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The mechanisms for reducing olanzapineinduced weight gain/obesity by betahistine: clinical implications

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THE MECHANISMS FOR REDUCING OLANZAPINE-INDUCED WEIGHT GAIN/OBESITY BY BETAHISTINE: CLINICAL IMPLICATIONS

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ABSTRACT

Olanzapine, a second generation antipsychotic drug, is widely used to treat multiple domains of schizophrenia and other mental disorders. However, it is associated with substantial body weight gain/obesity side-effects. Since the antagonistic affinity to histaminergic H_1 receptor (H_1R) has been identified as a major contributor for antipsychotic-induced weight gain, this thesis investigated the effects and mechanisms of co-treatment with betahistine (a histaminergic H_1R agonist and H_3 receptor antagonist) for ameliorating olanzapine-induced weight gain/obesity in a series of four experiments using a female rat model.

The first experiment showed that short-term (2 weeks) combination treatment of betahistine and olanzapine (O+B) reduced (-45%) body weight gain and feeding efficiency caused by olanzapine in drug-naïve rats. Olanzapine significantly upregulated expressions of H_1R, Neuropeptide Y (NPY), and AMP-activated protein kinase α (AMPKα) phosphorylation, that were reversed by O+B co-treatment. Hypothalamic pro-opiomelanocortin (POMC) expression was decreased by olanzapine, but not affected by O+B co-treatment. These results suggest that O+B co-treatment may reduce olanzapine-induced weight gain *via* the H_1R-NPY and H_1R-pAMPKα pathways.

Since patients suffering with schizophrenia and other mental disorders often face chronic, even life-time, antipsychotic treatment, I further investigated effects of chronic O+B co-treatment on preventing olanzapine-induced weight gain. Chronic co-administration of O+B significantly reduced (-51.4%) weight gain, feeding efficiency, liver and fat mass induced by olanzapine. Consistently, the chronic olanzapine-only

treatment increased expressions of hypothalamic H_1R, pAMPKα and NPY, while reducing uncoupling protein 1 (UCP_1) and peroxisome proliferator-activated receptor gamma coactivator1-alpha (PGC-1a) levels in brown adipose tissue. These olanzapine-induced changes could be reversed by chronic O+B co-treatment.

Following experiments investigated the effects of O+B co-treatment on the primary therapeutic receptor binding sites of olanzapine in various brain regions. Both short-term olanzapine-only and O+B co-treatment significantly decreased 5-HT_{2A} receptor (5-HT_{2A}R) bindings in the prefrontal cortex (PFC), cingulate cortex (Cg), and nucleus accumbens (NAc), but had no effects on dopamine D_2 receptors (D_2R). Olanzapine also significantly decreased 5-HTT bindings in the ventral tegmental area (VTA) and substantia nigra (SN). The results confirmed the important role of 5-HT_{2A}R in the efficacy of olanzapine, which was not influenced by short-term O+B co-treatment.

Both chronic olanzapine-only and O+B co-treatment significantly decreased the bindings of 5-HT_{2A}R, 5-HT_{2C}R, and 5-HTT in the PFC, Cg and NAc. The chronic olanzapine-only treatment significantly increased the D_2R bindings in the Cg, NAc, and CPu (which might be attributed to "dopaminergic supersensitivity"), while the chronic betahistine-only treatment reduced D_2R bindings. Chronic O+B co-treatment reversed the D_2R bindings in the NAc and CPu that were increased by chronic olanzapine treatment. Therefore, chronic O+B co-treatment has similar effects on serotonin neurotransmission as olanzapine-only treatment, but reverses the D_2R binding that is upregulated by chronic olanzapine treatment.

In brief, this thesis provided sound evidence that both short-term and chronic co-treatment with betahistine would be effective combination therapy to reduce olanzapine-induced weight gain without affecting its therapeutic effects. These results support further clinical trials to test the effectiveness of betahistine co-treatment for controlling weight gain/obesity side-effects in schizophrenia patients with antipsychotic treatment.

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STATEMENT FOR THE STYLE OF THE THESIS

In accordance with the University of Wollongong thesis committee "Guidelines for Preparation and Submission of HDR theses" (2014) and "Higher Degree Research (HDR) Thesis by Compilation Rules" (2014), this PhD thesis is presented in "Journal Article Compilation Style Format". This comprises a series of four original studies published in peer-reviewed journals, including *Psychoneuroendocrinology, PloS One* and *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. I am the first author of the four publications. I hereby declare that I am the primary designer of these studies, and have carried out all experiments, data analysis and manuscript preparation.

Jiamei Lian

2014

I consent to the presentation of this PhD in 'Journal Article Style' and I acknowledge the above statement pertaining to student contribution to be correct.

Associate Professor Chao Deng, Principal Supervisor

2014

LIST OF PUBLICATIONS INCLUDED AS PART OF THE THESIS

The following four refereed journal papers are included as part of the thesis:

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Lian J., Huang X-F., Pai N., Deng C. (2014) Betahistine ameliorates olanzapine-induced weight gain through modulation of histaminergic, NPY and AMPK pathways. *Psychoneuroendocrinology*. 48:77-86.

Lian J., Huang X-F., Pai N., Deng C. (2014) Preventing olanzapine-induced weight gain using betahsitine: a study in a rat model with chronic olanzapine treatment. *PLoS ONE*. 9(8): e104160.

Lian J., Huang X-F., Pai N., Deng C. (2013) Effects of olanzapine and betahistine co-treatment on serotonin transporter, 5-HT_{2A} and dopamine D_2 receptor binding density. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. 47:62-68.

Other publications and presentations related to the thesis:

Published Abstract

Lian J., Huang X-F., Pai N., Deng C. (2012) Preventing and treating olanzapine-induced obesity with betahistine: a chronic animal model study. Collegium Internationale Neuro-Psychopharmacoloicum XXVI World Congress, Stockholm, Sweden, 3-7th June 2012. *International Journal of Neuropsychopharmacology*. 15 (S1): 56.

Conference Oral Presentation

Lian J., Huang X-F., Pai N., Deng C. (2014) Betahistine Ameliorates Olanzapine-Induced Weight Gain through Histaminergic, NPY and AMPK Pathways. *Biological Psychiatry Australia*, 13rd-14th October, Melbourne, Australia. Oral presentation.

Conference Proceedings

Lian J., Huang X-F., Pai N., Deng C. (2014) Preventing olanzapine-induced obesity with betahistine: a chronic animal model study. *Australian Neuroscience Society 34th Meeting*, 28th-31st January, Adelaide, Australia.

Lian J., Huang X-F., Pai N., Deng C. (2013) Long term effects of olanzapine and betahistine on serotonin 5-HT_{2A} receptor binding in the rat brain. *Australian Neuroscience Society 33rd Meeting*, 3rd-6th February, Melbourne, Australia, p. 171.

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Additional Publications from other projects that I have involved in during my doctoral studies:

Publications in Referred Journals

Zhang Q., He M., Deng C., Wang H., **Lian J.**, Huang X-F. (2014) Hypothalamic ghrelin signalling mediates olanzapine induced hyperphagia and weight gain in female rats. *The International Journal of Neuropsychopharmacology*. 17 (5): 807-818.

Zhang Q., **Lian J.**, He M., Deng C., Wang H., Huang X-F. (2014) Olanzapine reduced brown adipose tissue thermogenesis and locomotor activity in female rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 51: 172-180.

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Conference Proceedings

Deng C., **Lian J.** (2014) Differential effects of antipsychotics on serotonin 5-HT_{2A}R, $5HT_{2C}$R, 5HTT binding density in the brain of male and female juvenile rats. *Society for Neuroscience*, 15^{th}-19^{th} November, 2014, Washington, DC, USA.

Deng C., **Lian J.**, De Santis M., Huang X-F (2013) Differential effects of antipsychotic drugs on food intake, weight gain and locomotor activity in male and female juvenile rats. In: *Society for Neuroscience*, 9^{th}-13^{rd} November, 2013 San Diego, California, USA.

He M., Zhang Q., Wang H.Q., **Lian J**., Deng C., Huang X-F. (2013) Central administration of histamine H_1 receptor agonist, FMPH, blocks olanzapine-induced

activation of AMPK-CPT_1 signaling in the dorsal vagal complex in rats. *Australian Neuroscience Society 33rd Meeting*, 3-6 February 2013, Melbourne, Australia, p 120.

Zhang, Q., He, M., Wang, H., **Lian, J**., Deng, C., Huang, X-F. (2013). Brown adipose tissue thermogenesis is time-dependently downregulated by olanzapine in rats. *Proceedings of the 31st Annual Scientific Meeting of the Obesity Society*, November 2013, Atlanta, GA, USA: T-715-P.

Zhang, Q., He, M., Wang, H., **Lian, J**., Deng, C., Huang, X-F. (2013). Time-dependent ghrelinergic signaling and hyperphagia induced by olanzapine. *Australian Neuroscience Society 33rd Meeting*, 3-6 February 2013, Melbourne, Australia, p 48.

Zhang, Q., He, M., Wang, H., **Lian, J.**, Deng, C., Huang, X-F. (2012). Time-dependant alterations of hypothalamic energy regulatory network by olanzapine in rats. *Australian Neuroscience Society 32nd Meeting*, 29th January-1st February 2012, Gold Coast, Australia, p 150.

He M., Zhang Q., Wang H.Q., **Lian J**., Deng C., Huang X-F. (2012) Olanzapine treatment and time-dependent changes of hypothalamic AMPK-ACC-CPT_1 signalling, food intake and body weight in rats. *Australian Neuroscience Society 32nd Meeting*, 29th January-1st February 2012, Gold Coast, Australia, p 148.

STATEMENT OF CONTRIBUTION OF OTHERS

I, Jiamei Lian, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, University of Wollongong, is entirely my own work unless otherwise referenced or acknowledged. Three co-authors (Chao Deng, Xu-Feng Huang, and Nagesh Pai) of the four journal articles included in the thesis are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts.

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LIST OF ABBREVIATIONS

5-HT	Serotonin
5-HT_{1B}R	5-HT_{1B} receptor
5-HT_{2A}R	Serotonergic 5-HT_{2A} receptor
5-HT_{2C}R	Serotonergic 5-HT_{2C} receptor
5-HTT	Serotonergic 5-HT transporter
α_{1A}	Adrenergic α_{1A} receptor
α_{2A}	Adrenergic α_{2A} receptor
α-MSH	Alpha-melanocyte stimulating hormone
ACC	Acetyl-CoA carboxylase
ACTH	Adrenocorticotrophin
AgRP	Agouti-related protein
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
Arc	Arcuate nucleus
BAT	Brown adipose tissue
BMI	Body mass index
CART	Cocaine-and amphetamine-regulated transcript
CATIE	Clinical Antipsychotic Trials of Intervention Effectiveness
Cg	Cingulate cortex
CNS	Central nervous system
CPT_1	Carnitine palmitoyltransferase 1
CPu	Caudate putamen

D_2R	Dopaminergic D_2 receptor
db/db	Leptin receptor mutation
DMN	Dorsomedial nucleus
DVC	Dorsal vagal complex
EPS	Extrapyramidal symptoms
EUFEST	European First Episode Schizophrenia Trial
FGAs	First generation antipsychotics
FMPH	2-(3-trifluoromethylpehenyl)histamine
GABA	Gamma-aminobutyric acid
HDC	Histidine decarboxylase
HIP	Hippocampus
H_1R	Histaminergic H_1 receptor
H_3R	Histaminergic H_3 receptor
ICV	Intracerebroventricular
LH	Lateral hypothalamus
M_3R	Muscarinic M_3 receptor
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NAcC	Nucleus accumbens core
NAcS	Nucleus accumbens shell
NIH	National Institutes of Health
NPY	Neuropeptide Y
NPYRs	NPY receptors
O+B	Olanzapine and betahistine
ob/ob	Leptin deficiency

pAMPK	AMPK phosphorylation
PET	Positron emission tomography
PFC	Prefrontal cortex
PFO	Perifornical areas
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator1-alpha
PGC-1β	Peroxisome proliferator-activated receptor gamma coactivator1-beta
POMC	Pro-opiomelanocortin
PVN	Paraventricular neucleus
SD	Sprague Dawley
SGAs	Second generation antipsychotic drugs
SN	Substantia nigra
SNS	Sympathetic nervous system
SPECT	Single photon emission computed tomography
TD	Tardive dyskinesia
t.i.d.	Three times daily
TMN	Tuberomammilary nucleus
UCP_1	Uncoupling protein 1
VMH	Ventromedial hypothalamic nucleus
VTA	Ventral tegmental area
WAT	White adipose tissue

CHAPTER 1

GENERAL INTRODUCTION

Schizophrenia is a devastating mental disorder influencing functions of the central nervous system (van Os and Kapur, 2009). As one of the first line second generation antipsychotic drugs (SGAs), olanzapine is widely used to treat multiple domains of schizophrenia symptoms and other serious mental disorders (Meltzer, 2013). However, it is associated with substantial body weight gain/obesity and other troublesome metabolic side-effects such as type II diabetes and cardiovascular disease (Coccurello and Moles, 2010; Deng, 2013). The antagonistic affinity to histaminergic H_1 receptor (H_1R) of SGAs has been identified as one of the main contributors to weight gain/obesity side-effects, suggesting H_1R as a potential target for controlling SGA-induced weight gain side-effects (Dwyer et al., 2005; Deng et al., 2010). Therefore, this thesis investigated the effects and mechanisms of co-treatment with betahistine (a histaminergic H_1R agonist and H_3 receptor antagonist) for ameliorating olanzapine-induced weight gain/obesity in a female rat model.

