zai, C. C., Sicard, M., Remington, E., Souza, R. P., Tiwari, A. K., et al. (2012). Systematic analysis of dopamine receptor genes (DRD1-DRD5) in antipsychotic-induced weight gain. *The Pharmacogenomics Journal, 12*(2), 156-164.

Murray,M,R, & Bramon, E.,. (2005).
Developmental model of schizophrenia In Sadock,B,J, & Sadock,V,A, (Eds.), *Comprehensive textbook of psychiatry* (8th ed., pp. 1381). Philadelphia: Lippincot Williams and Wilkins.

Murrell, A., Heeson, S., Bowden, L., Constancia, M., Dean, W., Kelsey, G., et al. (2001). An intragenic methylated region in the imprinted Igf2 gene augments transcription. *EMBO Reports, 2*(12), 1101-1106.

Musil, R., Spellmann, I., Riedel, M., Dehning, S., Douhet, A., Maino, K., et al. (2008). SNAP-25 gene polymorphisms and weight gain in schizophrenic patients. *Journal of Psychiatric Research, 42*(12), 963-970.

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., et al. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature, 393*(6683), 386-389.

Nestler, E. J. (1997). Schizophrenia. an emerging pathophysiology. *Nature, 385*(6617), 578-579.

Neve,K,A, & Neve,R,L,. (1997). Molecular biology of dopamine receptors. In Neve,K,A, & Neve,R,L, (Eds.), *Dopamine receptors* (pp. 27-76). Totawa: Humana Press.

Neve, K. A., Seamans, J. K., & Trantham-Davidson, H. (2004). Dopamine receptor signaling. *Journal of Receptor and Signal Transduction Research, 24*(3), 165-205.

Newcomer, J. W. (2005). Second-generation (atypical) antipsychotics and metabolic effects: A comprehensive literature review. *CNS Drugs, 19 Suppl 1*, 1-93.

Nichols, D. E., & Nichols, C. D. (2008). Serotonin receptors. *Chemical Reviews, 108*(5), 1614-1641.

Nicholson, J. R., Peter, J. C., Lecourt, A. C., Barde, Y. A., & Hofbauer, K. G. (2007). Melanocortin-4 receptor activation stimulates hypothalamic brain-derived neurotrophic factor release to regulate food intake, body temperature and cardiovascular function. *Journal of Neuroendocrinology, 19*(12), 974-982.

Nie, L., Wu, H. J., Hsu, J. M., Chang, S. S., Labaff, A. M., Li, C. W., et al. (2012). Long non-coding RNAs: Versatile master regulators of gene expression and crucial players in cancer. *American Journal of Translational Research, 4*(2), 127-150.

Nikolac Perkovic, M., Nedic Erjavec, G., Zivkovic, M., Sagud, M., Uzun, S., Mihaljevic-Peles, A., et al. (2014). Association between the brain-derived neurotrophic factor Val66Met polymorphism and therapeutic response to olanzapine in schizophrenia patients. *Psychopharmacology, 231*(18), 3757-3764.

Noble, E. E., Billington, C. J., Kotz, C. M., & Wang, C. (2011). The lighter side of BDNF. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 300*(5), R1053-69.

Norman, D., Isidori, A. M., Frajese, V., Caprio, M., Chew, S. L., Grossman, A. B., et al. (2003). ACTH and alpha-MSH inhibit leptin expression and secretion in 3T3-L1 adipocytes: Model for a central-peripheral melanocortin-leptin pathway. *Molecular and Cellular Endocrinology, 200*(1-2), 99-109.

Obeid, R., & Herrmann, W. (2009). Homocysteine and lipids: S-adenosyl methionine as a key intermediate. *FEBS Letters, 583*(8), 1215-1225.

Oh, J. E., Cho, Y. M., Kwak, S. N., Kim, J. H., Lee, K. W., Jung, H., et al. (2012). Inhibition of mouse brown adipocyte differentiation by second-generation antipsychotics. *Experimental & Molecular Medicine, 44*(9), 545-553.

Okano, M., Bell, D. W., Haber, D. A., & Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell, 99*(3), 247-257.

Okpokoro, U., & Sampson, S. (2014). Brief family intervention for schizophrenia. *Schizophrenia Bulletin,* 40(3), 497-498.

Olney, J. W., & Farber, N. B. (1995). Glutamate receptor dysfunction and schizophrenia. *Archives of General Psychiatry, 52*(12), 998-1007.

Olney, J. W., Newcomer, J. W., & Farber, N. B. (1999). NMDA receptor hypofunction model of schizophrenia. *Journal of Psychiatric Research, 33*(6), 523-533.

Orthen-Gambill, N. (1988). Antihistaminic drugs increase feeding, while histidine suppresses feeding in rats. *Pharmacology, Biochemistry, and Behavior, 31*(1), 81-86.

Ota, V. K., Noto, C., Gadelha, A., Santoro, M. L., Spindola, L. M., Gouvea, E. S., et al. (2014). Changes in gene expression and methylation in the blood of patients with first-episode psychosis. *Schizophrenia Research, 159*(2-3), 358-364.

Pahlman, S., Odelstad, L., Larsson, E., Grotte, G., & Nilsson, K. (1981). Phenotypic changes of human neuroblastoma cells in culture induced by 12-O-tetradecanoyl-phorbol-13-acetate. *International Journal of Cancer.Journal International Du Cancer, 28*(5), 583-589.

Pahlman, S., Ruusala, A. I., Abrahamsson, L., Mattsson, M. E., & Esscher, T. (1984). Retinoic acid-induced differentiation of cultured human neuroblastoma cells: A comparison with phorbolester-induced differentiation. *Cell Differentiation, 14*(2), 135-144.

Panariello, F., De Luca, V., & de Bartolomeis, A. (2011). Weight gain, schizophrenia and antipsychotics: New findings from animal model and pharmacogenomic studies. *Schizophrenia Research and Treatment, 2011*, 459284.

Panariello, F., Perruolo, G., Cassese, A., Giacco, F., Botta, G., Barbagallo, A. P., et al. (2012). Clozapine impairs insulin action by up-regulating akt phosphorylation and Ped/Pea-15 protein abundance. *Journal of Cellular Physiology, 227*(4), 1485-1492.

Pappas, J. J., Toulouse, A., & Clarke Bradley, Walter, Edward,. (2013). The bisulfite genomic sequencing protocol. *Advances in Lung Cancer, 2*(1), 21-25.

Parada, M. A., Hernandez, L., & Hoebel, B. G. (1988). Sulpiride injections in the lateral hypothalamus induce feeding and drinking in rats. *Pharmacology, Biochemistry, and Behavior, 30*(4), 917-923.

Park, Y. M., Chung, Y. C., Lee, S. H., Lee, K. J., Kim, H., Byun, Y. C., et al. (2006). Weight gain associated with the alpha2a-adrenergic receptor -1,291 C/G polymorphism and olanzapine treatment. *American Journal of Medical Genetics.Part B, Neuropsychiatric Genetics : The Official Publication of the International Society of Psychiatric Genetics, 141B*(4), 394-397.

Park, Y. M., Chung, Y. C., Lee, S. H., Lee, K. J., Kim, H., Choi, J. E., et al. (2009). G-protein beta3 subunit gene 825C/T polymorphism is not associated with olanzapine-induced weight gain in korean schizophrenic patients. *Psychiatry Investigation, 6*(1), 39-43.

Parsons, M. J., D'Souza, U. M., Arranz, M. J., Kerwin, R. W., & Makoff, A. J. (2004). The -1438A/G polymorphism in the 5-hydroxytryptamine type 2A receptor gene affects promoter activity. *Biological Psychiatry, 56*(6), 406-410.

Pasqualini, C., Weltzien, F. A., Vidal, B., Baloche, S., Rouget, C., Gilles, N., et al. (2009). Two distinct dopamine D2 receptor genes in the european eel: Molecular characterization, tissue-specific transcription, and regulation by sex steroids. *Endocrinology, 150*(3), 1377-1392.

Patel, J. K., Buckley, P. F., Woolson, S., Hamer, R. M., McEvoy, J. P., Perkins, D. O., et al. (2009). Metabolic profiles of second-generation antipsychotics in early psychosis: Findings from the CAFE study. *Schizophrenia Research, 111*(1-3), 9-16.

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., et al. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science (New York, N.Y.), 269*(5223), 540-543.

Penner, M. R., Roth, T. L., Chawla, M. K., Hoang, L. T., Roth, E. D., Lubin, F. D., et al. (2011). Age-related changes in arc transcription and DNA methylation within the hippocampus. *Neurobiology of Aging, 32*(12), 2198-2210.

Perez-Cornago, A., Mansego, M. L., Zulet, M. A., & Martinez, J. A. (2014). DNA hypermethylation of the serotonin receptor type-2A gene is associated with a worse response to a weight loss intervention in subjects with metabolic syndrome. *Nutrients, 6*(6), 2387-2403.

Perez-Iglesias, R., Mata, I., Amado, J. A., Berja, A., Garcia-Unzueta, M. T., Martinez Garcia, O., et al. (2010). Effect of FTO, SH2B1, LEP, and LEPR polymorphisms on weight gain associated with antipsychotic treatment. *Journal of Clinical Psychopharmacology, 30*(6), 661-666.

Perez-Iglesias, R., Vazquez-Barquero, J. L., Amado, J. A., Berja, A., Garcia-Unzueta, M. T., Pelayo-Teran, J. M., et al. (2008). Effect of antipsychotics on peptides involved in energy balance in drug-naive psychotic patients after 1 year of treatment. *Journal of Clinical Psychopharmacology, 28*(3), 289-295.

Perng, W., Mora-Plazas, M., Marin, C., Rozek, L. S., Baylin, A., & Villamor, E. (2013). A prospective study of LINE-1DNA methylation and development of adiposity in school-age children. *PloS One, 8*(4), e62587.

Perng, W., Rozek, L. S., Mora-Plazas, M., Duchin, O., Marin, C., Forero, Y., et al. (2012). Micronutrient status and global DNA methylation in school-age children. *Epigenetics : Official Journal of the DNA Methylation Society, 7*(10), 1133-1141.

Perng, W., Villamor, E., Shroff, M. R., Nettleton, J. A., Pilsner, J. R., Liu, Y., et al. (2014). Dietary intake, plasma homocysteine, and repetitive element DNA methylation in the multi-ethnic study of atherosclerosis (MESA). *Nutrition, Metabolism, and Cardiovascular Diseases : NMCD, 24*(6), 614-622.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29*(9), e45.

Philibert, R. A., Plume, J. M., Gibbons, F. X., Brody, G. H., & Beach, S. R. (2012). The impact of recent alcohol use on genome wide DNA methylation signatures. *Frontiers in Genetics, 3*, 54.

Pilowsky, L. S. (2001). Probing targets for antipsychotic drug action with PET and SPET receptor imaging. *Nuclear Medicine Communications, 22*(7), 829-833.

Pinter, S. F., Sadreyev, R. I., Yildirim, E., Jeon, Y., Ohsumi, T. K., Borowsky, M., et al. (2012). Spreading of X chromosome inactivation via a hierarchy of defined polycomb stations. *Genome Research, 22*(10), 1864-1876.

Pi-Sunyer, X. (2009). The medical risks of obesity. *Postgraduate Medicine, 121*(6), 21-33.

Piyathilake, C. J., Frost, A. R., Bell, W. C., Oelschlager, D., Weiss, H., Johanning, G. L., et al. (2001). Altered global methylation of DNA: An epigenetic difference in susceptibility for lung cancer is associated with its progression. *Human Pathology, 32*(8), 856-862.

Polanczyk, G., Zeni, C., Genro, J. P., Guimaraes, A. P., Roman, T., Hutz, M. H., & Rohde, L. A. (2007). Association of the adrenergic alpha2A receptor gene with methylphenidate improvement of inattentive symptoms in children and adolescents with attention-deficit/hyperactivity disorder. *Archives of General Psychiatry,* 64(2), 218-224.

Ponting, C. P., Oliver, P. L., & Reik, W. (2009). Evolution and functions of long noncoding RNAs. *Cell, 136*(4), 629-641.

Qi, Q., Kilpelainen, T. O., Downer, M. K., Tanaka, T., Smith, C. E., Sluijs, I., et al. (2014). FTO genetic variants, dietary intake and body mass index: Insights from 177 330 individuals. *Human Molecular Genetics, 23*(25), 6961-6972.

Qiu, J., Xue, C., Bosch, M. A., Murphy, J. G., Fan, W., Ronnekleiv, O. K., et al. (2007). Serotonin 5-hydroxytryptamine2C receptor signaling in hypothalamic proopiomelanocortin neurons: Role in energy homeostasis in females. *Molecular Pharmacology, 72*(4), 885-896.

Rahn, E. J., Guzman-Karlsson, M. C., & David Sweatt, J. (2013). Cellular, molecular, and epigenetic mechanisms in non-associative conditioning: Implications for pain and memory. *Neurobiology of Learning and Memory, 105*, 133-150.

Ranasinghe, C., Gamage, P., Katulanda, P., Andraweera, N., Thilakarathne, S., & Tharanga, P. (2013). Relationship between body mass index (BMI) and body fat percentage, estimated by bioelectrical impedance, in a group of sri lankan adults: A cross sectional study. *BMC Public Health, 13*, 797-2458-13-797.

Ravussin, E., Lillioja, S., Anderson, T. E., Christin, L., & Bogardus, C. (1986). Determinants of 24-hour energy expenditure in man. methods and results using a respiratory chamber. *The Journal of Clinical Investigation, 78*(6), 1568-1578.

Ravussin, E., Lillioja, S., Knowler, W. C., Christin, L., Freymond, D., Abbott, W. G., et al. (1988). Reduced rate of energy expenditure as a risk factor for body-weight gain. *The New England Journal of Medicine, 318*(8), 467-472.

Redrobe, J. P., Dumont, Y., Fournier, A., Baker, G. B., & Quirion, R. (2005). Role of serotonin (5-HT) in the antidepressant-like properties of neuropeptide Y (NPY) in the mouse forced swim test. *Peptides, 26*(8), 1394-1400.

Reynolds, G. P. (2012). Pharmacogenetic aspects of antipsychotic drug-induced weight gain - A critical review. *Clinical Psychopharmacology and Neuroscience : The Official Scientific Journal of the Korean College of Neuropsychopharmacology, 10*(2), 71-77.