The study in Chapter 3 showed that a short-term (2 weeks) combination treatment of betahistine (2.67 mg/kg, t.i.d.) and olanzapine (1 mg/kg, t.i.d.) (O+B) reduced (-45%) body weight gain induced by olanzapine in drug-naïve rats. To reveal the mechanisms underlying these effects, a number of experiments were performed to investigate the effects of co-treatment of O+B on the expressions of H_1R, AMP-activated protein kinase (AMPK), neuropeptide Y (NPY), and proopiomelanocortin (POMC) in the hypothalamus associated with reducing olanzapine-induced weight gain. Olanzapine

significantly upregulated mRNA and protein expressions of H_1R, while O+B co-treatment significantly downregulated H_1R levels, compared to the olanzapine-only treatment group. NPY mRNA expression was significantly enhanced by olanzapine, but it was significantly reversed by O+B co-treatment. Hypothalamic H_1R expression was positively correlated with total food intake, and NPY expression. Olanzapine also increased AMPKα activation measured by the AMPKα phosphorylation (pAMPKα)/AMPKα ratio compared with controls, whereas O+B co-treatment decreased the pAMPKα/AMPKα ratio, compared with olanzapine only treatment. The pAMPKα/AMPKα ratio was positively correlated with total food intake and H_1R expression. Although olanzapine administration decreased the POMC mRNA level, this level was not affected by O+B co-treatment. Therefore, these results suggested that co-treatment (2 weeks) with betahistine may reverse olanzapine-induced body weight gain *via* the H_1R-NPY and H_1R-pAMPKα pathways.

Another key issue is that clinical patients suffering with schizophrenia, bipolar disease and other mental disorders often face chronic, even life-time, antipsychotic treatment, in which they have often had previous antipsychotic exposure (Maayan et al., 2010). Therefore, in Chapter 4, we investigated the effects of chronic (5 weeks) O+B co-treatment in controlling body weight in female rats with chronic and repeated exposure to olanzapine. Rats were treated with olanzapine (1 mg/kg, t.i.d.) or vehicle for 3.5 weeks, and then olanzapine treatment was withdrawn for 19 days. From week 6, the two groups were divided into 4 groups (n=12) for 5 weeks' treatment: (1) olanzapine-only (1 mg/kg, t.i.d.), (2) betahistine-only (9.6 mg/kg, t.i.d.), (3) olanzapine and betahistine co-treatment (O+B), and (4) vehicle. The results showed that 5 weeks co-administration of O+B significantly reduced (-51.4%) weight gain induced by olanzapine. Co-treatment

of O+B also led to a decrease in feeding efficiency, liver and fat mass. Consistently, the olanzapine-only treatment increased hypothalamic H_1R protein levels, as well as hypothalamic pAMPKα, AMPKα and NPY protein levels, while reducing hypothalamic POMC, and uncoupling protein 1 (UCP_1) and peroxisome proliferator-activated receptor gamma coactivator1-alpha (PGC-1a) protein levels in brown adipose tissue (BAT). The olanzapine induced changes in hypothalamic H_1R, pAMPKα, BAT UCP_1 and PGC-1a could be reversed by co-treatment of O+B. These results supported further clinical trials to test the effectiveness of co-treatment of O+B for controlling weight gain/obesity side-effects in schizophrenia with chronic antipsychotic treatment.

The study presented in Chapter 5 investigated the effects of short-term (2 weeks) co-treatment of O+B on the primary therapeutic receptor binding sites of olanzapine (Meltzer, 2013), that are serotonergic 5-HT_{2A} receptor (5-HT_{2A}R), 5-HT transporter (5-HTT) and dopaminergic D_2 receptor (D_2R) bindings in various brain regions involved in antipsychotic efficacy including the prefrontal cortex (PFC), cingulate cortex (Cg), nucleus accumbens (NAc), and caudate putamen (CPu) (using samples from Chapter 3 experiments). Quantitative autoradiography was used to detect the density of [^{3}H]ketanserin, [^{3}H]paroxetine and [^{3}H]raclopride binding sites to 5-HT_{2A}R, 5-HTT and D_2R. Compared to the controls, olanzapine significantly decreased [^{3}H]ketanserin bindings to 5-HT_{2A}R in the PFC, Cg, and NAc. Similar changes in 5-HT_{2A}R bindings in these nuclei were also observed in the O+B co-treatment group. Olanzapine also significantly decreased [^{3}H]paroxetine binding to 5-HTT in the ventral tegmental area (VTA) and substantia nigra (SN), however, neither olanzapine only nor O+B co-treatment affected [^{3}H]raclopride binding to D_2R. The results confirmed the important

role of 5-HT_{2A}R in the efficacy of olanzapine, which is not influenced by O+B co-treatment.

The study in Chapter 6 investigated the effects of chronic (5 weeks) treatment of olanzapine and/or betahistine on the binding density of serotonergic 5-HT_{2A}R and 5-HT_{2C}R, 5-HTT, and dopaminergic D_2R in the PFC, Cg, NAc, and CPu. Compared to the control, the olanzapine-only treatment significantly decreased the bindings of 5-HT_{2A}R, 5-HT_{2C}R, and 5-HTT in the PFC, Cg and NAc. Similar changes were observed in the rats receiving the O+B co-treatment. The olanzapine-only treatment significantly increased the D_2R binding in the Cg, NAc, and CPu, while the betahistine-only treatment reduced D_2R binding. Co-treatment with betahistine reversed the D_2R bindings in the NAc and CPu that were increased by olanzapine. Therefore, chronic O+B co-treatment has similar effects on serotonin neurotransmission as the olanzapine-only treatment, but reverses the D_2R that is upregulated by chronic olanzapine treatment.

To sum up, this thesis systematically revealed the mechanisms of co-treatment with betahistine in reducing olanzapine-induced body weight gain *via* modulation of the hypothalamic H_1R-AMPKα, NPY, and BAT UCP_1-PGC-1α pathways. Understanding the mechanisms of betahistine in the prevention and treatment of olanzapine-induced obesity through these signalling pathways will potentially lead to a new treatment strategy for schizophrenia with the development of more effective antipsychotic drugs with fewer side-effects. On the other hand, in both short-term/drug-naïve and chronic/drug-repeated treatment subjects, because both olanzapine-only and O+B co-treatment have similar effects in attenuating 5-HT_{2A}R, 5-HT_{2C}R and 5-HTT levels, betahistine may be safely co-administered with olanzapine without influencing

olanzapine's therapeutic action on serotonin neurotransmission. Additionally, since chronic olanzapine treatment with betahistine can reverse the elevated D_2R binding caused by chronic olanzapine treatment, co-treatment with betahistine may improve therapeutic effects by preventing the "dopaminergic supersensitivity" caused by chronic antipsychotic treatment. These results provided solid evidence supporting further clinical trials in treating antipsychotics-induced weight gain using betahistine in patients with schizophrenia and other mental disorders.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Schizophrenia remains a chronic, severe and complicated psychotic disorder impairing the function of the central nervous system (CNS), and is one of the most costly diseases to sufferers and their families (Tandon et al., 2008; van Os and Kapur, 2009). Broadly, it is characterised by positive symptoms (such as delusions, hallucinations), negative symptoms (such as apathy, avolition and poverty of speech), as well as cognitive deficits (such as deficits in memory, attention and executive function) (van Os and Kapur, 2009). The onset of the disorder is normally during late adolescence or early adulthood (Laruelle et al., 2003; Robinson et al., 2004), with a world-wide prevalence of 1-2% in the general population (McGrath et al., 2003; Perala et al., 2007). The significant medical co-morbidity and mortality of schizophrenia may shorten the average life-span by 10-30 years (Goff et al., 2005). It is believed that factors such as genetics and environmental vulnerability can affect multiple neurotransmitter systems, such as the dopaminergic, glutamatergic and muscarinic systems, which cause schizophrenia (Lieberman, 2006; Deng and Dean, 2013). In order to attenuate the symptoms of schizophrenia, pharmacological interventions, psychosocial rehabilitation and nutritional supplements have been supplied (Kohler et al., 2014). To date, pharmacological intervention using antipsychotic drugs plays the most critical role in schizophrenia treatment. Unfortunately, current antipsychotic drugs have limited efficacy for treating this complex disease (Meltzer, 2013), but cause some serious side-effects (Deng, 2013; Werner and Covenas, 2014).

Antipsychotic drugs have brought a significant improvement in the treatment of schizophrenia since the 1950s, and other psychiatric disorders, and can be broadly classified into two generations. First generation antipsychotic drugs (FGAs), also called "typical antipsychotics", such as chlorpromazine and haloperidol, can ameliorate the positive symptoms such as delusions and hallucinations although they are less effective on the negative symptoms such as apathy, avolition, and cognitive deficits of schizophrenia. Indeed, FGAs work mostly by blocking the dopaminergic D_2 receptor (D_2R) (Seeman, 2011; Ginovart and Kapur, 2012). However, D_2R blockade by FGAs also causes extra-pyramidal symptoms (EPS) side-effects, such as tardive dyskinesia and akathisia, as well as hyperprolactinemia and body weight gain to some extent, which is problematic for long-term use (Bishara and Taylor, 2008). For example, clinical studies demonstrated that 1 year of haloperidol treatment in first-episode patients led to a substantial weight gain side-effects (between 7.3 and 9.56 kg) (Kahn et al., 2008; Perez-Iglesias et al., 2008), although haloperidol was originally believed to have far fewer body weight gain side-effects compared with second generation antipsychotics (Bobes et al., 2003b; Tardy et al., 2014).

Second generation antipsychotic drugs (SGAs), also called "atypical antipsychotics", such as olanzapine and clozapine currently form the first line of treatment for schizophrenia and other serious mental disorders, and are effective to some degree in relieving the positive and negative symptoms, as well as cognitive deficits of schizophrenia, with fewer EPS side-effects at clinically effective doses (Kane and Correll, 2010; Lambert, 2011; Meltzer, 2013). Interestingly, meta-analysis showed that SGAs are not a homogeneous class of drugs and are associated with distinct efficacy

and side-effects profiles (Leucht et al., 2009; Kane and Correll, 2010). The pharmacological properties of SGAs are predominantly potent as a serotonergic 5-HT_{2A} receptor (5-HT_{2A}R) antagonist, and dopaminergic D_2R antagonist, as well as, in some cases such as aripiprazole, as D_2R and 5-HT_{1A} receptor (5-HT_{1A}R) partial agonists (DeLeon et al., 2004). SGAs normally cause serious metabolic side-effects, especially weight gain and obesity. Among the SGAs, olanzapine and clozapine are associated with the most severe weight gain/obesity side-effects, and with other prominent metabolic diseases such as dyslipidaemia, gluco-regulatory abnormalities and insulin resistance, and type II diabetes (Milano et al., 2013), and are currently of great interest to clinicians due to their widespread use in clinics (Correll et al., 2011; Osuntokun et al., 2011). More importantly, these side-effects are associated with relapse of psychosis due to non-compliance, increased morbidity and mortality, as well as reduced quality of life (Lieberman et al., 2005; Spelman et al., 2007). However, to date, there is no effective way to prevent or treat SGA-induced weight gain/obesity side-effects.

Accumulated evidence has demonstrated that antipsychotics effects encompass a wide range of non-dopaminergic G-protein-coupled receptors including histaminergic H_1 (H_1R), serotonergic 5-HT_{2C} (5-HT_{2C}R), and muscarinic M_3 (M_3R) receptors, contributing to weight gain/obesity side-effects (Harris et al., 2013) (Table 2.1). Among them, H_1R antagonism has been identified as one main indicator for predicting weight gain-induced by SGAs (Kroeze et al., 2003; Deng et al., 2010). As a potential target for treating weight gain side-effects, therefore, this PhD study investigated whether co-treatment with betahistine (an H_1R agonist and H_3 receptor (H_3R) antagonist) can ameliorate olanzapine-induced weight gain, and also elucidated the underlying mechanisms, using the established animal models. Furthermore, it has also detected

whether co-treatment with betahistine affects the key receptor binding sites (e.g. 5-HT_{2A}R and D_2R) involved in the therapeutic effects of olanzapine.

2.2 Literature Review

2.2.1 Neuropharmacological mechanisms of therapeutic efficacy of antipsychotics

SGAs, including olanzapine and clozapine, have a pharmacological profile with various antagonistic and/or agonistic binding affinities with various neurotransmitter receptors such as serotonergic (5-hydroxytryptamine, 5-HT), dopaminergic, muscarinic, adrenergic and histaminergic receptors, which may play a significant role in their therapeutic efficacy and side-effects (Fulton and Goa, 1997; Meltzer, 1999; Milano et al., 2013; Urs et al., 2014) (Table 2.1). It has been proposed that the interaction between 5-HT and dopamine systems, also called the "serotonin-dopamine hypothesis", plays a significant role in the therapeutic action of SGAs, in that most SGAs such as olanzapine have greater affinity for 5-HT_{2A} (5-HT_{2A}R) compared with dopamine D_2 (D_2R) receptors (Kuroki et al., 2008; Meltzer and Massey, 2011). On the other hand, 5-HT_{2C}R, histaminergic H_1 receptor (H_1R) and muscarinic M_3R have been reported to be involved in SGA-induced weight gain and other metabolic side-effects (Deng et al., 2010; Correll et al., 2011; Roerig et al., 2011).

Table 2.1 Weight gain and receptor binding affinities for antipsychotics.

Receptor	Haloperidol	Clozapine	Olanzapine	Quetiapine	Risperidone	Aripiprazole
5-HT$_{2C}$	10,000	17	6.8	2,502	35	22.4
5-HT$_{2A}$	53	5.4	2	101	0.17	8.7
D$_2$	4	256	34	245	6.5	0.66
H$_1$	**1,800**	**1.2**	**2**	**11**	**15**	**29.7**
H$_3$	>10,000	6,357	3,713	>10,000	>10,000	>10,000
M$_3$	10,000	25	105	10,000	10,000	4,677
α$_{1A}$	12	1.64	115	22	5	26
α$_{2A}$	1,130	142	314.1	3,630	150.8	74
Weight Gain (kg/10wks)	0.48	4.00	**3.51**	2.61	1.67	0.71

The receptor affinity values were reported as Ki (nM). 5-HT$_{2C}$, serotonin$_{2C}$; 5-HT$_{2A}$, serotonin$_{2A}$; $\alpha_{1A,}$ adrenergic α_{1A}; $\alpha_{2A,}$ adrenergic α_{2A}; D_2, dopamine$_2$; H_1, histamine$_1$; M_3, muscarinic$_3$. (Data adapted from Allison et al., 1999, Kroeze et al., 2003). For histamine$_3$ (H_3) receptors, Ki determination was generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # NO1MH32004 (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscol at NIMH, Bethesda MD, USA.

2.2.1.1 The role of dopamine neurotransmission in antipsychotic efficacy

From the 1960s, abnormal dopaminergic signalling has been recognised as a key contributor in the pathophysiology of schizophrenia (van Rossum, 1966). It was reported in the 1970s that utilizing competition binding experiments with [^{3}H]haloperidol and [^{3}H]dopamine, the property of all antipsychotics was attributed to their ability to bind with dopamine receptors in striatal homogenates (Creese et al., 1976; Seeman et al., 1976). The "dopamine hypothesis of schizophrenia" supposed the hyperactivity of dopaminergic neurotransmission at D_2R, which was proven in both clinic and animal models *via* examining the ability of dopamine agonist, amphetamine, in stimulating dopamine release (Miyamoto et al., 2003; Tenn et al., 2003).

There are four major dopaminergic neuron projections that are derived from the mesencephalon: 1) the mesolimbic pathway, dopaminergic neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), which is related to the positive symptoms of schizophrenia; 2) the mesocortical pathway, dopaminergic neurons projecting from the VTA to the frontal cortex including the prefrontal cortex (PFC) and cingulate cortex (Cg), which is associated with the negative symptoms and cognitive deficits of schizophrenia; 3) the nigrostriatal pathway, dopaminergic neurons projecting from the substantia nigra (SN) to the caudate putamen (CPu), which is involved in motor control; and 4) the tuberoinfundibular pathway, dopaminergic neurons projecting from the hypothalamus to the pituitary gland, which is associated with hyperprolactinaemia side-effects of FGAs (Ginovart and Kapur, 2012; Russo and Nestler, 2013).

Dopaminergic D_2Rs are also expressed at the presynaptic level as autoreceptors that regulate the synthesis and release of dopamine, as well as the firing of dopamine neurons (Ginovart and Kapur, 2012). It has been revealed that D_2R performs its physiological function throughout both G-protein-dependent and independent (the scaffolding protein β-arrestin 2-dependent) signalling (Beaulieu et al., 2005; Miyamoto et al., 2012). D_2R occupancy plays a critical role in predicting antipsychotic responses and side-effects, which has been supported using PET (positron emission tomography) and SPECT (single photon emission computed tomography) (Remington and Kapur, 1999; Seeman, 2011; Ginovart and Kapur, 2012). It was demonstrated that for FGAs, antipsychotic efficacy requires 65-70% D_2R occupancy, while the >80% D_2R occupancy significantly increased the risk of EPS side-effects (Remington and Kapur, 1999).

Although SGAs target multiple receptors (particularly 5-HT_2R) and have reduced EPS side-effects (but cause serious metabolic disorders), some authors suggested that their antagonistic action at the dopaminergic D_2R was sufficient for antipsychotic activity (Tarazi et al., 2001; Ginovart and Kapur, 2012). It has been indicated that chronic/sub-chronic administration of haloperidol (an FGA), as well as olanzapine and clozapine (SGAs), induce upregulation of D_2R binding levels and dopamine release in the PFC, NAc, CPu and hippocampus (O'Dell et al., 1990). However, short-term SGA treatment often revealed no alteration in D_2R levels (Kusumi et al., 2000). Another theory suggested that some SGAs including aripiprazole have faster dissociation rates (k_{off} values), while other SGAs including olanzapine and risperidone have slower dissociation from the D_2R (Seeman, 2002). As a partial D_2R agonist, aripiprazole has lower D_2R affinity compared with full agonists, which inhibits endogenous dopamine

activity and prevents excessive D_2R activation (Han et al., 2009; Ginovart and Kapur, 2012; Miyamoto et al., 2012). However, SGAs such as clozapine and quetiapine exhibited less striatal D_2R occupancy (<60%) compared to FGAs, which indicated that besides the D_2R blockade, other neurotransmission systems may also be involved in the therapeutic efficacy of SGAs such as olanzapine and clozapine (Ginovart and Kapur, 2012; Miyamoto et al., 2012).

2.2.1.2 The role of serotonin neurotransmission in therapeutic efficacy of SGAs

Besides the vital role of the dopamine system in the pathological theory and treatment of schizophrenia, over the past decades greater attention has been paid to other neurotransmissions including the serotonergic system (Matsumoto et al., 2005; Meltzer, 2012). The serotonergic system is believed to modulate numerous sensory, motor and behavioural processes in the mammalian nervous system, and is also implicated in the pathology of schizophrenia and other mental disorders such as depression and bipolar disorder (Carlsson, 1987; Tecott et al., 1995; Davis and Chen, 2001; Sawa and Snyder, 2002; Carlsson et al., 2004; Dolzan et al., 2008). The serotonin (5-HT) is one of major monoaminergic neurotransmitters in the brain, and acts through 5-HT receptors, including the $5\text{-}HT_{1A\text{-}F}$, $5\text{-}HT_{2A\text{-}C}$ and $5\text{-}HT_{3\text{-}7}$ receptor subtypes (Meltzer et al., 2003). Among them, the $5\text{-}HT_{2A}$, $5\text{-}HT_{2C}$, $5\text{-}HT_3$, $5\text{-}HT_6$ and $5\text{-}HT_7$ receptors are associated with the therapeutic efficacy of SGAs (Meltzer and Huang, 2008).

SGAs such as olanzapine, clozapine, quetiapine, risperidone, and ziprasidone treat schizophrenia through direct or indirect effects on distinct 5-HT receptors (Meltzer et al., 2003; Meltzer, 2007). In particular, as shown in Table 2.1, the $5\text{-}HT_{2A}R$ and $5\text{-}HT_{2C}R$ are G-protein-coupled receptors involved in the therapeutic effects of SGAs including

olanzapine and clozapine (Horacek et al., 2006; Meltzer, 2012). However, it has also been reported that clozapine and risperidone, but not olanzapine, significantly enhanced extracellular 5-HT release in the NAc and PFC, which contribute to the SGAs' affinity in improving mood disorders and cognition (Ichikawa et al., 1998).

In addition, the 5-HT neurons originating from the raphe nuclei of the midbrain, innervate both the SN and VTA, in which there exist higher densities of 5-HT immune-reactive fibres connecting synaptically with both dopamine and non-dopamine neurons (Di Matteo et al., 2001). In particular, it was reported that 5-HT_{2A}R, 5-HT_{1A}R and 5-HT_{2C}R contributed to serotonergic modulation, which can enhance dopamine output in the striatum and PFC (Horacek et al., 2006). Therefore, another explanation regarding the therapeutic efficacy of SGAs is the interaction between the 5-HT and dopamine receptors (Meltzer, 2012).

A. The role of 5-HT_{2A}R in therapeutic efficacy of SGAs

Serotonergic 5-HT_{2A}R is widely expressed throughout the CNS, particularly in most of the serotonergic terminal rich areas, including the PFC, NAc, and in the cell bodies of dopamine neurons in the VTA and SN, as well as in most cortical pyramidal neurons (Jakab and Goldman-Rakic, 1998; Doherty and Pickel, 2000). The 5-HT_{2A}R is a G-protein-coupled receptor linked to the intracellular molecular signal-transduction cascade, which plays a crucial role in the therapeutic action of SGAs and psychiatric disorders (Kusumi et al., 2000; Horacek et al., 2006; Meltzer and Massey, 2011). The accumulated evidence has demonstrated that SGAs such as olanzapine and clozapine attenuate 5-HT_{2A}R binding and mRNA expression (which are involved in the therapeutic effects of SGAs) in the PFC, NAc, Cg, and SN after both acute and chronic

administration (Kusumi et al., 2000; Kuroki et al., 2003; Huang et al., 2006b; Kuroki et al., 2008; Meltzer and Massey, 2011; Yadav et al., 2011).

It has been suggested that to some extent a higher 5-HT_{2A}R binding affinity, but a lower D_2R binding affinity, was the differentiation of SGAs from FGAs (Meltzer and Massey, 2011). It has also been reported that the blockade of 5-HT_{2A}R, by olanzapine for example, causes changes in dopaminergic output in the PFC (increase) and NAc (decrease) throughout the nigrostriatal or mesolimbic dopaminergic pathways (Kuroki et al., 2008). It should be noted that Richtand and colleagues reported that there was no relation between the 5-HT_{2A}R/D_2R ratio and efficacy of SGAs (Richtand et al., 2007). Some SGAs, such as amisulpride, have weaker 5-HT_{2A}R affinity (Leucht et al., 2009).

B. The role of 5-HT_{2C}R in therapeutic efficacy of SGAs

The 5-HT_{2C}Rs are located throughout the CNS, including the VTA and NAc (Pazos et al., 1985), and are widely considered to be another of the serotonergic receptors involved in the pathology of schizophrenia and response to SGAs (Dwyer et al., 2005; Reynolds et al., 2005; Sodhi et al., 2005; Meltzer and Massey, 2011). Similar to the 5-HT_{2A}R antagonists, treatment by SGAs including olanzapine reduced the 5-HT_{2C}R binding level in the PFC, Cg and NAc (Tarazi et al., 2002; Huang et al., 2006b).