Reynolds, G. P., Abdul-Monim, Z., Neill, J. C., & Zhang, Z. J. (2004). Calcium binding protein markers of GABA deficits in schizophrenia--postmortem studies and animal models. *Neurotoxicity Research, 6*(1), 57-61.

Reynolds, G. P., & Kirk, S. L. (2010). Metabolic side effects of antipsychotic drug treatment--pharmacological mechanisms. *Pharmacology & Therapeutics, 125*(1), 169-179.

Reynolds, G. P., Yevtushenko, O. O., Gordon, S., Arranz, B., San, L., & Cooper, S. J. (2013). The obesity risk gene FTO influences body mass in chronic schizophrenia but not initial antipsychotic drug-induced weight gain in first-episode patients. *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP), 16*(6), 1421-1425.

Reynolds, G. P., Zhang, Z. J., & Zhang, X. B. (2002). Association of antipsychotic drug-induced weight gain with a 5-HT2C receptor gene polymorphism. *Lancet, 359*(9323), 2086-2087.

Rice, D. P. (1999). The economic impact of schizophrenia. *The Journal of Clinical Psychiatry, 60 Suppl 1*, 4-6; discussion 28-30.

Richardson, M. A., Read, L. L., Taylor Clelland, C. L., Reilly, M. A., Chao, H. M., Guynn, R. W., et al. (2005). Evidence for a tetrahydrobiopterin deficit in schizophrenia. *Neuropsychobiology, 52*(4), 190-201.

Richelson, E., & Souder, T. (2000). Binding of antipsychotic drugs to human brain receptors focus on newer generation compounds. *Life Sciences, 68*(1), 29-39.

Riggs, A.,D., Martienssen, R.,A., & Russo, V. (1996).
Introduction In A. Riggs D., R. Martienssen A. & V. Russo (Eds.),

Epigenetic mechanisms of gene regulation (). New York: Cold Spring Harbor Laboratory Press.

Risselada, A. J., Vehof, J., Bruggeman, R., Wilffert, B., Cohen, D., Al Hadithy, A. F., et al. (2010). Association between the 1291-C/G polymorphism in the adrenergic alpha-2a receptor and the metabolic syndrome. *Journal of Clinical Psychopharmacology, 30*(6), 667-671.

Rittenhouse, P. A., Bakkum, E. A., Levy, A. D., Li, Q., Carnes, M., & van de Kar, L. D. (1994). Evidence that ACTH secretion is regulated by serotonin2A/2C (5-HT2A/2C) receptors. *The Journal of Pharmacology and Experimental Therapeutics, 271*(3), 1647-1655.

Rivera, H. M., Santollo, J., Nikonova, L. V., & Eckel, L. A. (2012). Estradiol increases the anorexia associated with increased 5-HT(2C) receptor activation in ovariectomized rats. *Physiology & Behavior, 105*(2), 188-194.

Roepke, T. A., Smith, A. W., Ronnekleiv, O. K., & Kelly, M. J. (2012). Serotonin 5-HT2C receptor-mediated inhibition of the M-current in hypothalamic POMC neurons. *American Journal of Physiology.Endocrinology and Metabolism, 302*(11), E1399-406.

Rohm, B., Holik, A. K., Somoza, M. M., Pignitter, M., Zaunschirm, M., Ley, J. P., et al. (2013). Nonivamide, a capsaicin analog, increases dopamine and serotonin release in SH-SY5Y cells via a TRPV1-independent pathway. *Molecular Nutrition & Food Research, 57*(11), 2008-2018.

Rosen, E. D., & Spiegelman, B. M. (2000). Molecular regulation of adipogenesis. *Annual Review of Cell and Developmental Biology, 16*, 145-171.

Rosmond, R., Bouchard, C., & Bjorntorp, P. (2002a). A C-1291G polymorphism in the alpha2A-adrenergic receptor gene (ADRA2A) promoter is associated with cortisol escape from dexamethasone and elevated glucose levels. *Journal of Internal Medicine, 251*(3), 252-257.

Rosmond, R., Bouchard, C., & Bjorntorp, P. (2002b). 5-HT2A receptor gene promoter polymorphism in relation to abdominal obesity and cortisol. *Obesity Research, 10*(7), 585-589.

Rossi, J., Balthasar, N., Olson, D., Scott, M., Berglund, E., Lee, C. E., et al. (2011). Melanocortin-4 receptors expressed by cholinergic neurons regulate energy balance and glucose homeostasis. *Cell Metabolism, 13*(2), 195-204.

Roth, B. L., Sheffler, D. J., & Kroeze, W. K. (2004). Magic shotguns versus magic bullets: Selectively non-selective drugs for mood disorders and schizophrenia. *Nature Reviews.Drug Discovery, 3*(4), 353-359.

Rubenstein, A. H. (2005). Obesity: A modern epidemic. *Transactions of the American Clinical and Climatological Association, 116*, 103-11; discussion 112-3.

Russo-Neustadt, A. (2003). Brain-derived neurotrophic factor, behavior, and new directions for the treatment of mental disorders. *Seminars in Clinical Neuropsychiatry, 8*(2), 109-118.

Ryden, M., Faulds, G., Hoffstedt, J., Wennlund, A., & Arner, P. (2002). Effect of the (C825T) gbeta(3) polymorphism on adrenoceptor-mediated lipolysis in human fat cells. *Diabetes, 51*(5), 1601-1608.

Sachs, G. S., & Guille, C. (1999). Weight gain associated with use of psychotropic medications. *J Clin Psychiatry*, 60 Suppl 21, 16-19.

Saiz, P. A., Susce, M. T., Clark, D. A., Kerwin, R. W., Molero, P., Arranz, M. J., et al. (2008). An investigation of the alpha1A-adrenergic receptor gene and antipsychotic-induced side-effects. *Human Psychopharmacology, 23*(2), 107-114.

Salam, M. T., Byun, H. M., Lurmann, F., Breton, C. V., Wang, X., Eckel, S. P., et al. (2012). Genetic and epigenetic variations in inducible nitric oxide synthase promoter, particulate pollution, and exhaled nitric oxide levels in children. *The Journal of Allergy and Clinical Immunology, 129*(1), 232-9.e1-7.

Sambrook, F., & Russel, D. (Eds.). (2001). *Molecular cloning : A laboratory manual* (3rd ed.). N.Y.: Cold Spring Harbor Laboratory Press.

Santini, V., Kantarjian, H. M., & Issa, J. P. (2001). Changes in DNA methylation in neoplasia: Pathophysiology and therapeutic implications. *Annals of Internal Medicine, 134*(7), 573-586.

Sarvari, A. K., Vereb, Z., Uray, I. P., Fesus, L., & Balajthy, Z. (2014). Atypical antipsychotics induce both proinflammatory and adipogenic gene expression in human adipocytes in vitro. *Biochemical and Biophysical Research Communications, 450*(4), 1383-1389.

Satta, R., Maloku, E., Zhubi, A., Pibiri, F., Hajos, M., Costa, E., et al. (2008). Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons. *Proceedings of the National Academy of Sciences of the United States of America, 105*(42), 16356-16361.

Sawaguchi, T., Matsumura, M., & Kubota, K. (1989). Delayed response deficits produced by local injection of bicuculline into the dorsolateral prefrontal cortex in japanese macaque monkeys. *Experimental Brain Research*, 75(3), 457-469.

Schmitz, K. M., Mayer, C., Postepska, A., & Grummt, I. (2010). Interaction of noncoding RNA with the rDNA promoter mediates recruitment of DNMT3b and silencing of rRNA genes. *Genes & Development, 24*(20), 2264-2269.

Schwartz, M. W., Woods, S. C., Porte, D.,Jr, Seeley, R. J., & Baskin, D. G. (2000). Central nervous system control of food intake. *Nature, 404*(6778), 661-671.

Schwartz, R. S., Jaeger, L. F., & Veith, R. C. (1988). Effect of clonidine on the thermic effect of feeding in humans. *The American Journal of Physiology, 254*(1 Pt 2), R90-4.

Seeman, P., & Lee, T. (1975). Antipsychotic drugs: Direct correlation between clinical potency and presynaptic action on dopamine neurons. *Science (New York, N.Y.), 188*(4194), 1217-1219.

Selhub, J. (2008). Public health significance of elevated homocysteine. *Food and Nutrition Bulletin, 29*(2 Suppl), S116-25.

Selten, J. P., & Slaets, J. P. (1994). Evidence against maternal influenza as a risk factor for schizophrenia. *The British Journal of Psychiatry : The Journal of Mental Science, 164*(5), 674-676.

Sentissi, O., Epelbaum, J., Olie, J. P., & Poirier, M. F. (2008). Leptin and ghrelin levels in patients with schizophrenia during different antipsychotics treatment: A review. *Schizophrenia Bulletin, 34*(6), 1189-1199.

Sentissi, O., Grouselle, D., Viala, A., Bourdel, M. C., Olie, J. P., Epelbaum, J., et al. (2009). Ghrelin and leptin levels in schizophrenic patients treated with antipsychotic monotherapy. *Journal of Clinical Psychopharmacology, 29*(3), 304-306.

Sertie, A. L., Suzuki, A. M., Sertie, R. A., Andreotti, S., Lima, F. B., Passos-Bueno, M. R., et al. (2011). Effects of antipsychotics with different weight gain liabilities on human in vitro models of adipose tissue differentiation and metabolism. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 35*(8), 1884-1890.

Shahid, M., Walker, G. B., Zorn, S. H., & Wong, E. H. (2009). Asenapine: A novel psychopharmacologic agent with a unique human receptor signature. *Journal of Psychopharmacology (Oxford, England), 23*(1), 65-73.

Shao, P., Zhao, J. P., Chen, J. D., Wu, R. R., & He, Y. Q. (2008). Association of HTR2C-759C/T and -697G/C polymorphisms with antipsychotic agent-induced weight gain. *Zhong Nan Da Xue Xue Bao.Yi Xue Ban = Journal of Central South University.Medical Sciences*, 33(4), 312-315.

Sharp, A. J., Stathaki, E., Migliavacca, E., Brahmachary, M., Montgomery, S. B., Dupre, Y., et al. (2011). DNA methylation profiles of human active and inactive X chromosomes. *Genome Research, 21*(10), 1592-1600.

Shastry, B. S. (2009). SNPs: Impact on gene function and phenotype. *Methods in Molecular Biology (Clifton, N.J.)*, 578, 3-22.

Shaul, U., Ben-Shachar, D., Karry, R., & Klein, E. (2003). Modulation of frequency and duration of repetitive magnetic stimulation affects catecholamine levels and tyrosine hydroxylase activity in human neuroblastoma cells: Implication for the antidepressant effect of rTMS. *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP), 6*(3), 233-241.

Shi, Y. Y., & He, L. (2005). SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Research, 15*(2), 97-98.

Shih, J. C., Zhu, Q., & Chen, K. (1996). Determination of transcription initiation sites and promoter activity of the human 5-HT2A receptor gene. *Behavioural Brain Research, 73*(1-2), 59-62.

Shimabukuro, M., Jinno, Y., Fuke, C., & Okazaki, Y. (2006). Haloperidol treatment induces tissue- and sex-specific changes in DNA methylation: A control study using rats. *Behavioral and Brain Functions : BBF, 2*, 37.

Shing, E. C., Tiwari, A. K., Brandl, E. J., Zai, C. C., Lieberman, J. A., Meltzer, H. Y., et al. (2014). Fat mass- and obesity-associated (FTO) gene and antipsychotic-induced weight gain: An association study. *Neuropsychobiology, 69*(1), 59-63.

Sickert, L., Muller, D. J., Tiwari, A. K., Shaikh, S., Zai, C., De Souza, R., et al. (2009). Association of the alpha 2A adrenergic receptor -1291C/G polymorphism and antipsychotic-induced weight gain in european-americans. *Pharmacogenomics, 10*(7), 1169-1176.

Siffert, W., Forster, P., Jockel, K. H., Mvere, D. A., Brinkmann, B., Naber, C., et al. (1999). Worldwide ethnic distribution of the G protein beta3 subunit 825T allele and its association with obesity in caucasian, chinese, and black african individuals. *Journal of the American Society of Nephrology : JASN, 10*(9), 1921-1930.

Siffert, W., Rosskopf, D., Siffert, G., Busch, S., Moritz, A., Erbel, R., et al. (1998). Association of a human G-protein beta3 subunit variant with hypertension. *Nature Genetics, 18*(1), 45-48.

Sikich, L., Frazier, J. A., McClellan, J., Findling, R. L., Vitiello, B., Ritz, L., et al. (2008). Double-blind comparison of first- and second-generation antipsychotics in early-onset schizophrenia and schizo-affective disorder: Findings from the treatment of early-onset schizophrenia spectrum disorders (TEOSS) study. *The American Journal of Psychiatry, 165*(11), 1420-1431.

Silvestri, S., Seeman, M. V., Negrete, J. C., Houle, S., Shammi, C. M., Remington, G. J., et al. (2000). Increased dopamine D2 receptor binding after long-term treatment with antipsychotics in humans: A clinical PET study. *Psychopharmacology, 152*(2), 174-180.

Simpson, M. D., Lubman, D. I., Slater, P., & Deakin, J. F. (1996). Autoradiography with [3H]8-OH-DPAT reveals increases in 5-HT(1A) receptors in ventral prefrontal cortex in schizophrenia. *Biological Psychiatry, 39*(11), 919-928.

Singer, H., Walier, M., Nusgen, N., Meesters, C., Schreiner, F., Woelfle, J., et al. (2012). Methylation of L1Hs promoters is lower on the inactive X, has a tendency of being higher on autosomes in smaller genomes and shows inter-individual variability at some loci. *Human Molecular Genetics, 21*(1), 219-235.

Singh, J., & Kaur, G. (2007). Transcriptional regulation of polysialylated neural cell adhesion molecule expression by NMDA receptor activation in retinoic acid-differentiated SH-SY5Y neuroblastoma cultures. *Brain Research, 1154*, 8-21.

Smith, I. M., Mydlarz, W. K., Mithani, S. K., & Califano, J. A. (2007). DNA global hypomethylation in squamous cell head and neck cancer associated with smoking,

alcohol consumption and stage. *International Journal of Cancer.Journal International Du Cancer, 121*(8), 1724-1728.

Sohn, J. W., Harris, L. E., Berglund, E. D., Liu, T., Vong, L., Lowell, B. B., et al. (2013). Melanocortin 4 receptors reciprocally regulate sympathetic and parasympathetic preganglionic neurons. *Cell, 152*(3), 612-619.

Sohn, J. W., Xu, Y., Jones, J. E., Wickman, K., Williams, K. W., & Elmquist, J. K. (2011). Serotonin 2C receptor activates a distinct population of arcuate pro-opiomelanocortin neurons via TRPC channels. *Neuron, 71*(3), 488-497.

Soma, T., Kaganoi, J., Kawabe, A., Kondo, K., Imamura, M., & Shimada, Y. (2006). Nicotine induces the fragile histidine triad methylation in human esophageal squamous epithelial cells. *International Journal of Cancer.Journal International Du Cancer, 119*(5), 1023-1027.

Song, X., Pang, L., Feng, Y., Fan, X., Li, X., Zhang, W., et al. (2014). Fat-mass and obesity-associated gene polymorphisms and weight gain after risperidone treatment in first episode schizophrenia. *Behavioral and Brain Functions : BBF, 10*(1), 35.

Souza, R. P., De Luca, V., Muscettola, G., Rosa, D. V., de Bartolomeis, A., Romano Silva, M., et al. (2008). Association of antipsychotic induced weight gain and body mass index with GNB3 gene: A meta-analysis. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 32*(8), 1848-1853.

Speakman, J. R., Rance, K. A., & Johnstone, A. M. (2008). Polymorphisms of the FTO gene are associated with variation in energy intake, but not energy expenditure. *Obesity (Silver Spring, Md.), 16*(8), 1961-1965.

Speliotes, E. K., Willer, C. J., Berndt, S. I., Monda, K. L., Thorleifsson, G., Jackson, A. U., et al. (2010). Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nature Genetics, 42*(11), 937-948.

Spraul, M., Ravussin, E., Fontvieille, A. M., Rising, R., Larson, D. E., & Anderson, E. A. (1993). Reduced sympathetic nervous activity. A potential mechanism predisposing to body weight gain. *The Journal of Clinical Investigation, 92*(4), 1730-1735.

Spurlock, G., Heils, A., Holmans, P., Williams, J., D'Souza, U. M., Cardno, A., et al. (1998). A family based association study of T102C polymorphism in 5HT2A and schizophrenia plus identification of new polymorphisms in the promoter. *Molecular Psychiatry, 3*(1), 42-49.

Srivastava, V., Deshpande, S. N., Nimgaonkar, V. L., Lerer, B., & Thelma, B. (2008). Genetic correlates of olanzapine-induced weight gain in schizophrenia subjects from north india: Role of metabolic pathway genes. *Pharmacogenomics, 9*(8), 1055-1068.

Stan, A. D., & Lewis, D. A. (2012). Altered cortical GABA neurotransmission in schizophrenia: Insights into novel therapeutic strategies. *Current Pharmaceutical Biotechnology*, 13(8), 1557-1562.

Stedman, T., & Welham, J. (1993). The distribution of adipose tissue in female in-patients receiving psychotropic drugs. *The British Journal of Psychiatry : The Journal of Mental Science, 162*, 249-250.

Stern, L. L., Mason, J. B., Selhub, J., & Choi, S. W. (2000). Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. *Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 9*(8), 849-853.

Stunes, A. K., Reseland, J. E., Hauso, O., Kidd, M., Tommeras, K., Waldum, H. L., et al. (2011). Adipocytes express a functional system for serotonin synthesis, reuptake and receptor activation. *Diabetes, Obesity & Metabolism, 13*(6), 551-558.

Stutzmann, F., Cauchi, S., Durand, E., Calvacanti-Proenca, C., Pigeyre, M., Hartikainen, A. L., et al. (2009). Common genetic variation near MC4R is associated with eating behaviour patterns in european populations. *International Journal of Obesity (2005), 33*(3), 373-378.

Sugden, C. (2006). One-carbon metabolism in psychiatric illness. *Nutrition Research Reviews, 19*(1), 117-136.

Sumiyoshi, T., Stockmeier, C. A., Overholser, J. C., Dilley, G. E., & Meltzer, H. Y. (1996). Serotonin1A receptors are increased in postmortem prefrontal cortex in schizophrenia. *Brain Research, 708*(1-2), 209-214.

Susser, E., Lin, S. P., Brown, A. S., Lumey, L. H., & Erlenmeyer-Kimling, L. (1994). No relation between risk of schizophrenia and prenatal exposure to influenza in holland. *The American Journal of Psychiatry, 151*(6), 922-924.

Susser, E., Neugebauer, R., Hoek, H. W., Brown, A. S., Lin, S., Labovitz, D., et al. (1996). Schizophrenia after prenatal famine. further evidence. *Archives of General Psychiatry, 53*(1), 25-31.

Tang, B., Dean, B., & Thomas, E. A. (2011). Disease- and age-related changes in histone acetylation at gene promoters in psychiatric disorders. *Translational Psychiatry, 1*, e64.

Tang, H., Dalton, C. F., Srisawat, U., Zhang, Z. J., & Reynolds, G. P. (2014). Methylation at a transcription factor-binding site on the 5-HT1A receptor gene correlates with negative symptom treatment response in first episode schizophrenia. *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP), 17*(4), 645-649.

Tao, Y. X. (2010). The melanocortin-4 receptor: Physiology, pharmacology, and pathophysiology. *Endocrine Reviews, 31*(4), 506-543.

Tapia-Arancibia, L., Rage, F., Givalois, L., & Arancibia, S. (2004). Physiology of BDNF: Focus on hypothalamic function. *Frontiers in Neuroendocrinology, 25*(2), 77-107.

Tarantini, L., Bonzini, M., Apostoli, P., Pegoraro, V., Bollati, V., Marinelli, B., et al. (2009). Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. *Environmental Health Perspectives, 117*(2), 217-222.

Tataranni, P. A., Harper, I. T., Snitker, S., Del Parigi, A., Vozarova, B., Bunt, J., et al. (2003). Body weight gain in free-living pima indians: Effect of energy intake vs expenditure. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity, 27*(12), 1578-1583.

Tecott, L. H., Sun, L. M., Akana, S. F., Strack, A. M., Lowenstein, D. H., Dallman, M. F., et al. (1995). Eating disorder and epilepsy in mice lacking 5-HT2c serotonin receptors. *Nature, 374*(6522), 542-546.

Templeman, L. A., Reynolds, G. P., Arranz, B., & San, L. (2005). Polymorphisms of the 5-HT2C receptor and leptin genes are associated with antipsychotic drug-induced weight gain in caucasian subjects with a first-episode psychosis. *Pharmacogenetics and Genomics, 15*(4), 195-200.

Terruzzi, I., Senesi, P., Fermo, I., Lattuada, G., & Luzi, L. (2007). Are genetic variants of the methyl group metabolism enzymes risk factors predisposing to obesity? *Journal of Endocrinological Investigation, 30*(9), 747-753.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., et al. (1999). Housekeeping genes as internal standards: Use and limits. *Journal of Biotechnology, 75*(2-3), 291-295.

Thomas, G. N., Tomlinson, B., Chan, J. C., Young, R. P., & Critchley, J. A. (2000). The Trp64Arg polymorphism of the beta3-adrenergic receptor gene and obesity in chinese subjects with components of the metabolic syndrome. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity, 24*(5), 545-551.

Tiihonen, J., Lonnqvist, J., Wahlbeck, K., Klaukka, T., Niskanen, L., Tanskanen, A., et al. (2009). 11-year follow-up of mortality in patients with schizophrenia: A population-based cohort study (FIN11 study). *Lancet, 374*(9690), 620-627.

Timpson, N. J., Emmett, P. M., Frayling, T. M., Rogers, I., Hattersley, A. T., McCarthy, M. I., et al. (2008). The fat mass- and obesity-associated locus and dietary intake in children. *The American Journal of Clinical Nutrition, 88*(4), 971-978.

Tiwari, A. K., Brandl, E. J., Weber, C., Likhodi, O., Zai, C. C., Hahn, M. K., et al. (2013). Association of a functional polymorphism in neuropeptide Y with antipsychotic-induced weight gain in schizophrenia patients. *Journal of Clinical Psychopharmacology, 33*(1), 11-17.

Tiwari, A. K., Rodgers, J. B., Sicard, M., Zai, C. C., Likhodi, O., Freeman, N., et al. (2010b). Association study of polymorphisms in cholecystokinin gene and its receptors with antipsychotic induced weight gain in schizophrenia patients. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 34*(8), 1484-1490.

Tiwari, A. K., Zai, C. C., Likhodi, O., Lisker, A., Singh, D., Souza, R. P., et al. (2010a). A common polymorphism in the cannabinoid receptor 1 (CNR1) gene is associated with antipsychotic-induced weight gain in schizophrenia. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 35*(6), 1315-1324.

Tiwari, H. K., Patki, A., Lieberman, J., Stroup, T. S., Allison, D. B., Leibel, R. L., et al. (2011). Association of allelic variation in genes mediating aspects of energy homeostasis with weight gain during administration of antipsychotic drugs (CATIE study). *Frontiers in Genetics, 2*, 56.

Toperoff, G., Aran, D., Kark, J. D., Rosenberg, M., Dubnikov, T., Nissan, B., et al. (2012). Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. *Human Molecular Genetics, 21*(2), 371-383.

Toriya, M., Maekawa, F., Maejima, Y., Onaka, T., Fujiwara, K., Nakagawa, T., et al. (2010). Long-term infusion of brain-derived neurotrophic factor reduces food intake and body weight via a corticotrophin-releasing hormone pathway in the paraventricular nucleus of the hypothalamus. *Journal of Neuroendocrinology, 22*(9), 987-995.

Tsai, A., Liou, Y. J., Hong, C. J., Wu, C. L., Tsai, S. J., & Bai, Y. M. (2011). Association study of brain-derived neurotrophic factor gene polymorphisms and body weight change in schizophrenic patients under long-term atypical antipsychotic treatment. *Neuromolecular Medicine, 13*(4), 328-333.

Tsai, S. J., Yu, Y. W., Lin, C. H., Wang, Y. C., Chen, J. Y., & Hong, C. J. (2004). Association study of adrenergic beta3 receptor (Trp64Arg) and G-protein beta3 subunit gene (C825T) polymorphisms and weight change during clozapine treatment. *Neuropsychobiology, 50*(1), 37-40.

Tsao, D., Thomsen, H. K., Chou, J., Stratton, J., Hagen, M., Loo, C., et al. (2008). TrkB agonists ameliorate obesity and associated metabolic conditions in mice. *Endocrinology, 149*(3), 1038-1048.

Tschritter, O., Haupt, A., Preissl, H., Ketterer, C., Hennige, A. M., Sartorius, T., et al. (2011). An obesity risk SNP (rs17782313) near the MC4R gene is associated with cerebrocortical insulin resistance in humans. *Journal of Obesity, 2011*, 283153.

Tukey, J. W. (Ed.). (1977). *Exploratory data analysis* (First Edition), Reading, Mass: Addison-Wesley Publishing Co.

Turcot, V., Tchernof, A., Deshaies, Y., Perusse, L., Belisle, A., Marceau, S., et al. (2012). LINE-1 methylation in visceral adipose tissue of severely obese individuals is associated with metabolic syndrome status and related phenotypes. *Clinical Epigenetics, 4*(1), 10-7083-4-10.

Turker, M. S., & Bestor, T. H. (1997). Formation of methylation patterns in the mammalian genome. *Mutation Research, 386*(2), 119-130.

Ueland, P. M., Hustad, S., Schneede, J., Refsum, H., & Vollset, S. E. (2001). Biological and clinical implications of the MTHFR C677T polymorphism. *Trends in Pharmacological Sciences, 22*(4), 195-201.

Ujike, H., Nomura, A., Morita, Y., Morio, A., Okahisa, Y., Kotaka, T., et al. (2008). Multiple genetic factors in olanzapine-induced weight gain in schizophrenia patients: A cohort study. *The Journal of Clinical Psychiatry, 69*(9), 1416-1422.

Unger, T. J., Calderon, G. A., Bradley, L. C., Sena-Esteves, M., & Rios, M. (2007). Selective deletion of bdnf in the ventromedial and dorsomedial hypothalamus of adult mice results in hyperphagic behavior and obesity. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 27*(52), 14265-14274.

van der Put, N. M., Gabreels, F., Stevens, E. M., Smeitink, J. A., Trijbels, F. J., Eskes, T. K., et al. (1998). A second common mutation in the methylenetetrahydrofolate reductase gene: An additional risk factor for neural-tube defects? *American Journal of Human Genetics,* 62(5), 1044-1051.

van Winkel, R., Moons, T., Peerbooms, O., Rutten, B., Peuskens, J., Claes, S., et al. (2010b). MTHFR genotype and differential evolution of metabolic parameters after initiation of a second generation antipsychotic: An observational study. *International Clinical Psychopharmacology, 25*(5), 270-276.

van Winkel, R., Rutten, B. P., Peerbooms, O., Peuskens, J., van Os, J., & De Hert, M. (2010a). MTHFR and risk of metabolic syndrome in patients with schizophrenia. *Schizophrenia Research, 121*(1-3), 193-198.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology, 3*(7), RESEARCH0034.

Venkatasubramanian, G., Chittiprol, S., Neelakantachar, N., Shetty, T. K., & Gangadhar, B. N. (2010). A longitudinal study on the impact of antipsychotic treatment on serum leptin in schizophrenia. *Clinical Neuropharmacology, 33*(6), 288-292.

Vermeulen, E. G., Niessen, H. W., Bogels, M., Stehouwer, C. D., Rauwerda, J. A., & van Hinsbergh, V. W. (2001). Decreased smooth muscle cell/extracellular matrix ratio of media of femoral artery in patients with atherosclerosis and hyperhomocysteinemia. *Arteriosclerosis, Thrombosis, and Vascular Biology, 21*(4), 573-577.

Vestri, H. S., Maianu, L., Moellering, D. R., & Garvey, W. T. (2007). Atypical antipsychotic drugs directly impair insulin action in adipocytes: Effects on glucose transport, lipogenesis, and antilipolysis. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 32*(4), 765-772.

Voigt, J. P., Schade, R., Fink, H., & Hortnagl, H. (2002). Role of 5-HT1A receptors in the control of food intake in obese zucker rats of different ages. *Pharmacology, Biochemistry, and Behavior, 72*(1-2), 403-409.