The 5-HT_{2C}R plays an important role in regulating dopamine release (Meltzer and Huang, 2008). For example, the firing rate of dopamine neurons in the VTA is inhibited by 5-HT_{2C}R agonists, but stimulated by 5-HT_{2C}R antagonists (Meltzer and Huang, 2008). In addition, it was considered that 5-HT_{2C}R inhibited dopamine release in the PFC and NAc, which was consistent with microanalysis studies, in which the 5-HT_{2C}R

antagonist increased extracellular dopamine concentrations in the NAc and PFC (Millan et al., 1998; Di Matteo et al., 2001; De Deurwaerdere et al., 2004). Furthermore, there was evidence that a combined 5-HT_{2A}R and 5-HT_{2C}R blockade is more efficient than a 5-HT_{2A}R blockade by itself to increase dopamine release in the NAc and PFC; the combined blockade can interact reciprocally to modulate the activity of the mesolimbic and the mesocortical dopaminergic pathways, and resulted in an improvement in cognitive deficits (Meltzer et al., 2003). In addition, SGAs such as olanzapine, clozapine, risperidone and ziprasidone showed potent inverse agonistic affinity at 5-HT_{2C}R in both humans and rats (Herrick-Davis et al., 2000). The 5-HT_{2C}R agonist, WAY-163909, is present in antipsychotic action in a variety of preclinical models (Marquis et al., 2007). In addition, an association between the gene coding for 5-HT_{2C}R and olanzapine-induced weight gain (Sicard et al., 2010).

C. The role of 5-HTT in therapeutic efficacy of SGAs

The serotonin transporter (5-HTT) plays a key role in serotonergic neurotransmission that terminates the action of serotonin and recycles it in a sodium-dependent manner *via* transportation from the synaptic spaces into presynaptic neurons (Zhang and Malhotra, 2011). The 5-HTT gene (SLC6A4-solute carrier family 6, member 4) is associated with various normal and pathological human behaviours including various psychiatric disorders such as schizophrenia (Serretti et al., 2006; Dolzan et al., 2008). A number of studies identified an association between 5-HTT gene polymorphism and the symptomatology of schizophrenia, although there were some conflicting results (Fan and Sklar, 2005; Dolzan et al., 2008; Zaboli et al., 2008). For example, using a haplotype analysis, 5-HTT variants (5-HTTLPR, STin 2, rs104701, and rs1042173) were reported to have a significant association with schizophrenia (Zaboli et al., 2008).

Furthermore, the variations of 5-HTT gene polymorphisms and presence of 5-HTTLPR L allele were relevant to the treatment response to SGAs including olanzapine (Serretti et al., 2006; Bozina et al., 2007; Zhang and Malhotra, 2011). In particular, it has been reported that, the short allele of 5-HTTLPR is linked with poor response to olanzapine, clozapine and risperidone treatment, and affects the rate of 5-HT uptake (Serretti et al., 2006; Zhang and Malhotra, 2011). On the other hand, 5-HTT, as the major route of inactivation of 5-HT neurotransmission, affects the efficacy and tolerability of antipsychotics; this may explain the ability of selective 5-HT reuptake inhibitors and some antidepressants to inhibit 5-HT reuptake and cause EPS side-effects (Meltzer et al., 2003). However, the exact role of 5-HTT in the SGA therapeutics of schizophrenia is not clear.

2.2.2 Second generation antipsychotic drugs (SGAs) and weight gain/obesity side-effects

2.2.2.1 Clinical evidence in SGA-induced weight gain

A series of clinical trials have shown that SGA treatment causes serious metabolic side-effects (summarized by Deng, 2013, Allison et al., 2009). Clinical studies have indicated that patients gain 4-5 kg of weight during the first 10 weeks' of treatment with some SGAs, such as olanzapine and clozapine, and may continue to gain weight throughout the treatment period (Allison et al., 1999; Nasrallah, 2008). The NIH (National Institutes of Health, USA) funded CATIE study (Clinical Antipsychotic Trials of Intervention Effectiveness) reported the effects of 18 months' of treatment with SGAs including olanzapine, quetiapine, risperidone and ziprasidone on body weight

gain side-effects; olanzapine treatment caused significant weight gain (≥7% from baseline) (30% of schizophrenia patients, 0.9 kg/month), compared with quetiapine (16% and 0.23 kg/month, respectively), risperidone (14% and 0.18 kg/month respectively) and ziprasidone (7% and 0.14 kg/month respectively) (Lieberman et al., 2005). The CAFE study (Comparison of Atypical Antipsychotics in First Episode Psychosis) suggested that first-episode schizophrenia patients treated with olanzapine exhibited a significant weight gain side-effects (59.8%), compared with risperidone (32.5%) and quetiapine (29.2%) after 12 weeks' of treatment (Patel et al., 2009). Furthermore, the same investigators found that after 52 weeks' of treatment, 80% of olanzapine-treated patients (1.76 kg/month), compared to 57.6% of risperidone (1.28 kg/month) and 50% of quetiapine-treated patients (1.29 kg/month), gained ≥ 7% body weight (Patel et al., 2009). The European First Episode Schizophrenia Trial (EUFEST) showed a marked weight gain in the first episode schizophrenia patients after one year treatment of olanzapine (13.9 kg), quetiapine (10.5 kg), ziprasidone (4.8 kg), or haloperidol (7.3 kg) (McQuade et al., 2004). A drug-naïve first episode psychosis study reported that the 7% from base line weight gain was observed in 80% of olanzapine-treated patients, 58% of risperidone and 50% of quetiapine patients (Zimmermann et al., 2003).

A number of clinical reports suggested that increasing appetite and food intake, as well as delayed satiety signalling, are crucial behavioural changes associated with SGA-induced weight gain (Blouin et al., 2008; Sentissi et al., 2009; Deng, 2013). A 6 weeks randomized double-blind study found that olanzapine-treated patients had higher rates of food craving compared with clozapine (48.9% *vs.* 23.3%), as well as more frequent and earlier occurring binge eating (16.7% and 1 week *vs.* 8.9% and 3 weeks) (Kluge et al., 2007). It has also been reported that olanzapine treatment for one week significantly

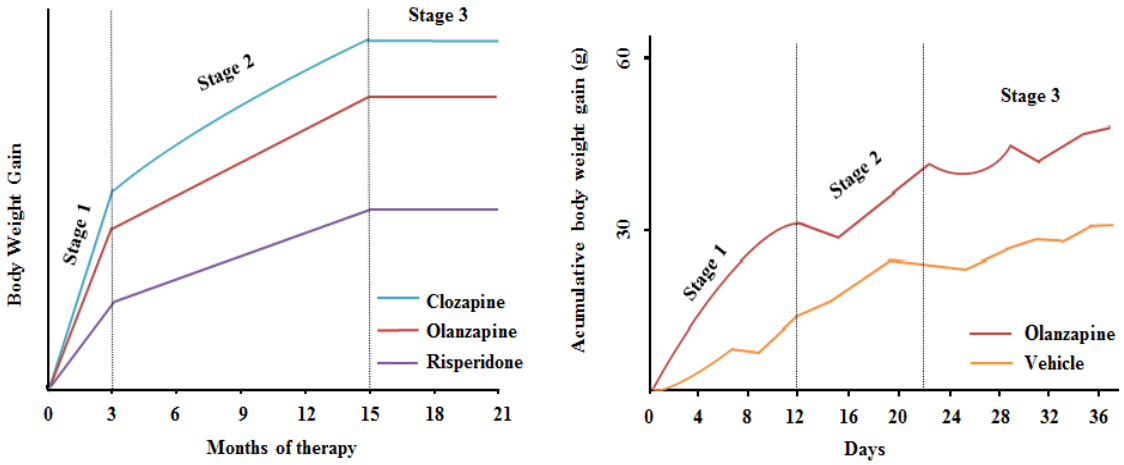
enhanced both anticipatory and consummatory reward responses to food rewards in the brain's reward circuitry including the SN, Cg and inferior frontal cortex, while inhibited feeding behaviour and attenuated activation in brain regions such as the lateral orbital frontal cortex (Mathews et al., 2012). Overweight and obesity are clinical conditions that are associated with increased body fat and body mass index (BMI, weight in kg/the square of height in meters, kg/m^2) (Allison et al., 2009), especially increased visceral fat in the abdomen which is associated with insulin resistance (Kenchaiah et al., 2004). Therefore, the clinical trials indicated that chronic SGA treatment may lead to substantial body weight gain/obesity side-effects; this may be due to increased energy intake and reduced energy expenditure.

The development of SGA-induced weight gain/obesity side-effects can be divided into three stages in humans (Figure 2.1A): firstly, the early acceleration stage in which SGAs cause a rapid increase in body weight gain (about 3 months' administration of olanzapine and clozapine); secondly, a middle, steady increase stage involving a steadier period of body weight gain lasting for up to 18 months; and lastly, weight maintenance throughout the treatment period stage in which the heavier weight gain level is maintained through the treatment period after 18 months (Zipursky et al., 2005; Pai et al., 2012; Deng, 2013).

2.2.2.2 Animal models in SGA-induced weight gain

Because of the ethical issues related to human studies, appropriate animal models mimicking the human scenario are important to examine the mechanisms of SGA-induced metabolic side-effects including weight gain (Choi et al., 2007; Tulipano et al., 2007; Smith et al., 2008; Coccurello and Moles, 2010). In fact, multiple studies reported

that olanzapine treatment has been modelled in rodents for investigating its metabolic side-effects including body weight gain/obesity, hyperphagia, hypolocomotor activity, hyperglycaemia and hyperinsulinaemia (Coccurello et al., 2006; Baptista et al., 2007; Cooper et al., 2008; Cooper et al., 2010; Albaugh et al., 2011; Shobo et al., 2011; Van Der Zwaal et al., 2014). In particular, animal models for olanzapine-induced weight-gain/obesity have been successfully established in female rats in our laboratory (Huang et al., 2006b; Han et al., 2008; Weston-Green et al., 2011; Deng et al., 2012), and other labs (Coccurello et al., 2006; Chintoh et al., 2008; Cooper et al., 2008; Boyda et al., 2012; Van Der Zwaal et al., 2014). The female rat models established in our laboratory successfully mimicked the development of olanzapine-induced body weight gain/obesity side-effects in humans (Huang et al., 2006a; Weston-Green et al., 2011). They show three stages of development: in Stage 1, olanzapine-induced positive energy balance is greatest and is characterised by an increased energy intake and rapid body weight gain during the first 2 weeks; in Stage 2, an elevated body weight gain is maintained during week 3-5, despite a lower increase in energy intake compared to Stage 1; Stage 3 is characterised by the maintenance of an elevated body weight gain and normal levels of energy intake (Figure 2.1B) (Huang et al., 2006b; Stefanidis et al., 2008; Pai et al., 2012; Van Der Zwaal et al., 2014). Other studies have also investigated animal rodent models for metabolic side-effects induced by other SGAs including risperidone, quetiapine, ziprasidone and sulpiride (Baptista et al., 2002; Smith et al., 2008; Savoy et al., 2010).



(A) (B)

Figure 2.1 (A): Body weight gain in schizophrenia patients treated with clozapine, olanzapine and risperidone; (B): Body weight gain in a rodent model with olanzapine treatment over 36 days compared to controls (Adapted from Pai et al., 2012; Huang et al., 2006).

The studies in animal models showed that olanzapine-induced weight gain is caused at least partly from increased food intake, reduced gross locomotor activity and thermogenesis (Arjona et al., 2004; Weston-Green et al., 2011; Deng, 2013; Zhang et al., 2014a). It has also been reported that female rats are more sensitive than male rats to SGA-induced weight gain side-effects (Albaugh et al., 2006; Wu et al., 2007; Weston-Green et al., 2010), which is consistent with clinical observation that female schizophrenia patients are more sensitive to SGA-induced metabolic side-effects compared with males (Bobes et al., 2003a; Hakko et al., 2006; Wu et al., 2007). However, SGA-induced weight gain can only be modelled in male rats under certain feeding conditions (Hartfield et al., 2003; Minet-Ringuet et al., 2006a; Shobo et al., 2011), such as high carbohydrate/medium fat/low protein (54%/31%/14%) diets (Minet-Ringuet et al., 2006b; Shobo et al., 2011). Therefore, the female rat model for SGA-

induced weight gain has been widely used for investigating the mechanisms of the side-effects.