Volk, D. W., & Lewis, D. A. (2005). GABA targets for the treatment of cognitive dysfunction in schizophrenia. *Current Neuropharmacology*, 3(1), 45-62.

von Meyenburg, C., Langhans, W., & Hrupka, B. J. (2003a). Evidence for a role of the 5-HT2C receptor in central lipopolysaccharide-, interleukin-1 beta-, and leptin-induced anorexia. *Pharmacology, Biochemistry, and Behavior, 74*(4), 1025-1031.

von Meyenburg, C., Langhans, W., & Hrupka, B. J. (2003b). Evidence that the anorexia induced by lipopolysaccharide is mediated by the 5-HT2C receptor. *Pharmacology, Biochemistry, and Behavior, 74*(2), 505-512.

Wabitsch, M., Blum, W. F., Muche, R., Braun, M., Hube, F., Rascher, W., et al. (1997). Contribution of androgens to the gender difference in leptin production in obese children and adolescents. *The Journal of Clinical Investigation, 100*(4), 808-813.

Waddington, J. L., Corvin, A. P., Donohoe, G., O'Tuathaigh, C. M., Mitchell, K. J., & Gill, M. (2007). Functional genomics and schizophrenia: Endophenotypes and mutant models. *The Psychiatric Clinics of North America, 30*(3), 365-399.

Wade, P. A., Gegonne, A., Jones, P. L., Ballestar, E., Aubry, F., & Wolffe, A. P. (1999). Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nature Genetics, 23*(1), 62-66.

Walsh, C. P., Chaillet, J. R., & Bestor, T. H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nature Genetics, 20*(2), 116-117.

Wang, G. J., Volkow, N. D., Logan, J., Pappas, N. R., Wong, C. T., Zhu, W., et al. (2001). Brain dopamine and obesity. *Lancet, 357*(9253), 354-357.

Wang, H. C., Yang, Y. K., Chen, P. S., Lee, I. H., Yeh, T. L., & Lu, R. B. (2007b). Increased plasma leptin in antipsychotic-naive females with schizophrenia, but not in males. *Neuropsychobiology, 56*(4), 213-215.

Wang, P., Yang, F. J., Du, H., Guan, Y. F., Xu, T. Y., Xu, X. W., et al. (2011). Involvement of leptin receptor long isoform (LepRb)-STAT3 signaling pathway in brain fat mass- and obesity-associated (FTO) downregulation during energy restriction. *Molecular Medicine (Cambridge, Mass.), 17*(5-6), 523-532.

Wang, X., Qin, X., Demirtas, H., Li, J., Mao, G., Huo, Y., et al. (2007a). Efficacy of folic acid supplementation in stroke prevention: A meta-analysis. *Lancet, 369*(9576), 1876-1882.

Wang, Y., Chen, S., Yao, T., Li, D., Wang, Y., Li, Y., et al. (2014). Homocysteine as a risk factor for hypertension: A 2-year follow-up study. *PloS One, 9*(10), e108223.

Wang, Y. C., Bai, Y. M., Chen, J. Y., Lin, C. C., Lai, I. C., & Liou, Y. J. (2005a). C825T polymorphism in the human G protein beta3 subunit gene is associated with long-term clozapine treatment-induced body weight change in the chinese population. *Pharmacogenetics and Genomics, 15*(10), 743-748.

Wang, Y. C., Bai, Y. M., Chen, J. Y., Lin, C. C., Lai, I. C., & Liou, Y. J. (2005b). Polymorphism of the adrenergic receptor alpha 2a -1291C>G genetic variation and clozapine-induced weight gain. *Journal of Neural Transmission (Vienna, Austria : 1996), 112*(11), 1463-1468.

Wangsri, S., Subbalekha, K., Kitkumthorn, N., & Mutirangura, A. (2012). Patterns and possible roles of LINE-1 methylation changes in smoke-exposed epithelia. *PloS One, 7*(9), e45292.

Wardle, J., Carnell, S., Haworth, C. M., Farooqi, I. S., O'Rahilly, S., & Plomin, R. (2008). Obesity associated genetic variation in FTO is associated with diminished satiety. *The Journal of Clinical Endocrinology and Metabolism, 93*(9), 3640-3643.

Watanabe, S. Y., Iga, J., Numata, S., Nakataki, M., Tanahashi, T., Itakura, M., et al. (2012). Association study of fat-mass and obesity-associated gene and body mass index in japanese patients with schizophrenia and healthy subjects. *Clinical Psychopharmacology and Neuroscience : The Official Scientific Journal of the Korean College of Neuropsychopharmacology, 10*(3), 185-189.

Watanabe, T., Takeda, A., Tsukiyama, T., Mise, K., Okuno, T., Sasaki, H., et al. (2006). Identification and characterization of two novel classes of small RNAs in the mouse germline: Retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. *Genes & Development, 20*(13), 1732-1743.

Weigle, D. S., Bukowski, T. R., Foster, D. C., Holderman, S., Kramer, J. M., Lasser, G., et al. (1995). Recombinant ob protein reduces feeding and body weight in the ob/ob mouse. *The Journal of Clinical Investigation, 96*(4), 2065-2070.

Weisberg, I., Tran, P., Christensen, B., Sibani, S., & Rozen, R. (1998). A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Molecular Genetics and Metabolism, 64*(3), 169-172.

Wellman, P. J., & Davies, B. T. (1992). Reversal of cirazoline- and phenylpropanolamine-induced anorexia by the alpha 1-receptor antagonist prazosin. *Pharmacology, Biochemistry, and Behavior, 42*(1), 97-100.

Wellman, P. J., Davies, B. T., Morien, A., & McMahon, L. (1993). Modulation of feeding by hypothalamic paraventricular nucleus alpha 1- and alpha 2-adrenergic receptors. *Life Sciences, 53*(9), 669-679.

Wilding,John,P, (Ed.). (2010). *Pathophysiology and aetiology of obesity* (1st ed.) Elsevier Ltd.

Wilhelm, C. S., Kelsey, K. T., Butler, R., Plaza, S., Gagne, L., Zens, M. S., et al. (2010). Implications of LINE1 methylation for bladder cancer risk in women. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 16*(5), 1682-1689.

Willer, C. J., Speliotes, E. K., Loos, R. J., Li, S., Lindgren, C. M., Heid, I. M., et al. (2009). Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. *Nature Genetics, 41*(1), 25-34.

Williams, J., Spurlock, G., McGuffin, P., Mallet, J., Nothen, M. M., Gill, M., et al. (1996). Association between schizophrenia and T102C polymorphism of the 5-hydroxytryptamine type 2a-receptor gene. european multicentre association study of schizophrenia (EMASS) group. *Lancet, 347*(9011), 1294-1296.

Wirshing, D. A., Wirshing, W. C., Kysar, L., Berisford, M. A., Goldstein, D., Pashdag, J., et al. (1999). Novel antipsychotics: Comparison of weight gain liabilities. *The Journal of Clinical Psychiatry, 60*(6), 358-363.

Wongtrakoongate, P. (2015). Epigenetic therapy of cancer stem and progenitor cells by targeting DNA methylation machineries. *World Journal of Stem Cells, 7*(1), 137-148.

Woolley, D. W., & Shaw, E. (1954). A biochemical and pharmacological suggestion about certain mental disorders. *Proceedings of the National Academy of Sciences of the United States of America, 40*(4), 228-231.

Wu, Q., Saunders, R. A., Szkudlarek-Mikho, M., Serna Ide, L., & Chin, K. V. (2010). The obesity-associated fto gene is a transcriptional coactivator. *Biochemical and Biophysical Research Communications, 401*(3), 390-395.

Wu, R., Zhao, J., Shao, P., Ou, J., & Chang, M. (2011). Genetic predictors of antipsychotic-induced weight gain: A case-matched multi-gene study. *Zhong Nan Da Xue Xue Bao.Yi Xue Ban = Journal of Central South University.Medical Sciences, 36*(8), 720-723.

Xi, B., Chandak, G. R., Shen, Y., Wang, Q., & Zhou, D. (2012). Association between common polymorphism near the MC4R gene and obesity risk: A systematic review and meta-analysis. *PloS One, 7*(9), e45731.

Xiao, W., Wang, J., Li, H., Guan, W., Xia, D., Yu, G., et al. (2013). Fibulin-1 is down-regulated through promoter hypermethylation and suppresses renal cell carcinoma progression. *The Journal of Urology, 190*(1), 291-301.

Xie, E., Zhu, L., Zhao, L., & Chang, L. S. (1996). The human serotonin 5-HT2C receptor: Complete cDNA, genomic structure, and alternatively spliced variant. *Genomics, 35*(3), 551-561.

Xie, H. R., Hu, L. S., & Li, G. Y. (2010). SH-SY5Y human neuroblastoma cell line: In vitro cell model of dopaminergic neurons in parkinson's disease. *Chinese Medical Journal, 123*(8), 1086-1092.

Xie, S., Wang, Z., Okano, M., Nogami, M., Li, Y., He, W. W., et al. (1999). Cloning, expression and chromosome locations of the human DNMT3 gene family. *Gene, 236*(1), 87-95.

Xu, M., Li, S., Xing, Q., Gao, R., Feng, G., Lin, Z., et al. (2010). Genetic variants in the BDNF gene and therapeutic response to risperidone in schizophrenia patients: A pharmacogenetic study. *European Journal of Human Genetics : EJHG, 18*(6), 707-712.

Xu, M. Q., St Clair, D., Feng, G. Y., Lin, Z. G., He, G., Li, X., et al. (2008). BDNF gene is a genetic risk factor for schizophrenia and is related to the chlorpromazine-induced extrapyramidal syndrome in the chinese population. *Pharmacogenetics and Genomics, 18*(6), 449-457.

Xu, Z., Chen, H., Liu, D., & Huo, J. (2015). Fibulin-1 is downregulated through promoter hypermethylation in colorectal cancer: A CONSORT study. *Medicine, 94*(13), e663.

Yakub, M., Schulze, K. J., Khatry, S. K., Stewart, C. P., Christian, P., & West, K. P. (2014). High plasma homocysteine increases risk of metabolic syndrome in 6 to 8 year old children in rural nepal. *Nutrients, 6*(4), 1649-1661.

Yamada, J., Sugimoto, Y., Hirose, H., & Kajiwara, Y. (2003). Role of serotonergic mechanisms in leptin-induced suppression of milk intake in mice. *Neuroscience Letters, 348*(3), 195-197.

Yamada, J., Sugimoto, Y., & Ujikawa, M. (1999). The serotonin precursor 5-hydroxytryptophan elevates serum leptin levels in mice. *European Journal of Pharmacology, 383*(1), 49-51.

Yang, J. W., & Kim, S. S. (2015). Ginsenoside rc promotes anti-adipogenic activity on 3T3-L1 adipocytes by down-regulating C/EBPalpha and PPARgamma. *Molecules (Basel, Switzerland), 20*(1), 1293-1303.

Yang, L. H., Chen, T. M., Yu, S. T., & Chen, Y. H. (2007). Olanzapine induces SREBP-1-related adipogenesis in 3T3-L1 cells. *Pharmacological Research : The Official Journal of the Italian Pharmacological Society, 56*(3), 202-208.

Yang, M., Xu, Y., Liang, L., Fu, J., Xiong, F., Liu, G., et al. (2014). The effects of genetic variation in FTO rs9939609 on obesity and dietary preferences in chinese han children and adolescents. *PloS One, 9*(8), e104574.

Yang, N., & Kazazian, H. H.,Jr. (2006). L1 retrotransposition is suppressed by endogenously encoded small interfering RNAs in human cultured cells. *Nature Structural & Molecular Biology, 13*(9), 763-771.

Yang, Y., Li, W., Zhao, J., Zhang, H., Song, X., Xiao, B., et al. (2012). Association between ghrelin gene (GHRL) polymorphisms and clinical response to atypical antipsychotic drugs in han chinese schizophrenia patients. *Behavioral and Brain Functions : BBF, 8*, 11-9081-8-11.

Yang, Z., Yin, J. Y., Gong, Z. C., Huang, Q., Chen, H., Zhang, W., et al. (2009). Evidence for an effect of clozapine on the regulation of fat-cell derived factors. *Clinica Chimica Acta; International Journal of Clinical Chemistry, 408*(1-2), 98-104.

Yanik, T., Kursungoz, C., Sutcigil, L., & Ak, M. (2013). Weight gain in risperidone therapy: Investigation of peripheral hypothalamic neurohormone levels in psychotic patients. *Journal of Clinical Psychopharmacology, 33*(5), 608-613.

Yevtushenko, O. O., Cooper, S. J., O'Neill, R., Doherty, J. K., Woodside, J. V., & Reynolds, G. P. (2008). Influence of 5-HT2C receptor and leptin gene polymorphisms, smoking and drug treatment on metabolic disturbances in patients with schizophrenia. *The British Journal of Psychiatry : The Journal of Mental Science, 192*(6), 424-428.

Yideng, J., Jianzhong, Z., Ying, H., Juan, S., Jinge, Z., Shenglan, W., et al. (2007). Homocysteine-mediated expression of SAHH, DNMTs, MBD2, and DNA hypomethylation potential pathogenic mechanism in VSMCs. *DNA and Cell Biology, 26*(8), 603-611.

Yoder, J. A., Walsh, C. P., & Bestor, T. H. (1997). Cytosine methylation and the ecology of intragenomic parasites. *Trends in Genetics : TIG, 13*(8), 335-340.

Yuan, X., Yamada, K., Ishiyama-Shigemoto, S., Koyama, W., & Nonaka, K. (2000). Identification of polymorphic loci in the promoter region of the serotonin 5-HT2C receptor gene and their association with obesity and type II diabetes. *Diabetologia, 43*(3), 373-376.

Zai, G. C., Zai, C. C., Chowdhury, N. I., Tiwari, A. K., Souza, R. P., Lieberman, J. A., et al. (2012). The role of brain-derived neurotrophic factor (BDNF) gene variants in antipsychotic response and antipsychotic-induced weight gain. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 39*(1), 96-101.

Zakharyan, R., & Boyajyan, A. (2014). Brain-derived neurotrophic factor blood levels are decreased in schizophrenia patients and associate with rs6265 genotypes. *Clinical Biochemistry, 47*(12), 1052-1055.

Zhang, F. F., Cardarelli, R., Carroll, J., Fulda, K. G., Kaur, M., Gonzalez, K., et al. (2011). Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. *Epigenetics : Official Journal of the DNA Methylation Society, 6*(5), 623-629.

Zhang, G., & Pradhan, S. (2014). Mammalian epigenetic mechanisms. *IUBMB Life, 66*(4), 240-256.

Zhang, H., Herman, A. I., Kranzler, H. R., Anton, R. F., Zhao, H., Zheng, W., et al. (2013a). Array-based profiling of DNA methylation changes associated with alcohol dependence. *Alcoholism, Clinical and Experimental Research, 37 Suppl 1*, E108-15.

Zhang, J. P., Lencz, T., Geisler, S., DeRosse, P., Bromet, E. J., & Malhotra, A. K. (2013b). Genetic variation in BDNF is associated with antipsychotic treatment resistance in patients with schizophrenia. *Schizophrenia Research, 146*(1-3), 285-288.

Zhang, Q., Zhu, Y., Zhou, W., Gao, L., Yuan, L., & Han, X. (2013c). Serotonin receptor 2C and insulin secretion. *PloS One, 8*(1), e54250.

Zhang, R., Lu, J., Kong, X., Jin, L., & Luo, C. (2013d). Targeting epigenetics in nervous system disease. *CNS & Neurological Disorders Drug Targets, 12*(1), 126-141.

Zhang, X. Y., Tan, Y. L., Zhou, D. F., Cao, L. Y., Wu, G. Y., Xu, Q., et al. (2007). Serum BDNF levels and weight gain in schizophrenic patients on long-term treatment with antipsychotics. *Journal of Psychiatric Research, 41*(12), 997-1004.

Zhang, X. Y., Zhou, D. F., Wu, G. Y., Cao, L. Y., Tan, Y. L., Haile, C. N., et al. (2008). BDNF levels and genotype are associated with antipsychotic-induced weight gain in patients with chronic schizophrenia. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 33*(9), 2200-2205.

Zhang, Y., Ng, H. H., Erdjument-Bromage, H., Tempst, P., Bird, A., & Reinberg, D. (1999). Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. *Genes & Development, 13*(15), 1924-1935.

Zhang, Z. J., Yao, Z. J., Liu, W., Fang, Q., & Reynolds, G. P. (2004). Effects of antipsychotics on fat deposition and changes in leptin and insulin levels. magnetic resonance imaging study of previously untreated people with schizophrenia. *The British Journal of Psychiatry : The Journal of Mental Science, 184*, 58-62.

Zhang, Z. J., Yao, Z. J., Mou, X. D., Chen, J. F., Zhu, R. X., Liu, W., et al. (2003). Association of -2548G/A functional polymorphism in the promoter region of leptin gene with antipsychotic agent-induced weight gain. *Zhonghua Yi Xue Za Zhi, 83*(24), 2119-2123.

Zhou, L., Sutton, G. M., Rochford, J. J., Semple, R. K., Lam, D. D., Oksanen, L. J., et al. (2007). Serotonin 2C receptor agonists improve type 2 diabetes via melanocortin-4 receptor signaling pathways. *Cell Metabolism, 6*(5), 398-405.

Zhou, W., Cunningham, K. A., & Thomas, M. L. (2002). Estrogen regulation of gene expression in the brain: A possible mechanism altering the response to psychostimulants in female rats. *Brain Research.Molecular Brain Research, 100*(1-2), 75-83.

Zornberg, G. L., Buka, S. L., & Tsuang, M. T. (2000). The problem of obstetrical complications and schizophrenia. *Schizophrenia Bulletin, 26*(2), 249-256.

Zurlo, F., Ferraro, R. T., Fontvielle, A. M., Rising, R., Bogardus, C., & Ravussin, E. (1992). Spontaneous physical activity and obesity: Cross-sectional and longitudinal studies in pima indians. *The American Journal of Physiology, 263*(2 Pt 1), E296-300.

Appendix

1. Raw data analyses all cases of Chinese Han schizophrenia patients

Table 1: Methylation levels at 5 CpGs in the *HTR2C* -697G/C promoter sequences comparing between two subgroups of weight increase in Chinese Han patients

***HTR2C* -697G/C promoter region**	**CpG1 -698**	**CpG2 -691**	**CpG3 -670**	**CpG4 -661**	**CpG5 -644**
Weight increase>7% (n=82)	20.51±7.81	20.39±5.74	19.12±6.07	21.60±6.88	17.66±3.99
Weight increase<7% (n=100)	22.59±9.82	21.19±5.70	20.62±6.13	23.28±6.44	19.80±5.18
p value	0.176	0.424	0.355	0.308	**0.004**

Data is expressed as mean±SD.

Table 2: Methylation levels at 4 CpGs in the Hs_*HTR2C*_01_PM sequences comparing between two subgroups of weight increase in Chinese Han patients

Hs_*HTR2C*_01_PM	**CpG1**	**CpG2**	**CpG3**	**CpG4**
Weight increase>7% (n=82)	7.48±4.88	7.38±3.71	9.52±4.84	5.50±2.87
Weight increase<7% (n=100)	7.88±3.70	8.36±3.74	10.86±4.52	6.26±3.13
p value	0.187	0.101	**0.050**	0.126

Data is expressed as mean±SD.

Table 3: Methylation levels at 5 CpGs in the *HTR2C* -697G/C promoter sequences comparing between two subgroups of the *HTR2C* -759C/T genotype in Chinese Han patients

***HTR2C* -759C/T**	**CpG1 -698**	**CpG2 -691**	**CpG3 -670**	**CpG4 -661**	**CpG5 -644**
C/CC (n=141)	22.15±8.78	20.41±5.45	19.28±5.57	22.14±6.61	18.23±4.63
T/CT/TT (n=38)	19.53±9.72	21.99±6.26	22.55±7.34	23.99±6.67	21.17±4.81
p value	**0.017**	0.370	**0.010**	0.362	**0.002**

Data is expressed as mean±SD.

Table 4: Methylation levels at 4 CpGs in the Hs_*HTR2C*_01_PM sequences comparing between two subgroups of the *HTR2C* -759C/T genotype in Chinese Han patients

***HTR2C* -759C/T**	**CpG1**	**CpG2**	**CpG3**	**CpG4**
C/CC (n=141)	7.37±3.89	7.70±3.62	9.86±4.58	5.70±2.85
T/CT/TT (n=38)	8.24±3.72	8.48±3.75	11.82±4.62	6.67±3.38
p value	0.116	0.345	**0.017**	0.063

Data is expressed as mean±SD.

2. Raw data analyses all cases of Spanish schizophrenia patients

Table 5: Methylation levels at 5 CpGs in the *HTR2C* -697G/C promoter sequences comparing between two subgroups of weight increase in Spanish patients

HTR2C -697G/C promoter region	CpG1 -698	CpG2 -691	CpG3 -670	CpG4 -661	CpG5 -644
Weight increase>7% (n=43)	18.79±16.90	15.00±9.31	17.37±11.54	19.05±16.33	15.67±9.15
Weight increase<7% (n=23)	20.17±17.54	17.61±11.38	18.09±12.87	24.87±20.04	18.09±10.04
p value	0.666	0.620	0.651	0.261	0.538

Data is expressed as mean±SD.

Table 6: Methylation levels at 4 CpGs in the Hs_*HTR2C*_01_PM sequences comparing between two subgroups of weight increase in Spanish patients

Hs_*HTR2C*_01_PM	CpG1	CpG2	CpG3	CpG4
Weight increase>7% (n=43)	5.28±2.80	5.71±3.56	7.65±4.26	4.19±1.97
Weight increase<7% (n=23)	7.36±4.46	6.87±3.16	9.11±4.02	4.77±2.24
p value	0.064	0.172	**0.043**	0.949

Data is expressed as mean±SD.

Table 7: Methylation levels at 5 CpGs in the *HTR2C* -697G/C promoter sequences comparing between two subgroups of the *FTO* rs9939609 genotype in Spanish patients.

FTO rs9939609	CpG1 -698	CpG2 -691	CpG3 -670	CpG4 -661	CpG5 -644
AA (n=14)	30.86±16.35	21.07±8.67	21.64±12.09	23.21±15.21	19.00±9.43
AT/TT (n=56)	16.16±15.78	14.52±10.11	16.29±12.04	20.21±18.08	15.73±9.67
p value	**0.004**	**0.001**	**0.007**	0.325	0.072

Data is expressed as mean±SD.

International Journal of Neuropsychopharmacology (2014), 17, 485–490. © CINP 2013
doi:10.1017/S1461145713001375

BRIEF REPORT

Methylenetetrahydrofolate reductase (MTHFR) 677C/T polymorphism is associated with antipsychotic-induced weight gain in first-episode schizophrenia

Umarat Srisawat[1], Gavin P. Reynolds[1], Zhi Jun Zhang[2], Xiang Rong Zhang[2], Belen Arranz[3], Luis San[3] and Caroline F. Dalton[1]

[1] Sheffield Hallam University, Sheffield, UK
[2] ZhongDa Hospital and SouthEast University, Nanjing, China
[3] Parc Sanitari Sant Joan de Deu, CIBERSAM, Barcelona, Spain

Abstract

Genetic variants of the methylenetetrahydrofolate reductase (MTHFR) gene involved in homocysteine metabolism may be important predictors of antipsychotic drug-induced weight gain (AIWG). We tested whether two functional MTHFR polymorphisms are related to AIWG. Weight gain was studied in two cohorts of first-episode, initially drug-naive schizophrenia patients; Chinese Han (n = 182) and Spanish Caucasians (n = 72) receiving antipsychotics for 10 wk and 3 months respectively. Blood DNA was genotyped for 677C/T and 1298A/C MTHFR polymorphisms. Patients with the 677 CC genotype had a significantly greater increase in BMI compared to T-allele carriers in both Chinese ($p = 0.012$) and Spanish ($p = 0.017$) samples. The 677C/T MTHFR polymorphism showed an additive effect, but no significant interaction, with the -759C/T HTR2C polymorphism previously associated with AIWG.

These results suggest that the 677C/T MTHFR polymorphism might, along with the -759C/T HTR2C polymorphism and other genetic factors, provide a useful marker for the important and limiting side effect of AIWG.

Received 24 July 2013; Reviewed 23 August 2013; Revised 14 September 2013; Accepted 15 October 2013;
First published online 13 November 2013

Key words: Antipsychotic, genotype, MTHFR polymorphism, schizophrenia, weight gain.

Introduction

Schizophrenia is a severe, complex and chronic disorder, which for many patients is inadequately treated. Antipsychotic drugs can, in many individuals, relieve the positive psychotic symptoms but have various adverse effects; notably several of the drugs can induce a substantial weight gain in susceptible individuals. This weight gain may not only increase treatment noncompliance, but also affect morbidity from metabolic consequences including lipid abnormalities, insulin resistance and diabetes mellitus (Henderson et al., 2000). Patients receiving antipsychotic treatment can develop metabolic abnormalities with increased risk of cardiovascular disease and mortality (Casey et al., 2004; De Hert et al., 2009).

Susceptibility to antipsychotic-induced weight gain varies substantially between individuals in ways that cannot be fully explained by differences between drug effects or other environmental factors. Thus genetic influences are strongly implicated, and associations between many genetic polymorphisms and antipsychotic-induced weight gain have been reported. The most consistently reported genetic factors involved in antipsychotic-induced weight gain include polymorphisms in genes for 5-hydroxytryptamine 2C (5-HT2C), 5-HT2A, adrenergic alpha 2A and melanocortin 4 receptors, as well as leptin and fat mass and obesity associated (FTO) genes (Reynolds, 2012).

Recently, genetic variants of the methylenetetrahydrofolate reductase (MTHFR) gene have been proposed as potential predictors for antipsychotic-induced metabolic side effects (Kuzman and Müller, 2012). MTHFR exerts an important role in folate and homocysteine metabolism by catalysing the reduction of 5,10-methylenetetrehydrofolate to 5-methylenetetrahydrofolate (5-MTHF), which is used in methionine synthesis from homocysteine. The methionine is further converted to S-adenosylmethionine (SAM), which is a major methyl donor in a wide variety of enzymatic processes including the methylation of DNA. MTHFR deficiency can increase serum homocysteine, whereas the decrease in 5-MTHF and SAM causes deficits in DNA methylation, DNA synthesis and repair, and may

Address for correspondence: U. Srisawat, Sheffield Hallam University, Sheffield S1 1WB, UK.
Tel.: +44 774 173 9517 Fax: +44 (0)114 225 4449
Email: labboom@hotmail.com

predispose to neurodevelopmental and oncogenic processes, resulting in the development of many disorders including cardiovascular disease, renal failure, cancer and congenital abnormalities (Ueland et al., 2001).

The association of MTHFR polymorphisms with metabolic syndrome has been reported in the general population. Obesity has been associated with MTHFR 1298A/C (Terruzzi et al., 2007) and 677C/T genotypes (Lewis et al., 2006). Carriage of the 677T allele is associated with insulin resistance (Chen et al., 2010; Lunegova et al., 2011). Association of the 677T allele with central obesity, hypertriglyceridemia and low levels of high-density lipoprotein cholesterol (HDL-C) was also reported in the latter study (Lunegova et al., 2011). A replicated study reported that the 677T allele but not the 1298A/C polymorphism of MTHFR was associated with a greater risk of developing metabolic syndrome and the TT genotype was associated with risk of insulin resistance with greater central adiposity induced by antipsychotic treatment (Ellingrod et al., 2008, 2012). Others have reported the association of metabolic syndrome in schizophrenia with the 1298A/C polymorphism in 518 Caucasian patients (van Winkel et al., 2010a). These authors also reported that the 1298C variant was associated with an increased weight and impaired glucose tolerance in 104 Caucasian patients who received antipsychotic treatment for 3 months (van Winkel et al., 2010b). In the present study, we examined the association of the MTHFR 677C/T and 1298A/C polymorphisms with antipsychotic-induced weight gain in first-episode drug-naive patients with schizophrenia.

Methods

Study population

Two cohorts of first-episode, initially antipsychotic drug-naive patients with schizophrenia receiving treatment according to normal clinical practice were studied; one main sample of Chinese Han (n = 182) and a replication sample of Spanish Caucasians (n = 72). All patients gave written informed consent to the procedure of the study, which was approved by local ethical committees. Height and weight to determined body-mass index (BMI) were measured on initiation of antipsychotic drug treatment and after 8 or 10 wk (Chinese cohort) or 3 months (Spanish cohort) and weight gain was determined by change in BMI over the treatment period. Initial antipsychotic drug treatment for Chinese Han patients consisted primarily of chlorpromazine (n = 60) risperidone (n = 114); eight patients received clozapine, fluphenazine or sulpiride. Patients in the Spanish cohort received primarily risperidone (n = 21) or olanzapine (n = 22) and two received both; others had quetiapine (n = 10), haloperidol (n = 8) or ziprasidone (n = 6) with three not receiving antipsychotics. In this group, as with a subsample of the Chinese cohort (Reynolds et al., 2002), association of the -759C/T polymorphism of HTR2C with weight gain had previously been identified (Templeman et al., 2005). These results were also included in a combined analysis with the MTHFR findings.