2.2.3 The neuropharmacological mechanisms of SGA-induced weight gain side-effects

It has been recognised that increased energy intake (hyperphagia) and/or decreased energy expenditure are the main reasons for body weight gain associated with SGAs (Deng, 2013). A number of studies have shown that the robust potency of the antagonistic binding affinity of SGAs for 5-HT_{2C}R and histaminergic H_1R is associated with the weight gain side-effects of SGAs (Richelson and Souder, 2000; Rege, 2008; Lencz and Malhotra, 2009; Deng et al., 2010; Reynolds and Kirk, 2010; He et al., 2013). It was also suggested that increased appetite and food intake, as well as delayed satiety signalling are the key factors related to SGA-induced weight gain/obesity side-effects (Deng, 2013).

2.2.3.1 The function of the hypothalamus in body weight regulation

The hypothalamus is the main regulation centre for controlling energy balance and feeding behaviour (Mercer et al., 2011). The distinct nuclei in the hypothalamus including the arcuate nucleus (Arc), ventromedial hypothalamus (VMH) and lateral hypothalamus (LH) share a reciprocal connection, and regulate body energy homeostasis (Coppari et al., 2005; Sousa-Ferreira et al., 2014). More importantly, the hypothalamic Arc is the vital modulation centre for energy balance, which projects to other hypothalamic nuclei such as the VMH and LH (Hillebrand et al., 2002; Matsui-Sakata et al., 2005; Dalvi et al., 2011).

Furthermore, the Arc neurons produce orexigenic neuropeptides including neuropeptide Y (NPY) and agouti-related protein (AgRP) that lead to upregulation of food intake, as well as anorexigenic neuropeptides including proopiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART) that induce downregulation of food intake (Mercer et al., 2011). It has been demonstrated that neurogenesis in the hypothalamus participated in the response of hypothalamic neuronal circuits to metabolic signals such as POMC and NPY (Kokoeva et al., 2005; Pierce and Xu, 2010; Gouaze et al., 2013).

Since the Arc is an anatomical structure lacking a blood-brain barrier, peripheral signals such as hormones and gastrointestinal peptides can reach the Arc (Schwartz, 2000; Kohno and Yada, 2012). In fact, high densities of receptors for insulin, leptin and ghrelin are identified in the Arc and other hypothalamic nuclei (Mercer et al., 2011). Therefore, peripheral signals such as insulin and amylin (from the pancreas), ghrelin (from the stomach), leptin and adiponectin (from adipose tissue) can be integrated to contribute to the regulation of energy balance (Elmquist et al., 1998; Davidowa et al., 2004; Davidowa and Plagemann, 2007; Mercer et al., 2011; Kohno and Yada, 2012).

2.2.3.2 The roles of hypothalamic neuropeptide and regulation of energy homeostasis in SGA-induced weight gain

SGA-induced weight gain is associated with alterations to the neuroendocrine network that controls appetite, food intake and satiety (Milano et al., 2013). As mentioned above, the hypothalamic Arc plays a crucial role in appetite and energy homeostasis *via* activation of 2 distinct neural populations, that are (1) anabolic/orexigenic neurons

expressing NPY and AgRP, and (2) catabolic/anorexigenic neurons expressing POMC and CART (Schwartz et al., 2000; Ak et al., 2013), which are involved in the regulation of SGA-induced weight gain.

A. The roles of NPY and AgRP

As a 36 amino acid peptide, NPY has emerged as one of the most potent orexigenic hypothalamic neuropeptides (Smitka et al., 2013; Zhang et al., 2014c), having been isolated originally from a porcine brain, and is synthesized in both the central and peripheral neurons (Tatemoto et al., 1983). In the CNS, NPY is mainly synthesized in the hypothalamus, brainstem and anterior pituitary (Zhang et al., 2014c). Specifically, the Arc NPY neurons project to adjacent hypothalamic regions including LH, the paraventricular nucleus (PVN), the dorsomedial nucleus (DMN), and the perifornical areas (PFO) (Morris, 1989). As the biomarker for obesity, NPY is a major mediator in promoting energy storage (Zhang et al., 2014c), which regulates fat deposition and metabolism *via* various G-protein-coupled NPY receptors (NPYRs) including NPY receptor subtype 1, 2, and 5 (Larhammar et al., 1993). For example, the NPY receptor sub-type 5 antagonist, Velneperit, has been identified in clinical tests as a potential anti-obesity drug (George et al., 2014).

It has been reported that a single intracerebroventricular (ICV) injection of NPY stimulated feeding in rats (Clark et al., 1984), sheep (Miner et al., 1989) and monkeys (Larsen et al., 1999). Furthermore, injection of NPY into the PVN repeatedly led to sustained hyperphagia, body weight gain and fat mass accumulation (Stanley and Thomas, 1993), while decreased heat production in brown adipose tissue (BAT) resulted in the anabolic effects of NPY *via* inhibition of the sympathetic nervous system

(SNS) (Stanley et al., 1986; Egawa et al., 1990; Billington et al., 1991). In addition, synthesis and secretion of Arc NPY resulted in response to energy deficiency and greater metabolic need such as increased exercise, cold and pregnancy (Leibowitz and Wortley, 2004; Mercer et al., 2011). Therefore, NPY promotes energy storage by hyperphagia and decreased energy expenditure (Morton and Schwartz, 2001). Moreover, the Arc NPY neurons play a role in integrating peripheral energy signals, such as blood glucose concentration, ghrelin, leptin and insulin (Kohno and Yada, 2012). In particular, food intake is stimulated by increasing NPY signalling, and inhibited by insulin and leptin (Morton and Schwartz, 2001).

AgRP is synthesised exclusively in the Arc that projects to adjacent hypothalamic regions such as the PVN, DMN and LH (Suzuki et al., 2012). The major function of AgRP is to stimulate feeding by antagonising melanocortins at the MC_3R (melanocortin 3 receptor) and MC_4R (melanocortin 4 receptor) in the hypothalamus (Broberger et al., 1998; Haskell-Luevano et al., 1999; Nijenhuis et al., 2001; Cone, 2005). Polymorphism of the AgRP gene is associated with inherited leanness in humans (Marks et al., 2004). Similar to NPY, both leptin deficiency (*ob/ob*) and leptin receptor mutation (*db/db*) resulted in overexpression of AgRP in the Arc of mice (Ollmann et al., 1997; Cone, 2005), which has also arisen from fasting in rats and mice (Shutter et al., 1997). AgRP is sustained for up to a week after a single ICV injection, compared with the effects of NPY which are sustained over hours (Hagan et al., 2000; Schwartz et al., 2000). Selective ablation of NPY/AgRP-expressing nuclei in adult mice resulted in acute reduction of feeding (Gropp et al., 2005).

The effects of SGAs on expression of NPY and AgRP have been addressed in previous studies. In particular, Fernø and colleagues demonstrated that short-term exposure to olanzapine (6 days) resulted in upregulated NPY and AgRP expressions in the Arc of both rats fed *ad libitum* and pair-fed rats (Fernø et al., 2011). Furthermore, the increased hypothalamic NPY and AgRP expressions were also observed by the study using ICV administration of olanzapine (Martins et al., 2010). A recent study in our group also reported that the NPY level was upregulated in short (7 days) and mid-term (15 days) olanzapine treatment in rats and in another 8 days of pair-fed experiment (Zhang et al., 2014a) (summarised in Figure 2.3). The same authors further revealed elevated NPY and AgRP expressions after an acute ICV injection of olanzapine, which was associated with increased food intake (Zhang et al., 2014a). Another study from our group also reported that olanzapine upregulated NPY mRNA expression in the hypothalamic Arc (Weston-Green et al., 2012). NPY upregulation has also been reported in other SGAs: clozapine enhanced hypothalamic NPY expression in rats (Kirk et al., 2006), while quetiapine increased the NPY level in cerebrospinal fluid from schizophrenia patients (Nikisch et al., 2012); which were associated with SGA-induced weight gain. The association between AgRP and fat mass as well as appetite have been examined and were found to be disrupted in olanzapine-treated patients, but not in patients treated with ziprasidone (Ehrlich et al., 2012). However, some studies reported that hypothalamic NPY expression following SGAs exposure either decreased or did not change (Huang et al., 2006a; Davoodi et al., 2009; Guesdon et al., 2010; Secher et al., 2010). Ak and colleagues also reported that the plasma NPY level was attenuated in first attack psychotic male patients treated with olanzapine (Ak et al., 2013). These conflicting observations may be attributed to methodological differences including treatment duration, drug dosage and delivery methods (oral, intraperitoneal, or ICV

injection) (Huang et al., 2006a; Kirk et al., 2006; Davoodi et al., 2009; Guesdon et al., 2010; Martins et al., 2010; Fernø et al., 2011). Other studies have observed the various effects of SGAs on NPY expression in different brain regions (Obuchowicz and Turchan, 1999; Obuchowicz et al., 2004; Huang et al., 2006a; Weston-Green et al., 2012).

On the other hand, Ruano and colleagues reported that a polymorphism (rs1468271) in the NPY gene had no association with body weight gain in patients treated with olanzapine and risperidone (Ruano et al., 2007). A recent study by Tiwari and colleagues suggested a significant association between the SNPs rs16147, rs5573, and rs5574 in NPY and weight gain in clozapine and olanzapine-treated patients (Tiwari et al., 2013). The same authors also revealed an association of rs6837793, near NPY5R, with the weight gain profile in patients treated with risperidone, which supported the role of the NPY system in SGA-induced weight gain.

B. The roles of POMC and CART

POMC is synthesized from the 285-amino-acid-long polypeptide precursor, and produces peptide hormones including β-endorphins and melanocortin (Morton and Schwartz, 2001; Cone, 2005). There are several subtypes of the melanocortin peptides, such as adrenocorticotrophin (ACTH) and alpha-melanocyte-stimulating hormone (α-MSH), which act at G-protein-coupled melanocortin receptors such as the MC_{1-5} receptors (Morton and Schwartz, 2001; Cone, 2005; Millington, 2006). In the brain, POMC is mainly expressed in the Arc (Morton and Schwartz, 2001; Millington, 2006). The POMC neurons project to the periventricular, paraventricular and perifornical regions of the hypothalamus (Jobst et al., 2004; Cone, 2005). It is also reported that

POMC mRNA expression is increased by leptin and co-localized with leptin receptors (Cheung et al., 1997; Schwartz et al., 1997; Thornton et al., 1997; Mizuno et al., 1998b; Fry et al., 2007).

Certain POMC gene mutations may contribute to obesity in healthy human populations (Krude et al., 1998; Baker et al., 2005; Ternouth et al., 2011), while mouse knockout for POMC also shows the obese phenotype (Yaswen et al., 1999; Millington, 2006). POMC mRNA expression is reduced by food restriction, while increased by overfed rats (Mizuno et al., 1998a; Hagan et al., 1999). Importantly, the activation of POMC neurons is regulated by the binding of 5-HT to 5-HT_2R (Reynolds and Kirk, 2010; Ak et al., 2013). Additionally, POMC expression in the Arc is also regulated by the dopaminergic system *via* D_2R (Tiligada and Wilson, 1989).

CART is a peptide (first isolated from the ovine hypothalamus), and is widely distributed throughout the central and peripheral nervous systems (Vicentic and Jones, 2007; Zhang et al., 2012). CART protein is encoded by the CARTPT gene, which functions in feeding, reward, stress and pain transmissions (Douglass and Daoud, 1996; Kristensen et al., 1998). Similar to POMC, CART also downregulates food intake and body weight (Nakhate et al., 2011). A previous study has revealed that CART has an effect on neuronal activities, including the hypothalamic VMH and PVN, which are involved in the neuroplasticity of hypothalamic feeding circuits (Davidowa et al., 2005). Furthermore, CART interacts with other important mediators such as the cannabinoid CB_1 receptor, insulin and leptin, involved in the regulation of feeding (Cota et al., 2006; Vicentic and Jones, 2007). On the other hand, ICV administration of CART was

accompanied by a decrease in plasma insulin and leptin levels and an increase in lipid oxidation, which limits fat storage (Rohner-Jeanrenaud et al., 2002).