Genotyping of MTHFR polymorphisms

Genomic deoxyribonucleic acid (DNA) was isolated from blood using standard techniques and was genotyped for MTHFR 677C/T (rs1801133) and 1298A/C (rs1801131) using TaqMan® SNP genotyping assays: assay ID C_1202883_20 and C_850486_20, respectively (Applied Biosystems, USA). The PCR conditions consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Assays were run on a Step One Plus Real-Time PCR System (Applied Biosystems, USA).

Statistical analysis

All statistical analysis of results was performed using SPSS version 18.0. Data were expressed as mean±S.D. Stepwise linear regression was used to determine the potential confounding effects of baseline BMI and age on antipsychotic-induced weight gain. Analysis of variance was used to determine the association between MTHFR genotypes and weight gain. Statistical significance was assumed for p values less than 0.05. The main Chinese cohort of 182 subjects had approximately 90% power to identify a significant genotype difference for a medium effect size of 0.5.

Results

Clinical characteristics and genotype results

The 182 (83 men, 99 women) Chinese Han patients had a mean age 26.24±7.35 years. The genotype distribution for 677C/T MTHFR was as follows: CC (n = 54), TT (n = 28), and CT (n = 94), six samples failing genotyping, and for 1298A/C was AA (n = 114), CC (n = 5), and AC (n = 56) with seven samples failing genotyping. On regression analyses, baseline BMI but not age had a significant confounding effect on weight gain after 8–10 wk treatment ($F = 24.189$, $p < 0.001$), whereas age had a significant confounding effect on baseline BMI ($F = 11.036$, $p = 0.001$). Therefore, the subsequent analyses were performed with adjustment for age or baseline BMI as covariates.

The second study sample of 72 (53 men, 19 women) Spanish patients had a mean age of 25.35±6.80 years. The 677C/T genotype distribution was CC (n = 20), TT (n = 13), and CT (n = 36) and the 1298A/C genotypes were AA (n = 45), CC (n = 3), and AC (n = 21) with three samples failing genotyping. On regression analysis, age but not baseline BMI had a significant confounding effect on weight gain at 3 months ($F = 7.026$, $p = 0.010$). Therefore, the subsequent analysis was performed with adjustment for age as a covariate.

Table 1. Sociodemographics, baseline body mass index (BMI) and change in BMI among methylenetetrahydrofolate reductase (MTHFR) 677C/T and 1298A/C genotypes in Spanish and Chinese Han samples. Data are expressed as Mean±S.D.

	MTHFR 677C/T genotype				MTHFR 1298A/C genotype			
	CC	CT	TT	p*	AA	AC	CC	p*
Chinese Han sample	n=54	n=94	n=28		n=114	n=56	n=5	
Age	25.04±6.84	26.21±7.18	28.45±8.60	0.136	26.54±7.77	25.61±6.61	27.20±5.54	0.710
Gender M/F (%male)	25/29 (46.3%)	57/65 (46.7%)	13/15 (46.4%)	0.998	53/61 (46.5%)	24/32 (42.9%)	4/1 (80%)	0.279
Baseline BMI (kg/m^2)	20.99±2.69	21.43±2.77	21.92±3.53	0.607	21.47±3.06	21.20±2.59	21.78±1.51	0.895
Change BMI (kg/m^2)	1.58±1.25	0.92±1.15	1.43±1.10	0.003	1.27±1.24	1.04±1.18	1.63±0.94	0.228
Spanish sample	n=20	n=36	n=13		n=45	n=21	n=3	
Age	27.60±8.34	24.25±5.69	23.92±6.96	0.168	25.36±7.36	24.76±6.38	25.00±1.73	0.949
Gender M/F (%male)	14/6 (70.0%)	29/7 (80.6%)	9/4(69.2%)	0.578	32/13 (71.1%)	17/4 (81%)	3/0(100%)	0.412
Baseline BMI (kg/m^2)	21.44±3.78	22.06±3.70	22.29±4.01	0.780	21.92±3.62	21.99±4.16	21.45±3.76	0.973
Change BMI (kg/m^2)	2.86±1.53	2.09±1.44	1.85±1.81	0.049	2.18±1.66	2.46±1.52	2.03±0.99	0.807

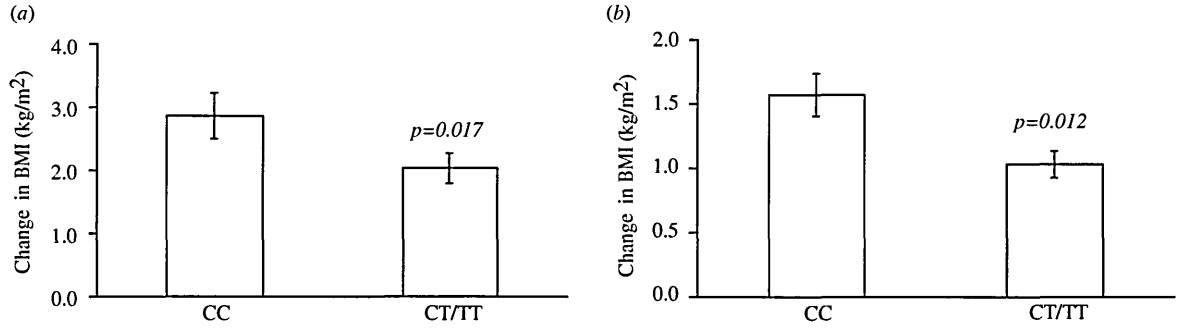


Fig. 1. The association between methylenetetrahydrofolate reductase (MTHFR) 677C/T genotype and weight gain for Spanish (a) and Chinese Han (b) schizophrenia patients. Data are expressed as mean±S.E.M. BMI: body mass index.

The two polymorphisms were found to be in strong linkage disequilibrium (D′=0.866 and 1.00, r^2=0.127 and 0.198 in Chinese and Spanish groups respectively) in which the 677T allele was almost exclusively carried with the 1298A allele.

Association of MTHFR 677C/T and 1298A/C polymorphisms with weight gain

As shown in Table 1, the baseline BMI, age, and gender distribution of both samples were not significantly different between genotypes of the 677C/T MTHFR polymorphism. The CC genotype had greater changes in BMI than T allele carriers: 1.58±1.25 vs. 1.04±1.16 kg/m^2 in Chinese (p=0.012) and 2.86±1.53 vs. 2.02±1.54 kg/m^2 in the Spanish sample (p=0.017) (Fig. 1).

Dividing the Spanish cohort into patients who received or did not receive olanzapine, and the Chinese cohort into those receiving either risperidone or chlorpromazine, resulted in the absence of a significant drug ×MTHFR genotype interaction.

The baseline BMI, age, and gender distribution of both study populations were not significantly associated with the 1298A/C MTHFR polymorphism. Nor were the changes in BMI of either study population significantly different between 1298A/C AA genotype and C carriers: 1.27±1.24 vs. 1.08±1.17 kg/m^2 in Chinese Han samples (p=0.242) and 2.18±1.66 vs. 2.40±1.45 kg/m^2 in Spanish samples (p=0.621) respectively.

Gene–gene interaction

Previous findings in these two cohorts (Reynolds et al., 2002; Templeman et al., 2005) showed that the T allele of the 5-HT2C receptor gene (HTR2C) -759C/T polymorphism had a protective effect against antipsychotic-induced weight gain. Association of this polymorphism with changes in BMI were as follows: in the Chinese cohort T carriers 0.71±1.11 kg/m^2 (n=38), C/CC genotype 1.33±1.21 kg/m^2 (n=141) p=0.004; in the Spanish cohort T carriers 1.24±1.46 kg/m^2 (n=16), C/CC genotype 2.48±1.54 kg/m^2 (n=50) p=0.012. The relationship between the effects of the HTR2C -759C/T and the MTHFR 677C/T polymorphisms was investigated. Analysing the association of weight gain with both polymorphisms together in each cohort, no significant interaction between the

polymorphisms was detected but a significant overall effect was observed (p=0.001 in the Chinese sample; p=0.019 in the Spanish sample), indicating an additive effect of the two polymorphisms. Thus carriage of two risk factors (HTR2C C/CC genotype and MTHFR 677 CC genotype) was associated with mean gains of 3.23 and 1.81 kg/m^2 in Spanish (n=14) and Chinese (n=41) cohorts respectively; equivalent values for subjects carrying neither risk factor were 1.35 (n=10) and 0.63 (n=25) kg/m^2.

Discussion

This study indicated that MTHFR 677C/T polymorphism is associated with antipsychotic-induced weight gain in first-episode patients with schizophrenia. Individuals carrying the T allele showed less weight gain compared to the common CC genotype after 8–10 wk or 3 months' treatment with antipsychotic drugs. This finding, observed in two patient cohorts of different ethnicity, indicates the effect to be a robust and reproducible one. The study had 90% power to identify a medium (0.50) effect size in the main cohort; previous studies of association of the well-replicated -759C/T polymorphism of HTR2C with antipsychotic drug-induced weight gain in a subgroup of the Chinese sample and in the Spanish sample have demonstrated substantially larger effect sizes of 0.90 and 0.86 respectively (Reynolds et al., 2002; Templeman et al., 2005). In order for pharmacogenetic risk factors to explain a good proportion of the variance and thereby to have substantial predictive value, strong effects are needed. In this we are aided substantially by the cohorts studied here; each only included first-episode patients who had never previously received antipsychotic drug treatment. This eliminates much of the variance associated with prior drug treatment, which can induce significant weight gain within a few weeks of initial treatment (Zhang et al., 2004).

The absence of an effect in the 1298A/C polymorphism, despite it being in strong linkage disequilibrium (high D′ values) with the significantly associated 677 genotype, presumably relates to the large differences in allele frequency between the two polymorphisms, as reflected by low r^2 values.

In two previous cross-sectional studies the 677C/T polymorphism is associated with metabolic syndrome following antipsychotic drug treatment (Ellingrod et al., 2008, 2012), although these authors find the 677T allele to be a risk factor, whereas we find a consistent effect of the 677T allele in protecting against antipsychotic drug-induced weight gain. This may well indicate the difference between effects on initial weight gain and its long-term consequences, in which differing pharmacogenetic influences are apparent (Reynolds et al., 2013). In another study the 1298A/C but not 677C/T polymorphism was associated with metabolic syndrome in schizophrenia (van Winkel et al., 2010a). The one previous longitudinal study of changes in weight and metabolic parameters following 3 months' treatment with second-generation antipsychotics also found an association with the 1298A/C but not 677C/T polymorphism (van Winkel et al., 2010b). This study differed from the present investigation of first-episode drug-naive patients in that weight but not BMI was measured, and the 104 patients were older (mean 31.3 yr) with first admission on average over 6yr previously; thus prior treatment may well have confounded subsequent weight gain. However, their finding that the 1298A allele is associated with less weight gain is not inconsistent with our finding given the close linkage disequilibrium between the two polymorphisms studied. As discussed by van Winkel et al. (2010b), there are no clinical or ethnic factors identified that may be responsible for the discrepancies between these findings, although it is notable that most studies were not powered to identify significant differences between the effects of the two closely linked polymorphisms. Nevertheless these various reports all indicate that functional genetic variation in MTHFR can influence antipsychotic drug-induced weight gain.

It is conceivable that pharmacogenetic associations such as that identified here may vary depending on the treatment regime. Different drugs may have differing mechanisms underlying their effect on body weight – certainly the greater effect of olanzapine over risperidone and several other antipsychotics supports this – and these pharmacological mechanisms may be differentially influenced by genetic polymorphisms. Our study was not powered to subdivide samples into treatment subgroups; however, further work needs to address the possible drug specificity of such pharmacogenetic findings.

There was no significant interaction between -759C/T of HTR2C and 677C/T of MTHFR on antipsychotic-induced weight gain, indicating that both polymorphisms exert independent influences on this side effect. However, the gene–gene analysis resulted in substantial increases in statistical significance, demonstrating an additive effect of the two polymorphisms. Clearly there are other genetic influences that are likely to contribute to determining initial weight gain associated with antipsychotic drug treatment, including polymorphisms for genes for leptin, melanocortin receptor 4, adrenoreceptor alpha2A and g-protein beta3 among probably many others (Reynolds, 2012).

The exact mechanism by which MTHFR polymorphisms might contribute to determining antipsychotic drug-induced weight gain is unclear. Both variant alleles of 677C/T and 1298A/C MTHFR polymorphisms cause decreased enzyme activity (Weisberg et al., 1998), although it is not easy to distinguish effects of two closely linked polymorphisms in vivo. MTHFR is an important enzyme in one-carbon metabolism and, via its role in DNA synthesis and methylation (Sugden, 2006), may influence gene expression (Jirtle and Skinner, 2007); such epigenetic effects could be involved in antipsychotic

drug-induced weight gain. Diminished levels of genomic DNA methylation (Stern et al., 2000) and gene-specific DNA methylation (Burghardt et al., 2012) have been reported to be associated with the 677TT genotype. It is therefore possible that decreased MTHFR enzyme activity in 677TT genotype results in decreased DNA methylation of genes involved in body weight regulation.

DNA methylation status is influenced by gene–nutrient interaction. It has been suggested that the MTHFR 677TT genotype affects DNA methylation status through an interaction with folate status (Friso and Choi, 2002). These authors found that genomic DNA methylation in peripheral blood mononuclear cells was directly correlated with folate status, inversely correlated with homocysteine levels, and only 677TT subjects with low folate accounted for decreased DNA methylation (Friso et al., 2002). Thus folate status in addition to the 677C/T MTHFR polymorphism might modulate DNA methylation of genes relating to the regulation of food intake, energy expenditure or body weight regulation, and thus could be an unexplored factor contributing to the variance in this and previous studies.

In conclusion, this present study indicates the association of the MTHFR 677C/T single polymorphism with weight gain following initial antipsychotic drug treatment in first-episode psychotic patients. Furthermore, the effect of the 677T allele appears to have a protective effect additional to that of the well-established HTR2C -759T allele against antipsychotic-induced weight gain. These two polymorphisms, in addition to several other possible genetic factors, might be valuable as pharmacogenetic markers of this important and limiting side effect.

Acknowledgment

Financial support for the study was provided by a PhD scholarship from the Royal Thai Government, Thailand to U. Srisawat.