It was shown that olanzapine treatment resulted in attenuation of POMC but not CART expressions in the hypothalamus of rats (Fernø et al., 2011; Sezlev et al., 2013; Zhang et al., 2014a). For example, a recent study in our group reported that olanzapine reduced hypothalamic POMC levels after short (7 days) and medium (15 days) term olanzapine treatment (Zhang et al., 2014a). Another study from our group also revealed that olanzapine significantly attenuated POMC mRNA expression in the Arc of rats treated with olanzapine (Weston-Green et al., 2012). Rats treated with olanzapine (6 days) showed a reduced hypothalamic POMC expression, but no change in CART (Fernø et al., 2011). Ak and colleagues also reported that the plasma POMC level was elevated in first attack psychotic male patients treated with olanzapine, but no changes in plasma CART level were observed (Ak et al., 2013). It should be noted that olanzapine suppresses POMC expression by blocking 5-HT_{2C}R, and indirectly blocking 5-HT_{1B} receptor (5-HT_{1B}R), while that suppression is abolished by GABA (gamma-aminobutyric acid) neurons (Donovan and Tecott, 2013) (summarised in Figure 2.3). However, it should also be noted that there are some conflicting reports. Two studies reported no alteration of POMC expression when treated with olanzapine, one in rats (Davoodi et al., 2009) and the other in patients (Chowdhury et al., 2014). A recent genetic study reported that neither the POMC single nucleotide polymorphisms (rs6713532, rs1047521, rs3754860) nor the CART (SNPs) (rs10515115, rs3763153, rs3857384, rs11575893, rs16871471) gene variants was associated with weight gain in chronic schizophrenia patients treated with SGAs (Chowdhury et al., 2014). In addition,

a genetic polymorphism study suggested that MC_4R is relevant to SGA-induced weight gain and related metabolic disorders (Malhotra et al., 2012).

2.2.3.3 The role of serotonin neurotransmission associated with SGA-induced weight gain

Serotonin neurotransmation plays a key role in regulating food intake (Meguid et al., 2000; De Vry et al., 2003). It has been reported that 5-HT_{2C}R gene knocked-out mice have been proven to develop hyperphagia, which further led to obesity and hyperinsulinemia (Tecott et al., 1995; Meier and Gressner, 2004). It was also reported that food intake was reduced by 5-HT_{2C}R agonist, but increased by a 5-HT_{2C}R antagonists (Kitchener and Dourish, 1994; Clifton et al., 2000; Schreiber and De Vry, 2002; Hayashi et al., 2005).

Over the past 2 decades, SGA-induced weight gain has been partially attributed to the 5-HT_{2C}R antagonist properties of SGAs (Reynolds and Kirk, 2010; Meltzer, 2013). Furthermore, some SGAs, including olanzapine and clozapine, are also inverse agonists on 5-HT_{2C}R, rather than full antagonists (Rauser et al., 2001; Kirk et al., 2009), which also plays a crucial role in SGA-induced weight gain (Kirk et al., 2009). Additionally, it has been reported that co-treatment of haloperidol (a D_2R antagonist) with SB243213 (a 5-HT_{2C}R antagonist) mimicked olanzapine induced body weight gain in rats (Berg et al., 2006; Kirk et al., 2009).

Furthermore, 5-HT has been found to influence appetite by activating anorexigenic POMC neurons and melanocortin-4 receptors (Lam et al., 2010). In particular, serotonergic neurons project to the hypothalamic POMC neurons where they co-express

5-HT_{2C}R (Donovan and Tecott, 2013). Therefore, as a potent 5-HT_{2C}R antagonist or inverse agonist, olanzapine may decrease expression of POMC by blocking the 5-HT_{2C}R that increases appetite (Xu et al., 2008; Lam et al., 2010) (summarised in Figure 2.3).

2.2.3.4 The role of histamine neurotransmission in SGA-induced weight gain

Histamine neurons originate from the tuberomammilary nucleus (TMN) of the posterior hypothalamus (which receives very dense of orexin innervations originating from the LH) and project to all brain regions including the hypothalamus itself (Schwartz et al., 2000; Brown et al., 2001; Haas et al., 2008; Masaki and Yoshimatsu, 2010). Since histamine cannot cross the blood-brain barrier, it is synthesised *in situ* in the brain from the precursor amino acid, $_{L}$-histidine and catalysed by the rate-limiting enzyme histidine decarboxylase (HDC) (Jorgensen et al., 2006).

Histamine exerts its actions *via* the specific histaminergic receptors, which have been classified into the H_1, H_2, H_3 and H_4 subtypes (Brown et al., 2001; Masaki and Yoshimatsu, 2006). All of them are G-protein-coupled receptors and widely expressed throughout the body. In the CNS, the H_1 receptors (H_1R) are mainly postsynaptically located and are found especially in the hypothalamus, cerebral cortex and limbic system (Lintunen et al., 1998; Brown et al., 2001), where they are well documented as involved in the regulation of body weight and food intake. H_2 receptors are also mainly postsynaptically located and are expressed in the hippocampus, amygdala and basal ganglia (Brown et al., 2001). H_3 receptors (H_3R) are exclusively presynaptically located and found in the NAc, striatum, basal ganglia, and hypothalamus (Arrang et al., 1983;

Brown et al., 2001). H_4 receptors are expressed in the hypothalamus and spinal cord (Strakhova et al., 2009).

Histamine plays a crucial role in the regulation of a wide range of behavioural and physiological functions in humans and animals, such as appetite, drinking, sleep, wakefulness, locomotor activity, learning and memory (Brown et al., 2001; Passani et al., 2011). In particular, the neurotransmitter histamine has been implicated in the regulation of energy homeostasis (Park et al., 1999; Deng et al., 2010). In other words, elevated hypothalamic histamine signalling contributed to decreased food intake and body weight gain in animals including rats (Clineschmidt and Lotti, 1973; Itowi et al., 1988; Lecklin et al., 1998; Masaki and Yoshimatsu, 2010), while reduction in histamine levels was associated with increased body weight gain and food intake (Brown et al., 2001; Passani et al., 2011). Histamine knockout mice exhibited predominantly obesity with increased visceral adiposity, hyperleptinemia and decreased glucose tolerance (Fulop et al., 2003; Jorgensen et al., 2006). It has also been reported that SGAs such as olanzapine can directly modulate histaminergic neurotransmission in the hypothalamus, which correlated with the regulation of feeding behaviour in rats (Davoodi et al., 2008).

A. H_1R and SGA-induced weight gain

The histaminergic H_1Rs are highly expressed in the hypothalamic Arc, VMH and PVN (Masaki et al., 2004), which are involved in the regulation of food intake and energy expenditure (Yoshimatsu et al., 2002; Poole et al., 2008; Masaki and Yoshimatsu, 2010). It has been reported that H_1R antagonists play a crucial role in increasing appetite and obesity development (Tecott et al., 1995; Deng et al., 2010), which were also observed in H_1R knockout (KO) mice (Masaki et al., 2004). Moreover, deprivation of food

predominantly led to activation of H_1R expression in the hypothalamic Arc (Umehara et al., 2010). It was also demonstrated that ICV injection of the H_1R agonist, 2-(3-trifluoromethylpehenyl)histamine (FMPH) inhibited food intake (He et al., 2014).

H_1R antagonist properties have been identified as playing a significant role in the development of SGA-induced body weight gain/obesity side-effects (approximately Clozapine = Olanzapine > Quetiapine > Risperidone > Haloperidol > Ziprasidone = Aripiprazole) (Kroeze et al., 2003; Matsui-Sakata et al., 2005; Correll, 2008; Coccurello and Moles, 2010) (Table 2.1). In particular, H_1R blockade is recognised as a predominant target for SGA-induced weight gain compared with other receptors (Dwyer et al., 2005; Stahl et al., 2009; Meltzer, 2013). A previous study in our laboratory showed that short (1 week) and long (12 weeks) term treatments with olanzapine (0.5 mg/kg, t.i.d.) significantly changed H_1R mRNA expression in the hypothalamic Arc and VMH, which was significantly correlated with food intake, body weight gain, feeding efficiency and fat pad mass (Han et al., 2008). Similarly, clinical studies demonstrated patients treated with antipsychotics showed a significant correlation between the genetic variants of H_1R (rs346074 – rs346070), BMI and obesity (Vehof et al., 2011).

Since histamine cannot pass the blood-brain barrier, direct peripheral H_1R antagonism by SGA treatment may also contribute to the obesity side-effects (He et al., 2013; He et al., 2014). The H_1R antagonistic affinity of SGAs is significantly correlated not only with increased body weight and adiposity, but also with insulin-resistance in schizophrenia patients (Erhart et al., 1998; Wirshing et al., 1999). Other antihistamine drugs such as loratadine and cyproheptadine have been associated with increased body weight and hyperphagia (Silverstone and Schuyler, 1975; Saleh et al., 1979; Chervinsky

et al., 1994). Furthermore, the H_1R antagonistic affinity of SGAs contributes to fat accumulation *via* downregulation of lipolysis, while upregulating lipogenesis in white adipose tissue (Lundius et al., 2010; Teff and Kim, 2011).

B. H_3R and SGA-induced weight gain

In addition to H_1R, the histaminergic H_3R is another significant target for regulating food intake and is highly expressed in the TMN of the hypothalamus (Pillot et al., 2002; Deng et al., 2010). Furthermore, H_3 heteroreceptors are also located on non-histaminergic neurons, regulating release of neurotransmitters such as acetylcholine, serotonin and dopamine, which may also be involved in food intake regulation (Threlfell et al., 2004; Passani et al., 2011).

SGAs maintain a very weak antagonistic potency at histaminergic H_3Rs in the brain (Schlicker and Marr, 1996) (Table 2.1). As a result, H_3R may play an indirect role in regulating the weight gain/obesity side-effects induced by SGAs. For example, it is possible that olanzapine can block postsynaptic H_1Rs, which may then lead to accumulation of histamine in the synaptic cleft (Deng et al., 2010) (summarised in Figure 2.3). Since the release and synthesis of histamine are regulated by presynaptic H_3 autoreceptors (Gomez-Ramirez et al., 2002), the accumulated histamine then activates the pre-synaptic H_3R slowing the synthesis and secretion of histamine and heightening feeding behaviour (Takahashi et al., 2002; Chiba et al., 2009; Deng et al., 2010). On the other hand, it was found that intraperitoneal injection of risperidone immediately increased hypothalamic histamine release, that was regulated by H_3R (Murotani et al., 2011).

2.2.4 The role of hypothalamic H_1R-AMPK signalling in SGA-induced weight gain

Hypothalamic AMP-activated protein kinase (AMPK) is highly expressed in the Arc, PVN, VMH and LH of the hypothalamus (Minokoshi et al., 2004; Kim et al., 2007; Meltzer, 2007; Kohno et al., 2011). It has been suggested that AMPK activity could be inhibited by histamine in hypothalamic tissue slices, while it is activated by H_1R antagonist, triprolidine in both hypothalamic tissue slices and the hypothalamus of knock-out mice (Kim et al., 2007). In addition, it is important to note that hyperphagia in diet-induced obese animals is attributed to the effect of hypothalamic AMPK signalling (Martin et al., 2006). Kahn and colleagues showed that AMPK activity in the hypothalamic Arc and PVN was inhibited by anorexigenic leptin and augmented by orexigenic AgRP (Kahn et al., 2005). Hypothalamic AMPK is also involved in feeding regulation and food intake by regulating the AMPK-ACC-Malonyl-CoA-CPT_1 axis (ACC: acetyl-CoA carboxylase; CPT_1: carnitine palmitoyltransferase 1) (Kola, 2008; Lage et al., 2008; Ronnett et al., 2009). A study using the CT1-1 cell line reported that the protein level of phosphor-AMPK (pAMPK) is activated by the H_1R antagonist, chlorpheniramine, while it is blocked by histamine (Kang et al., 2012).

It was found that olanzapine and clozapine activated hypothalamic AMPK by blocking H_1Rs to increase food intake and body weight gain (Kim et al., 2007; Meltzer, 2007; Sejima et al., 2011; Skrede et al., 2014) (Figure 2.2). In hypothalamic tissue slices, the level of pAMPK can be enhanced markedly by olanzapine, which indicates that the weight gain/obesity side-effects associated with olanzapine is mediated by activation of hypothalamic AMPK linked to blockade of the histaminergic H_1R (Kim et al., 2007; Meltzer, 2007). Our previous study found a time-dependent change in hypothalamic

AMPK signalling in SGA-induced obesity: the hypothalamic pAMPK level was increased after an 8-day administration of olanzapine (1 mg/kg, t.i.d.), followed a return to normal levels after 15 days' of treatment, and significantly reduced after 36 days of treatment (He et al., 2014). Additionally, administration of olanzapine by acute ICV infusion increased hypothalamic pAMPK expression (Martins et al., 2010). Therefore, besides H_1R itself, its downstream AMPK signals are also valuable targets for treating SGA-induced weight gain/obesity side-effects (Deng et al., 2010; He et al., 2013) (summarised in Figure 2.3).