Statement of Interest

G.P. Reynolds has received honoraria for educational lectures, advisory panel membership and/or travel support from the following pharmaceutical companies: Lundbeck, Janssen-Cilag, Otsuka and Sunovion.

References

Burghardt KJ, Pilsner JR, Bly MJ, Ellingrod VL (2012) DNA methylation in schizophrenia subjects: gender and MTHFR 677C/T genotype differences. Epigenomics 4:261–268.

Casey DE, Haupt DW, Newcomer JW, Henderson DC, Sernyak MJ, Davidson M, Lindenmayer JP, Manoukian SV, Banerji MA, Lebovitz HE, Hennekens CH (2004) Antipsychotic-induced weight gain and metabolic abnormalities: implications for increased mortality in patients with schizophrenia. J Clin Psychiatry 65 (Suppl. 7):4–18.

Chen AR, Zhang HG, Wang ZP (2010) C-reactive protein, vitamin B12 and C677T polymorphism of N-5,10-methylenetetrahydrofolate reductase gene are related to insulin resistance and risk factors for metabolic syndrome in Chinese population. Clin Invest Med 33:E290–E297.

De Hert M, Schreurs V, Vancampfort D, van Winkel R (2009) Metabolic syndrome in people with schizophrenia: a review. World Psychiatry 8:15–22.

Ellingrod VL, Miller DD, Taylor SF, Moline J, Holman T, Kerr J (2008) Metabolic syndrome and insulin resistance in schizophrenia patients receiving antipsychotics genotyped for the methylenetetrahydrofolate reductase (MTHFR) 677C/T and 1298A/C variants. Schizophr Res 98:47–54.

Ellingrod VL, Taylor SF, Dalack G, Grove TB, Bly MJ, Brook RD, Zöllner SK, Pop-Busui R (2012) Risk factors associated with metabolic syndrome in bipolar and schizophrenia subjects treated with antipsychotics: the role of folate pharmacogenetics. J Clin Psychopharmacol 32:261–265.

Friso S, Choi SW (2002) Gene-nutrient interactions and DNA methylation. J Nutr 132 (Suppl. 8):2382S–2387S.

Friso S, Choi SW, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, Olivieri O, Jacques PF, Rosenberg IH, Corrocher R, Selhub J (2002) A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci U S A 99:5606–5611.

Henderson DC, Cagliero E, Gray C, Nasrallah RA, Hayden DL, Schoenfeld DA, Goff DC (2000) Clozapine, diabetes mellitus, weight gain, and lipid abnormalities: a five-year naturalistic study. Am J Psychiatry 157:975–981.

Jirtle RL, Skinner MK (2007) Environmental epigenomics and disease susceptibility. Nat Rev Genet 8:253–262.

Kuzman MR, Müller DJ (2012) Association of the MTHFR gene with antipsychotic-induced metabolic abnormalities in patients with schizophrenia. Pharmacogenomics 13:843–846.

Lewis SJ, Lawlor DA, Davey Smith G, Araya R, Timpson N, Day IN, Ebrahim S (2006) The thermolabile variant of MTHFR is associated with depression in the British Women's Heart and Health Study and a meta-analysis. Mol Psychiatry 11:352–360.

Lunegova OS, Kerimkulova AS, Turdakmatov NB, Sovkhozova NA, Nabiev MP, Isakova ZhT, Iusupova ÉU, Moldokeeva ChB, Gotfrid Ilu, Mirrakhimov AÉ, Aldasheva NM, Kheĭer E, Aldashev AA, Mirrakhimov ÉM (2011) Association of C677T gene polymorphism of methylenetetrahydrofolate reductase with insulin resistance among Kirghizes. Kardiologiia 51:58–62.

Reynolds GP (2012) Pharmacogenetic aspects of antipsychotic drug-induced weight gain: a critical review. Clin Psychopharmacol Neurosci 10:71–77.

Reynolds GP, Zhang ZJ, Zhang XB (2002) Association of antipsychotic drug-induced weight gain with a 5-HT2C receptor gene polymorphism. Lancet 359:2086–2087.

Reynolds GP, Yevtushenko OO, Gordon S, Arranz B, San L, Cooper SJ (2013) The obesity risk gene FTO influences body mass in chronic schizophrenia but not initial antipsychotic drug-induced weight gain in first-episode patients. Int J Neuropsychopharmacol 16:1421–1425.

Stern LL, Mason JB, Selhub J, Choi SW (2000) Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. Cancer Epidemiol Biomarkers Prev 9:849–853.

Sugden C (2006) One-carbon metabolism in psychiatric illness. Nutr Res Rev 19:117–136.

Templeman LA, Reynolds GP, Arranz B, San L (2005) Polymorphisms of the 5-HT2C receptor and leptin genes are associated with antipsychotic drug-induced weight gain in Caucasian subjects with a first-episode psychosis. Pharmacogenet Genomics 15:195–200.

Terruzzi I, Senesi P, Fermo I, Lattuada G, Luzi L (2007) Are genetic variants of the methyl group metabolism enzymes risk factors predisposing to obesity? J Endocrinol Invest 30:747–753.

Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE (2001) Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci 22:195–201.

van Winkel R, Rutten BP, Peerbooms O, Peuskens J, van Os J, De Hert M (2010a) MTHFR and risk of metabolic syndrome in patients with schizophrenia. Schizophr Res 121:193–198.

van Winkel R, Moons T, Peerbooms O, Rutten B, Peuskens J, Claes S, van Os J, De Hert M (2010b) MTHFR genotype and differential evolution of metabolic parameters after initiation of a second generation antipsychotic: an observational study. Int Clin Psychopharmacol 25:270–276.

Weisberg I, Tran P, Christensen B, Sibani S, Rozen R (1998) A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 64:169–172.

Zhang ZJ, Yao ZJ, Liu W, Fang Q, Reynolds GP (2004) Effects of antipsychotics on fat deposition and changes in leptin and insulin levels. Magnetic resonance imaging study of previously untreated people with schizophrenia. Br J Psychiatry 184:58–62.

International Journal of Neuropsychopharmacology (2014), 17, 645–649. © CINP 2013
doi:10.1017/S1461145713001442

RAPID COMMUNICATION

Methylation at a transcription factor-binding site on the 5-HT1A receptor gene correlates with negative symptom treatment response in first episode schizophrenia

Hao Tang[1,2], Caroline F. Dalton[1], Umarat Srisawat[1], Zhi Jun Zhang[2] and Gavin P. Reynolds[1]

[1] Biomedical Research Centre, Sheffield Hallam University, Sheffield, UK

[2] Department of Neurology, ZhongDa Hospital and Institute of Neuropsychiatry, Southeast University, Nanjing, People's Republic of China

Abstract

Individual variability and inadequate response of negative symptoms are major limitations of antipsychotic treatment in schizophrenia. A functional polymorphism, rs6295, in the 5-HT1A-receptor gene (HTR1A) contributes to this variability in negative symptom response. The DNA sequence containing rs6295 is rich in cytosine methylation (CpG) sites; CpG methylation is an epigenetic factor that, like rs6295, can modify transcriptional control. To investigate whether DNA methylation influences response to antipsychotic treatment, we determined methylation at CpG sites close to rs6295 in DNA from 82 Chinese subjects with a first psychotic episode. Methylation of one CpG site within a recognition sequence for HES transcriptional repressors was found to correlate with changes in total PANSS score ($p=0.006$) and negative factor sub-score ($p<0.001$) following 10 wk initial antipsychotic treatment, as well as with baseline negative factor score ($p=0.019$); the effect on symptom change remained after correction for this baseline score. An effect of rs6295 on negative symptom response was not seen in this sample, which may not have provided sufficient power for the pharmacogenetic association. These preliminary results indicate that epigenetic modification of transcriptional regulation by specific cytosine methylation may modulate HTR1A expression, resulting in effects on emotional dysfunction and negative symptom response to antipsychotic treatment.

Received 16 August 2013; Reviewed 11 September 2013; Revised 8 October 2013; Accepted 30 October 2013; First published online 16 December 2013

Key words: Antipsychotic drugs, DNA methylation, HTR1A, negative symptoms, schizophrenia.

Introduction

Response to antipsychotic drug treatment shows substantial inter-individual variability, as well as a generally poorer improvement of negative than positive symptoms. In pharmacogenetic studies addressing this differential response, a replicated finding is the association of negative symptom response with the −1019C/G polymorphism (rs6295) in the 5-HT1A-receptor gene (HTR1A) (Reynolds et al., 2006; Wang et al., 2008; Mossner et al., 2009). This polymorphism, first found to be associated with depression and suicide (Lemonde et al., 2003), is also associated with response to antidepressant drugs (Lemonde et al., 2004; Yevtushenko et al., 2010). It is found to influence gene expression; the risk allele (G) disrupts the repressor activity of the DEAF1, HES1 and HES5 transcription factors, resulting in overexpression of the presynaptic 5-HT1A-receptor (Lemonde et al., 2003; Jacobsen et al., 2008).

Address for correspondence: Professor G. Reynolds, Biomedical Research Centre, Sheffield Hallam University, Howard St, Sheffield S1 1WB, UK.
Tel.: +44 7740 651500 Fax: +44 1142 254449
Email: gavin.reynolds@shu.ac.uk

Methylation of DNA sequences is another established influence on transcription factor binding (Sharma et al., 2010). Cytosine residues at CpG dinucleotide sequences are sites of DNA methylation, one of several epigenetic mechanisms for the modulation of DNA function. It is notable that rs6295 is found within an island of 13 CpG sites in the HTR1A promoter sequence; these adjacent methylation sites could conceivably contribute, along with rs6295, to modifying the binding of transcription factors such as DEAF1. We hypothesised that the percentage methylation of one or more CpG sites close to the rs6295 polymorphism in the promoter sequence of HTR1A may also influence symptom response to treatment with antipsychotic drugs.

Method

Chinese Han inpatients presenting with a first psychotic episode (45 male, 37 female; mean age 25.8 ± 7.1 years) participated in our study. All patients met criteria for a diagnosis of schizophrenia according to the Diagnostic and Statistical Manual of the American Psychiatric Association 4th Edition (DSM-IV). Exclusion criteria

included prior history of medication with antipsychotics, antidepressants or mood stabilisers, co-morbid DSM-IV diagnosis of substance abuse or dependence or other physical illness. Study subjects were treated according to standard clinical practice; initial treatment was with chlorpromazine (n=57), risperidone (n=18), clozapine (n=4) or fluphenazine (n=3) Drug treatment was reviewed after approximately 6 wk and modified as needed; this resulted in a further 26 subjects receiving clozapine: 21 from the chlorpromazine group and 5 from the risperidone group. Anticholinergic and benzodiazepine co-medication were administered as required for the alleviation of extrapyramidal symptoms and need for sedation, respectively. The Positive and Negative Syndrome Scale (PANSS) was used for assessment and evaluation of psychopathology and therapeutic response to antipsychotic treatment. All patients were assessed on the day of admission by a psychiatrist trained in the use of PANSS and subsequently reassessed after 10 wk of antipsychotic treatment. PANSS items were divided into five symptom factors according to the consensus scheme of Wallwork et al. (2012). The five factors are defined by the following PANSS items: Positive Factor: P1, P3, P5, G9; Negative Factor: N1, N2, N3, N4, N6, G7; Disorganised/Concrete Factor: P2, N5, G11; Excited Factor: P4, P7, G8, G14; Depressed Factor: G2, G3, G6. The Nanjing Brain Hospital Ethical Committee approved the study, and all patients gave written informed consent.

Genomic DNA, extracted from blood samples taken on the day of admission prior to initiation of drug treatment using a standard chloroform-phenol method, was bisulfite-modified to convert unmethylated cytosine residues to uracil using the EpiTect Fast Bisulfite Kit (UK) with a calculated mean conversion of 99%. For analysis, a sequence containing 13 CpGs in the HTR1A promoter (GRCh37.p10, Chromosome 5 bases 63258525–63258 684) including the rs6295 C/G polymorphism, (which enables/removes CpG12) was identified and amplified by PCR using primers, including a biotinylated reverse primer, as follows: 5′-AGTAAGGTTGGATTGTTAGATGA-3′ (forward) and 5′-[btn]CCTAAATCAATCTCCCAATTATTACTAA-3′ (reverse) (Eurofins MWG Operon). PCR reaction was carried out with 20 ng bisulfite-converted DNA using the PyroMark PCR kit (UK) in a final volume of 25 µl containing 12.5 µl 1x PyroMark PCR Master Mix, 2.5 µl 1x CoralLoad Concentrate, 1 µl of each primer in a final concentration of 0.05 µM, 7 µl RNase-free water. Amplification conditions were as follows: 95°C for 15 min, 45 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, finally, 72°C for 10 min. Methylation status of the sequence within the CpG island around rs6295, containing sites CpG9-13 (Fig. 1a), was determined with a PyroMark Q24 pyrosequencer (Qiagen UK) using 15–20 µl PCR product and employing a sequencing primer, 5′-TTTAGGTTGGAGTGTAATG-3′ (Eurofins MWG Operon). Pyrosequence setup and data

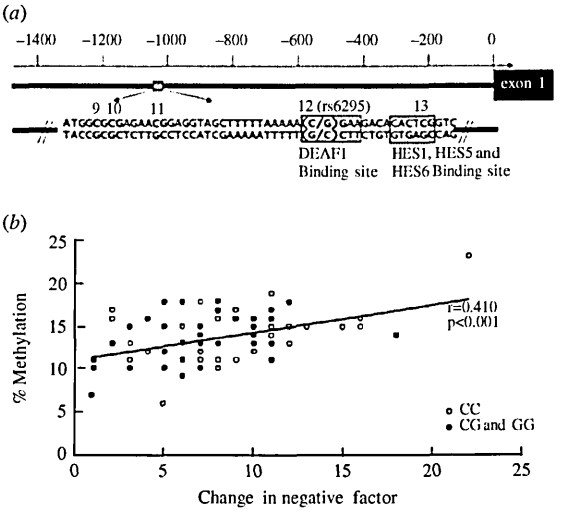


Fig. 1. (a) CpG sites (numbered 9–13) are shown in bold in the DNA sequence of HTR1A that underwent pyrosequencing, with the rs6295 polymorphism in brackets and binding sites for DEAF1 and HES transcription factors shown in frames. (b) Correlation between % methylation at CpG13 and improvement in the negative factor score.

reading were conducted by PyroMark Q24 2.0.6.20 software (UK). Samples underwent PCR and pyrosequencing in duplicate; any inconsistencies between samples were resolved following further repetition. Pyrosequencing also permitted determination of genotype for rs6295.

Data analysis was undertaken using SPSS version 16.0 (SPSS Inc., USA). Pearson's correlation was used to determine individual relationships between clinical measurements and percentage methylation. As we initially investigated five CpG sites adjacent to, and including that of, the rs6295 polymorphism for their correlation with symptom response, statistical significance was set at $p<0.01$ for a conservative Bonferroni correction; for subsequent, post-hoc analysis we applied $p<0.05$. Stepwise regression analysis was used to determine the influence of clinical and demographic factors on symptom measures. Power analysis demonstrated that the sample size was adequate to identify a medium effect size ($r=0.3$) with 80% power at $p<0.05$. Where shown, variance in data is expressed as standard deviation.

Results

Initial mean PANSS score on admission was 99.6±14.4; this reduced to 49.6±9.6 after 10 wk initial treatment with antipsychotic drugs. Mean methylation was determined at methylation sites CpG9, CpG10, CpG11 and CpG13 as 13.8, 18.1, 20.5 and 13.9%, respectively; at CpG12 (rs6295 C-allele carriers) it was 21.0%. Results for CpG9 and CpG13 each contained one extreme outlier ($z>5$) and were not normally distributed; removal of the two outlier data points normalised the distributions

Table 1. Methylation at CpG sites and their correlation with symptom changes after 10 wk' treatment with antipsychotic drugs

	CpG9 (n=81)		CpG10 (n=82)		CpG11 (n=82)		CpG12 at C allele (n=76)		CpG13 (n=81)	
Percentage methylation (mean±S.D.)	13.60±2.73		18.10±4.35		20.49±3.87		21.01±4.74		13.73±2.91	
Spearman's r and p values	r	p	r	p	r	p	r	p	r	p
Change in total PANSS	0.15	0.17	0.11	0.31	0.04	0.74	0.13	0.27	0.30	0.006
Changes in symptom factors										
Excited factor	0.25	0.024	0.15	0.18	0.08	0.49	0.07	0.53	0.16	0.17
Negative factor	0.09	0.41	0.11	0.35	0.08	0.49	0.13	0.26	0.41	0.000
Disorganised/concrete factor	0.01	0.92	−0.06	0.61	−0.08	0.46	−0.04	0.75	0.10	0.36
Positive factor	0.11	0.34	0.05	0.67	−0.02	0.88	−0.00	0.99	0.09	0.45
Depressed factor	0.15	0.17	0.17	0.13	0.09	0.40	0.07	0.53	0.21	0.065

(Shapiro-Wilk test; p=0.264, 0.234 respectively). Only methylation at CpG13 showed a significant correlation with clinical measures (Table 1). CpG13 methylation was significantly correlated with change in total PANSS (r=0.300, n=81, p=0.006). This reflected a highly significant positive correlation with change in the negative factor (r=0.410, p<0.001) (Fig. 1b). Stepwise linear regression indicated a significant effect of negative factor score before treatment on change in negative symptoms, but no significant effect of age, sex or whether patients received clozapine. A significant correlation with CpG13 methylation was also observed with this negative factor baseline score (r=0.261, p=0.019) but not with the baseline score of any other factor. After controlling for baseline negative symptom score the correlation between CpG13 methylation and change in negative symptoms remained highly significant (r=0.353, p=0.001). CpG13 methylation had a small effect on the change in the depressed factor (r=0.206, p=0.065); correlations with changes in other factors were also not significant (p>0.1).

The subjects were found to have the following rs6295 genotypes: 46 CC, 30 CG and 6 GG. Genotype had no significant influence on methylation of the invariant CpG sites, nor was genotype significantly related to PANSS measures of symptom response to treatment, including negative symptom response. Thus CC genotype and G allele carriers had mean values for change in negative score of 7.93±3.93 and 7.31±4.22, respectively. We investigated the relationship between CpG13 methylation and change in negative symptoms separately within two rs6295 genotype subgroups of the sample. The correlation remained in both the CC genotype (r=0.422, n=46, p=0.004) and the G carrier (r=0.421, n=35, p=0.012) subjects (Fig. 1b).

Discussion

In a hypothesis-driven search for effects of DNA methylation in the promoter region of a gene (HTR1A) known to be associated with symptom response to antipsychotic medication, we have identified a correlation of the change in negative symptoms following initial antipsychotic drug treatment with methylation, determined prior to onset of treatment, at a specific CpG site adjacent to the functional polymorphism rs6295. Thus epigenetic variation, as well as a genetic polymorphism, in a specific DNA sequence in the HTR1A promoter region can influence negative symptoms in schizophrenia and their subsequent response to antipsychotic drug treatment.

CpG13 is found within a recognition site for the repressor activity of transcription factors HES1 and HES5 (Fig. 1a); HES5 activity is also influenced by the nearby rs6295 allele (Jacobsen et al., 2008). The strong repressor activity of HES1 is thought to play an essential role in the developmental repression of HTR1A expression, while the inhibitory effect of HES6 on HES1, HES5 and DEAF1 repressor activity may regulate HTR1A expression (Jacobsen et al., 2008). Thus a methylation-induced variability in the balance between effects of these transcriptional regulators may modulate the developmental control of HTR1A transcription and its effects on affective circuitry (Richardson-Jones et al., 2011); leucocyte DNA methylation as determined here may well reflect epigenetic changes during, or even prior to, early development (Rosa et al., 2008). However, effects on expression of HTR1A in the adult may also be important; it is notable that a negative correlation between 5-HT1A-receptor binding potential in the amygdala and PANSS-derived negative and depression/anxiety symptom scores has been reported in drug-free schizophrenia (Yasuno et al., 2004). Certainly dysfunction of the amygdala and its connectivity with the prefrontal cortex are proposed to be involved in the emotional dysregulation underlying negative symptoms of schizophrenia (Aleman and Kahn, 2005).

By analogy with the association of rs6295 with antipsychotic drug response (Reynolds et al., 2006), we hypothesise that poor symptom response to drug treatment

is associated with loss of inhibitory control of HTR1A expression, with a consequent increase in 5-HT1A auto receptors and reduction in serotonin neurotransmission. That we find poorer response to be correlated with diminished methylation at CpG13, suggests that methylation might enhance transcriptional repressor activity, perhaps by recruiting methyl-binding repressor proteins. However, the finding of a greater reduction in amygdala 5-HT1A-receptors in patients with more severe negative symptoms (Yasuno et al., 2004), along with our observation of a positive correlation between CpG13 methylation and baseline negative factor score, would be consistent with an alternative view where greater methylation resulted in suppression of HTR1A expression. This could be brought about by disruption of the enhancer activity of DEAF1 on HTR1A expression that is found at post-synaptic sites (Czesak et al., 2006).

The 5-HT1A-receptor is increasingly being recognised as a potential target for antipsychotic drug action, particularly with respect to negative and cognitive symptoms of schizophrenia (Newman-Tancredi and Kleven, 2011). Certainly the 5-HT system is implicated in negative symptoms and their response to drugs; selective serotonin reuptake inhibitors as an adjunct to antipsychotic treatment can improve negative symptoms in some subjects (Silver, 2004). Our findings add further evidence for 5-HT1A receptor involvement in negative symptoms and response to antipsychotic drug treatment. These receptors mediate the action of atypical antipsychotic drugs on dopamine release in the frontal cortex (Diaz-Mataix et al., 2005), one mechanism proposed to underlie drug effects on negative symptoms (e.g. Ichikawa et al., 2001). Thus genetically or epigenetically determined differences in 5-HT1A-receptor density or its regulatory control may influence subsequent negative symptom response to antipsychotic drugs.

We recognise that the assessment of peripheral blood DNA, rather than DNA from brain tissue, is an inevitable limitation of our study, However, others have found psychiatric correlates of HTR1A methylation in blood DNA: methylation of the HTR1A proximal promoter region is reportedly increased in schizophrenia (Carrard et al., 2011). Notably, a site-specific hypomethylation of the 5-HT2A-receptor gene in schizophrenia and bipolar disorder is found in both brain- and saliva-derived DNA (Ghadirivasfi et al., 2011). We were unable to replicate in this sample the previously reported association of rs6295 with negative symptom response to treatment. This is not easily explained given that the original result was seen with a smaller sample (Reynolds et al., 2006), although the combination of sample size, lower G allele frequency and perhaps other factors associated with this Asian population may have contributed.

Nevertheless, the sample demonstrates a strong effect of methylation at the recognition site for transcription factors implicated in the effect of the rs6295 polymorphism on HTR1A gene expression (Jacobsen et al., 2008). This finding is replicated internally within the two genotype groups and represents a uniquely specific observation of an epigenetic factor relating to symptom response in the initial drug treatment of schizophrenia. As such the findings have an impact far beyond an understanding of the epigenetic influences on antipsychotic treatment response; as well as raising the question whether there might be an association of CpG13 methylation with schizophrenia, they immediately generate hypotheses relating to depression and antidepressant response, with which the adjacent HTR1A polymorphism has established associations (Lemonde et al., 2003; 2004).

Acknowledgments

No specific funding was received for this study. U. Srisawat is supported by a PhD scholarship from the Royal Thai Government, Thailand.

Statement of Interest

G.P. Reynolds has received honoraria for educational lectures, advisory panel membership and travel support from the following pharmaceutical companies: Lundbeck, Janssen-Cilag, Otsuka and Sunovion. No other authors have financial interests to disclose.

References

Aleman A, Kahn RS (2005) Strange feelings: do amygdala abnormalities dysregulate the emotional brain in schizophrenia? Prog Neurobiol 77:283–298.

Carrard A, Salzmann A, Malafosse A, Karege F (2011) Increased DNA methylation status of the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder. J Affect Disord 132:450–453.

Czesak M, Lemonde S, Peterson EA, Rogaeva A, Albert PR (2006) Cell-specific repressor or enhancer activities of Deaf-1 at a serotonin 1A receptor gene polymorphism. J Neurosci 26:1864–1871.

Diaz-Mataix L, Scorza MC, Bortolozzi A, Toth M, Celada P, Artigas F (2005) Involvement of 5-HT1A receptors in prefrontal cortex in the modulation of dopaminergic activity: role in atypical antipsychotic action. J Neurosci 25:10831–10843.

Ghadirivasfi M, Nohesara S, Ahmadkhaniha HR, Eskandari MR, Mostafavi S, Thiagalingam S, Abdolmaleky HM (2011) Hypomethylation of the serotonin receptor type-2A Gene (HTR2A) at T102C polymorphic site in DNA derived from the saliva of patients with schizophrenia and bipolar disorder. Am J Med Genet B Neuropsychiatr Genet 156B:536–545.

Ichikawa J, Ishii H, Bonaccorso S, Fowler WL, O'Laughlin IA, Meltzer HY (2001) 5-HT(2A) and D(2) receptor blockade increases cortical DA release via 5-HT(1A) receptor activation: a possible mechanism of atypical antipsychotic-induced cortical dopamine release. J Neurochem 76:1521–1531.

Jacobsen KX, Vanderluit JL, Slack RS, Albert PR (2008) HES1 regulates 5-HT1A receptor gene transcription at a functional

polymorphism: essential role in developmental expression. Mol Cell Neurosci 38:349–358.

Lemonde S, Turecki G, Bakish D, Du L, Hrdina PD, Bown CD, Sequeira A, Kushwaha N, Morris SJ, Basak A, Ou XM, Albert PR (2003) Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. J Neurosci 23:8788–8799.

Lemonde S, Du L, Bakish D, Hrdina P, Albert PR (2004) Association of the C(-1019)G 5-HT1A functional promoter polymorphism with antidepressant response. Int J Neuropsychopharmacol 7:501–506.

Mossner R, Schuhmacher A, Kuhn KU, Cvetanovska G, Rujescu D, Zill P, Quednow BB, Rietschel M, Wolwer W, Gaebel W, Wagner M, Maier W (2009) Functional serotonin 1A receptor variant influences treatment response to atypical antipsychotics in schizophrenia. Pharmacogenet Genomics 19:91–94.

Newman-Tancredi A, Kleven MS (2011) Comparative pharmacology of antipsychotics possessing combined dopamine D2 and serotonin 5-HT1A receptor properties. Psychopharmacology (Berl) 216:451–473.

Reynolds GP, Arranz B, Templeman LA, Fertuzinhos S, San L (2006) Effect of 5-HT1A receptor gene polymorphism on negative and depressive symptom response to antipsychotic treatment of drug-naive psychotic patients. Am J Psychiatry 163:1826–1829.

Richardson-Jones JW, Craige CP, Nguyen TH, Kung HF, Gardier AM, Dranovsky A, David DJ, Guiard BP, Beck SG, Hen R, Leonardo ED (2011) Serotonin-1A autoreceptors are necessary and sufficient for the normal formation of circuits underlying innate anxiety. J Neurosci 31:6008–6018.

Rosa A, Picchioni MM, Kalidindi S, Loat CS, Knight J, Toulopoulou T, Vonk R, van der Schot AC, Nolen W, Kahn RS, McGuffin P, Murray RM, Craig IW (2008) Differential methylation of the X-chromosome is a possible source of discordance for bipolar disorder female monozygotic twins. Am J Med Genet B Neuropsychiatr Genet 147B:459–462.

Silver H (2004) Selective serotonin re-uptake inhibitor augmentation in the treatment of negative symptoms of schizophrenia. Expert Opin Pharmacother 5:2053–2058.

Sharma RP, Gavin DP, Grayson DR (2010) CpG methylation in neurons: message, memory, or mask? Neuropsychopharmacology 35:2009–2020.

Wallwork RS, Fortgang R, Hashimoto R, Weinberger DR, Dickinson D (2012) Searching for a consensus five-factor model of the positive and negative syndrome scale for schizophrenia. Schizophr Res 137:246–250.

Wang L, Fang C, Zhang A, Du J, Yu L, Ma J, Feng G, Xing Q, He L (2008) The –1019 C/G polymorphism of the 5-HT(1)A receptor gene is associated with negative symptom response to risperidone treatment in schizophrenia patients. J Psychopharmacol 22:904–909.

Yasuno F, Suhara T, Ichimiya T, Takano A, Ando T, Okubo Y (2004) Decreased 5-HT1A receptor binding in amygdala of schizophrenia. Biol Psychiatry 55:439–444.

Yevtushenko OO, Oros MM, Reynolds GP (2010) Early response to selective serotonin reuptake inhibitors in panic disorder is associated with a functional 5-HT1A receptor gene polymorphism. J Affect Disord 123:308–311.