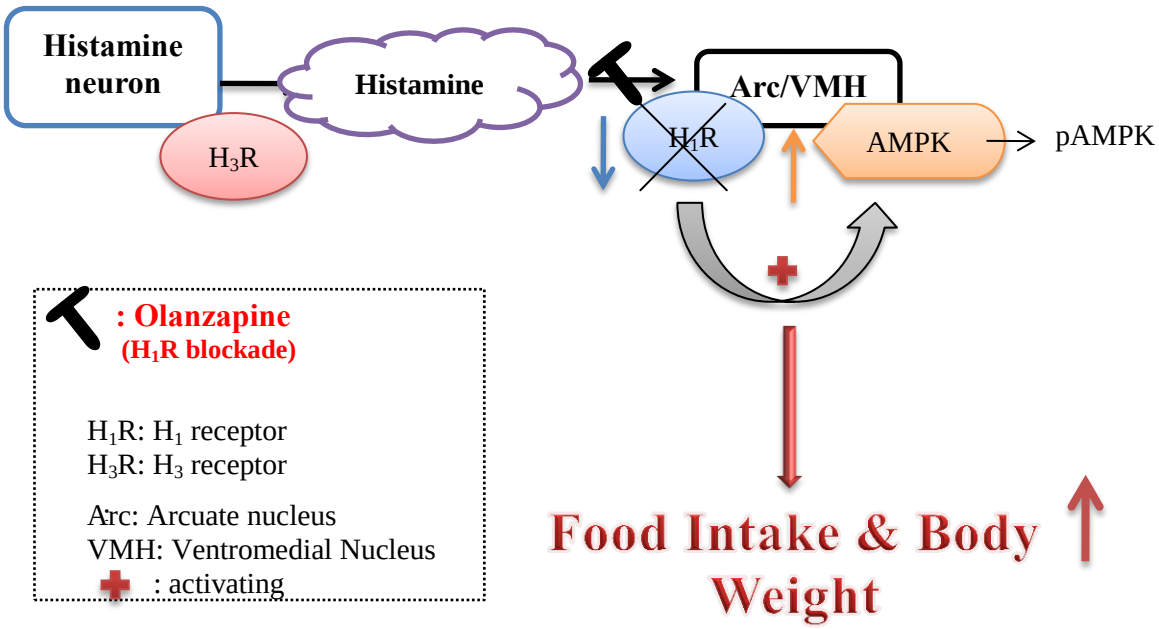


Figure 2.2 The proposed mechanisms of olanzapine-induced weight gain through histaminergic H_1 receptors. Olanzapine can block hypothalamic H_1 receptors and activate AMPK, which increases food intake and weight gain.

2.2.5 The role of brown adipose tissue in energy homeostasis associated with SGA-induced weight gain

Brown adipose tissue (BAT) functions by transferring energy from food into heat, burns lipids for thermogenesis and energy expenditure, and is also abundantly innervated by the sympathetic nervous system (SNS) (Oh et al., 2012). As another primary energy storage reservoir, BAT has an opposite role to white adipose tissue (WAT), which dissipates chemical energy producing heat generation *via* non-shivering thermogenesis, rather than storing and releasing energy in the form of triglycerides (Uldry et al., 2006; Stefanidis et al., 2008; Oh et al., 2012; Zhang et al., 2014c).

It has been indicated that treatment with obesogenic SGAs is associated with decreased energy expenditure both in patient and animal models (Blessing et al., 2006; Stefanidis et al., 2008; Skouroliakou et al., 2009; Cuerda et al., 2011; Zhang et al., 2014b) (summarised in Figure 2.3). In humans, BAT is distributed mainly in the interscapular of early neonates and adults and is predominately involved in energy balance such as regulation of body weight gain (Tam et al., 2012). In rodents, it was revealed that SGAs including clozapine, quetiapine and ziprasidone were prone to induce weight gain by inhibition of thermogenesis in BAT (Ota et al., 2002; Blessing et al., 2006; Oh et al., 2012). In addition, a study of psychotropic-induced obesity showed that lithium, a bipolar disorder therapy, downregulated the differentiation of mouse BAT (Rodriguez de la Concepcion et al., 2005).

2.2.5.1 Uncoupling peptide 1 (UCP_1) in SGA-induced weight gain

Thermogenesis of BAT is a response to cold exposure or diet, and occurs through the activation of uncoupling peptide 1 (UCP_1), which is a protein located in an inner-mitochondrial membrane that uncouples the mitochondrial proton gradient leading to oxygen consumption (Klingenberg and Huang, 1999; Rosen and Spiegelman, 2006). It has been reported that the noradrenaline released from the SNS nerve endings in BAT activates β-adrenergic receptors, BAT cell proliferation and mitochondriogenesis, and increased expression of UCP_1 (Himms-Hagen, 1990). As discussed above, hypothalamic AMPK co-operating with other neurotransmitters/neuropeptides, plays significant roles in regulating energy homeostasis such as hyperphagia and obesity (Lage et al., 2008; Lim et al., 2010). In addition, hypothalamic H_1R-pAMPK can regulate UCP_1 and BAT thermogenesis (Yasuda et al., 2004; Skrede et al., 2014).

It was recently demonstrated that clozapine significantly downregulated the expression of UCP_1 in BAT, while quetiapine suppressed the UCP_1 but less strongly compared with clozapine, and ziprasidone did not affect UCP_1 (an SGA with weight gain side-effects) (Oh et al., 2012). A more recent study in our lab also found long term (35 days) treatment of olanzapine reduced UCP_1 protein expression in the BAT of rats, which is associated with decreased BAT thermogenesis (Zhang et al., 2014b). Previous studies revealed that olanzapine treatment significantly reduced UCP_1 protein expression in BAT (Stefanidis et al., 2008; Hu et al., 2014). However, a weaker correlation between mRNA and protein levels of UCP_1 has been reported, having found that UCP_1 mRNA was unaltered after olanzapine treatment (Stefanidis et al., 2008; Nedergaard and Cannon, 2013); this suggested that olanzapine increases mainly UCP_1 protein production, not UCP_1 mRNA expression (summarised in Figure 2.3).

2.2.5.2 PGC-1 in SGA-induced weight gain

The peroxisome proliferator-activated receptor γ (pPPARγ)-coactivator 1α (PGC-1α) is expressed in the heart, kidneys, BAT, and brain; it was originally described as a cold-inducible coactivator controlling thermogenesis in the BAT and skeletal muscle by regulating the metabolism from mitochondrial biogenesis and respiration to hepatic gluconeogenesis (Houten and Auwerx, 2004). Besides PGC-1α, PGC-1β is also a member of the transcriptional coactivators playing a critical role in BAT thermogenesis (Houten and Auwerx, 2004; Uldry et al., 2006).

PGC-1α plays a pivotal role in brown adipose cells including mitochondrial biogenesis and UCP_1 activity in relation to thermogenesis of BAT (Puigserver et al., 1996; Puigserver et al., 1998). Furthermore, ectopic expression of PGC-1α is sufficient to promote several aspects of differentiation toward to the brown fat lineage, including the induction of UCP_1 gene expression (Uldry et al., 2006). PGC-1α is also rapidly and strongly induced by cold exposure, which is also confirmed by a mice study in which PGC-1α deficiency led to cold sensitivity and low UCP_1 expression (Lin et al., 2004). In addition, PGC-1α deficiency observed in the brain, hepatocytes and muscle contributed to deficient BAT function in PGC-1α$^{-/-}$ mice (Houten and Auwerx, 2004; Lin et al., 2004). The previous study suggested that deficiency of either PGC-1α or PGC-1β caused a significant decline in mitochondrial gene expression including UCP_1 during differentiation (Uldry et al., 2006). It is worth noting that, besides BAT thermogenesis and UCP_1, PGC-1a expression is also modulated by hypothalamic AMPK signalling (Lopez et al., 2010; Morrison et al., 2014).

PGC-1α is related to SGA-induced weight gain side-effects (Oh et al., 2012; Hu et al., 2014; Zhang et al., 2014b). For example, Zhang and colleagues reported a downregulated PGC-1α expression in the BAT of rats, treated by long-term administration of olanzapine, which was associated with reduced BAT temperature (Zhang et al., 2014b). It has been reported that clozapine significantly reduced the expression of mouse brown adipogenesis markers including PGC-1α (Oh et al., 2012) (summarised in Figure 2.3). In addition, a recent rat study also suggested that olanzapine significantly reduced PGC-1α expression in skeletal muscle, while gene expression of PGC-1α was increased after co-treatment with olanzapine and metformin, and co-treatment with berberine (a herbal alkaloid), compared with olanzapine treatment alone (Hu et al., 2014). To date, it is not clear whether PGC-1β also contributes to SGA-induced weight gain.

As discussed above, SGAs may induce body weight gain side-effects by elevating hypothalamic H_1R-AMPK pathway and NPY, AgRP expressions, while attenuating POMC levels. Furthermore, BAT thermogenesis biomarkers UCP_1 and PGC-1a, and locomotor activity, are also involved in regulation of SGA-induced weight gain (Figure 2.3).

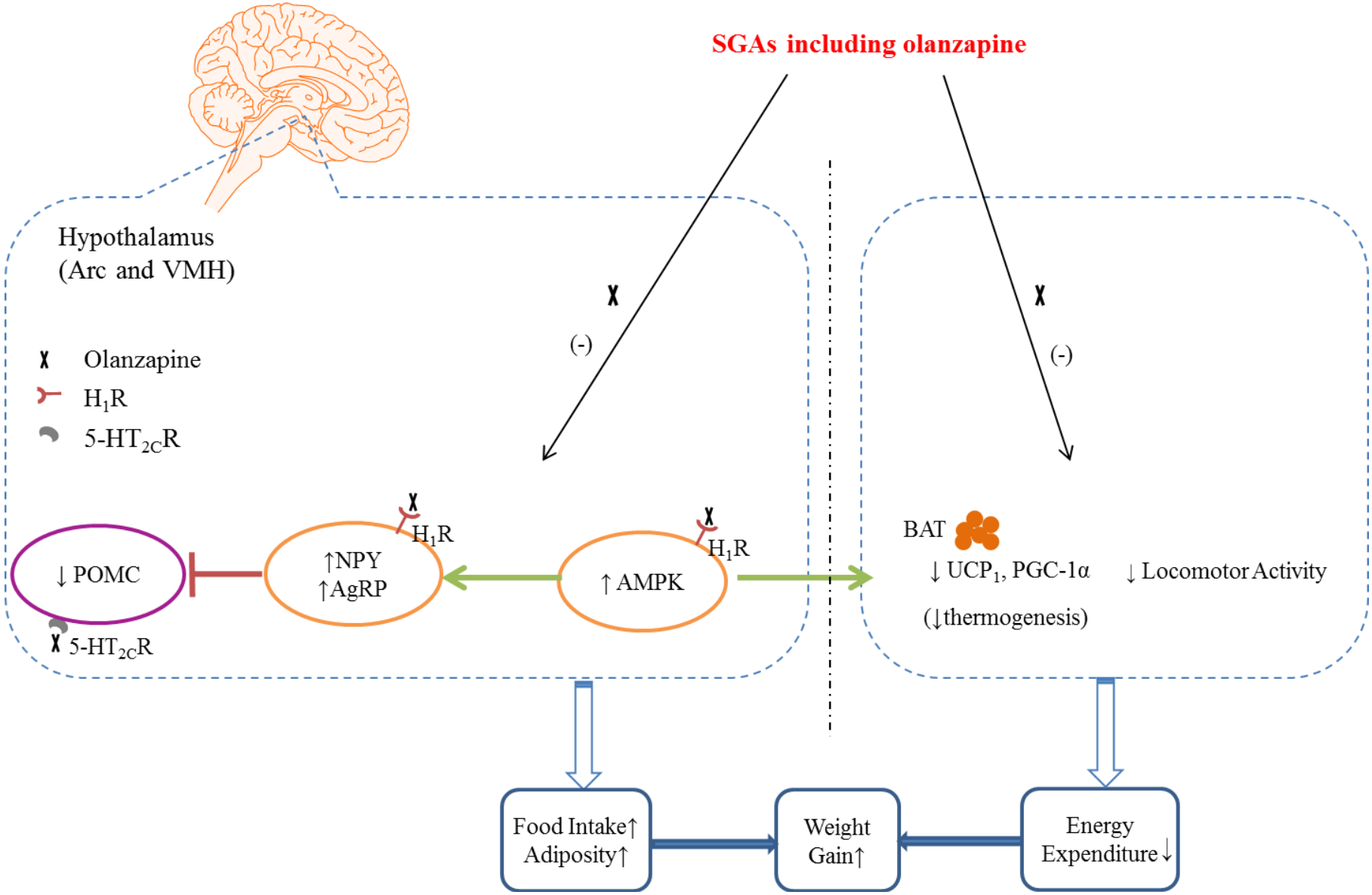

SGAs including olanzapine
Hypothalamus
(Arc and VMH)
Olanzapine
H1R
5-HT2CR
(-)
(-)
↓ POMC
↑NPY
↑AgRP
↑ AMPK
H1R
H1R
5-HT2CR
BAT
↓ UCP1, PGC-1α
↓ Locomotor Activity
(↓thermogenesis)
Food Intake↑
Adiposity↑
Weight
Gain↑
Energy
Expenditure↓

Figure 2.3 A proposed mechanism underlying SGA-induced body weight gain/obesity side-effects through regulation of energy intake and expenditure. On one hand, SGAs block the histamine H_1 receptors. The H_1R blockade by SGAs may cause a compensatory upregulation of H_1R density in the hypothalamus, and enhance hypothalamic AMPK, NPY and AgRP expressions. SGAs may downregulate POMC levels through acting on 5-$HT_{2C}R$. On the other hand, SGAs may reduce thermogenesis by attenuating UCP_1 and PGC-1α expressions in BAT, which could also be modulated by hypothalamic H_1R-AMPK signalling. In addition, decreased energy expenditure could also be due to reduced locomotor activity caused by SGA treatment.

Abbreviations: 5-$HT_{2C}R$, serotonin 5-HT_{2C} receptor; AgRP, agouti-related peptide; AMPK, active protein kinase; BAT, brown adipose tissue; H_1R, histamine H_1 receptor; NPY, neuropeptide Y; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; POMC, pro-opiomelanocortin; UCP_1, uncoupling protein 1.

2.2.6 Interventions/treatment for ameliorating SGA-induced weight gain side-effects

2.2.6.1 Current status of pharmacologic interventions for controlling SGA-induced weight gain

Regarding pharmacological interventions, a number of drugs have been trialled with some success in partially ameliorating SGA-induced weight gain side-effects. A meta-analysis study examined 25 pharmacologic weight loss intervention trials (n=1221) and revealed that amantadine, metformin, reboxetine, sibutramine and topiramate were effective in reducing SGA-induced weight gain (Baptista et al., 2008). Another meta-analysis of 32 placebo-controlled pharmacologic intervention trials involving 1482 subjects suggested that metformin had the most promising effect on weight loss, followed by fenfluramine, sibutramine, topiramate, and reboxetine (Maayan et al., 2010). Other clinical trials also showed a similar effect of metformin in attenuating antipsychotic-induced weight gain (Morrison et al., 2002; Baptista et al., 2008; Shin et al., 2009; Weaver et al., 2010; Jarskog et al., 2013). However, another study showed that co-treatment of metformin with risperidone had no significant effect in reducing weight gain (Arman et al., 2008). A recent study reported that both metformin and berberine treatment did not affect food intake, but significantly prevented olanzapine-induced brown fat loss (Hu et al., 2014). The same author further found that UCP_1 expression was significantly increased after co-treatment of metformin and olanzapine, compared with olanzapine only treatment (Hu et al., 2014). In addition, metformin and rosiglitazone can also reduce glucose intolerance and insulin resistance in patients treated with SGAs (Baptista et al., 2009; Ehret et al., 2010). The potential of zoisamide, sibutramine and topiramate have also been addressed as adjuvant treatments for weight

loss of schizophrenic patients treated with SGAs (Das et al., 2012; Fiedorowicz et al., 2012; Ghanizadeh et al., 2013).

It is worth noting that some pharmacological interventions can cause additional health risks (Maayan et al., 2010). For example, metformin led to lactic acidosis, especially in the elderly, nausea, vomiting and diarrhoea, while topiramate was associated with cognitive blunting (Maayan et al., 2010; Narula et al., 2010; Loke et al., 2011). However, to date, these pharmacological intervention studies were not based on the mechanism of SGA-induced weight gain, particularly considering H_1R and 5-$HT_{2C}R$ as key contributors for SGA-induced weight gain. Therefore, it is important to investigate the potential for targeting H_1R and 5-$HT_{2C}R$ to control SGA-induced weight gain, which has been addressed partially in the current study (by targeting H_1R).

2.2.6.2 The H_1R agonist as a target for controlling SGA-induced weight gain

As reviewed above, the antagonistic property of histaminergic H_1R is the major contributor to SGA-induced weight gain side-effects; therefore, there is great potential for controlling the weight gain by targeting H_1R. The question is therefore whether an H_1R agonist could be used to prevent and treat olanzapine-induced obesity. One candidate is FMPH, a selective H_1R agonist, which has been shown to have some potential to reverse olanzapine-induced hyperphagia after ICV injection (He et al., 2014). Unfortunately, it is unable to cross the blood-brain barrier (Malmberg-Aiello et al., 1998), and there is no other highly selective and orally deliverable H_1R agonist on the market.

Another significant candidate is betahistine ($C_8H_{12}N_2$), which is readily available in clinics with a highly safety profile (1:100000 reported adverse drug reactions), and has been used to treat more than 130 million patients suffering vestibular disorders such as vertigo and dizziness since the 1970s (Jeck-Thole and Wagner, 2006; Tighilet et al., 2007). It has been used as an anti-obesity drug in clinical trials. A randomised, double-blind placebo-controlled trial reported that 32 mg/day treatment of betahistine to 20 obese subjects for 28 days resulted in 1.1% weight loss, compared to 0.6% weight gain in the placebo group (Barak, 2008). Barak and colleagues also reported that 12 weeks' treatment of betahistine (16-48 mg/kg) in 281 adults led to significant weight loss in the subgroup of non-Hispanic women ≤50 years old with 48 mg/day betahistine treatment (Barak, 2008).

Betahistine acts as a modulator of the histaminergic system and has both H_1R-agonistic and H_3R-antagonistic properties in the brain (Yoshida et al., 2000; Fossati et al., 2001). Based on our previous study (Deng et al., 2012), it is proposed that, under normal conditions, histamine may activate H_1R on the hypothalamic neurons, leading to a decrease in food intake. However, olanzapine blocks histaminergic H_1R on the hypothalamic neurons causing an increase in food intake (Figure 2.4A). As an H_1R agonist, betahistine can directly activate H_1R and may compete with olanzapine for binding to H_1R, therefore reducing the H_1R antagonist property of olanzapine. On the other hand, betahistine, as an H_3R antagonist, increases histamine release by blocking presynaptic H_3R, which may augment its direct agonistic effects on H_1R (Figure 2.4B) (Deng et al., 2012).

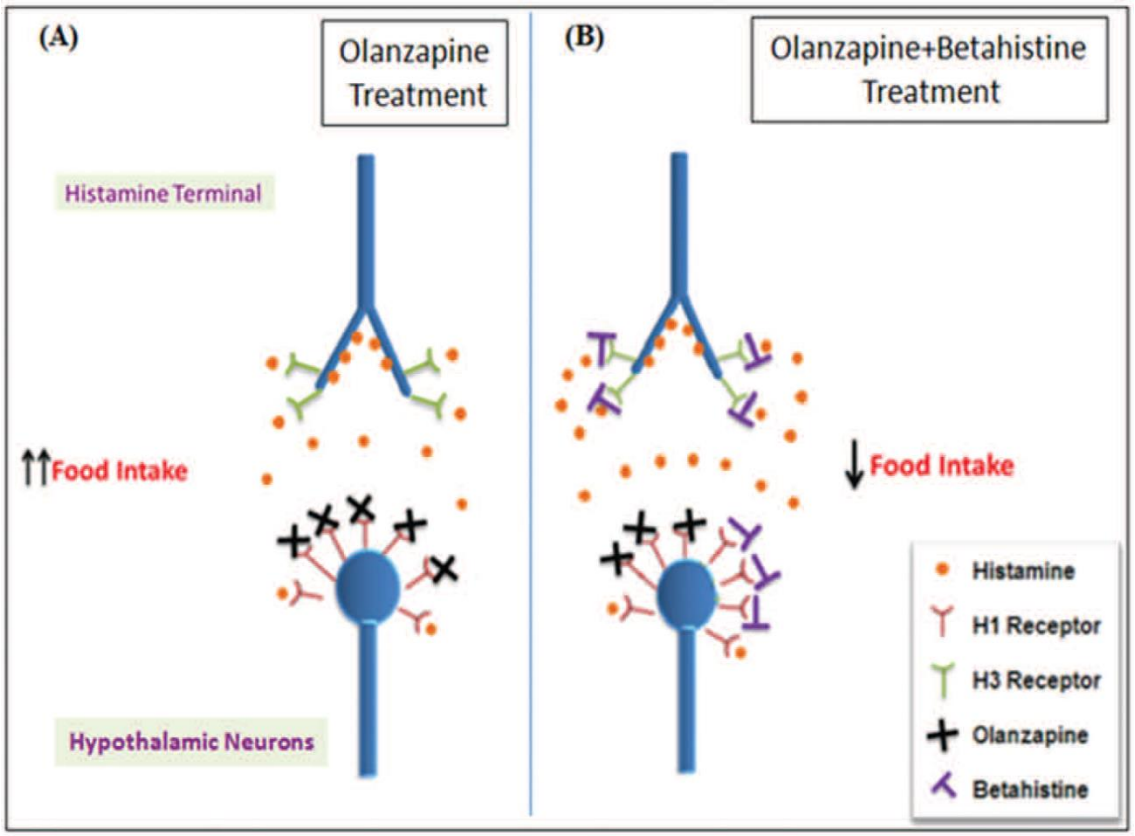


Figure 2.4 (A): The possible mechanism of Histamine H_1 and H_3 receptor regulation of food intake (Abbreviation: DVC-Dorsal vagal complex); (B): Olanzapine-induced body weight gain could be significantly reduced by co-administration with betahistine through activating postsynaptic H_1R and blocking presynaptic H_3R (From Deng et al., 2012, reprinted with permission of SAGE).

Interestingly, the combined histaminergic H_1R/H_3R action of betahistine has been proven to be efficient in increasing satiety and reducing the desire to eat fatty foods in rats (Szelag et al., 2001). Furthermore, a small clinical trial (in three first-episode schizophrenia patients) found that betahistine was able to reduce olanzapine-induced weight gain following 6 weeks' of co-treatment with olanzapine and betahistine (O+B) (Poyurovsky et al., 2005). The same author also reported that co-administration with olanzapine, betahistine and reboxetine (a selective norepinephrine reuptake inhibitor) in

first episode schizophrenia patients resulted in significantly attenuated weight gain compared with olanzapine-only treatment, while a betahistine and reboxetine combination treatment produced a two-fold larger weight attenuating effect, compared with reboxetine only combination (Poyurovsky et al., 2013).

Using the established rat model of olanzapine-induced weight gain, our previous study found that the short-term (2 weeks) co-treatment of O+B was effective to reduce (~45%) the weight-gain side-effect induced by olanzapine in drug-naïve rats (Deng et al., 2012) (Figure 2.5A). Food intake and feeding efficiency were also increased in rats treated with olanzapine compared to controls. Although it was not significant during the two weeks of treatment, rats co-administered with O+B consumed less food through the treatment period, and had significantly lower feeding efficiency than rats treated with olanzapine only (Figure 2.5B and C). However, no significant difference was observed in water intake (Figure 2.5D). These results illustrated that olanzapine can induce body weight gain, while co-treatment of O+B (2 weeks) may be used to reduce this side-effect (Deng et al., 2012).

Behavioural tests revealed that locomotor activity was decreased in olanzapine and O+B co-treatment compared with the control, while there was no difference between olanzapine and co-treatment groups (Figure 2.6A and B). In contrast, betahistine only treatment had no effect on locomotor activity compared with control (Figure 2.6A and B). The rats treated with both olanzapine-only and co-treatment of O+B had lower velocity compared with the control (Figure 2.6C) (Deng et al., 2012). These results suggested that reduced activity partially contributed to the weight gain side-effect induced by olanzapine. Co-treatment with betahistine did not improve locomotor

activities decreased by olanzapine. This finding may be one possible explanation of why betahistine can only partially improve olanzapine-induced weight gain (Deng et al., 2012).

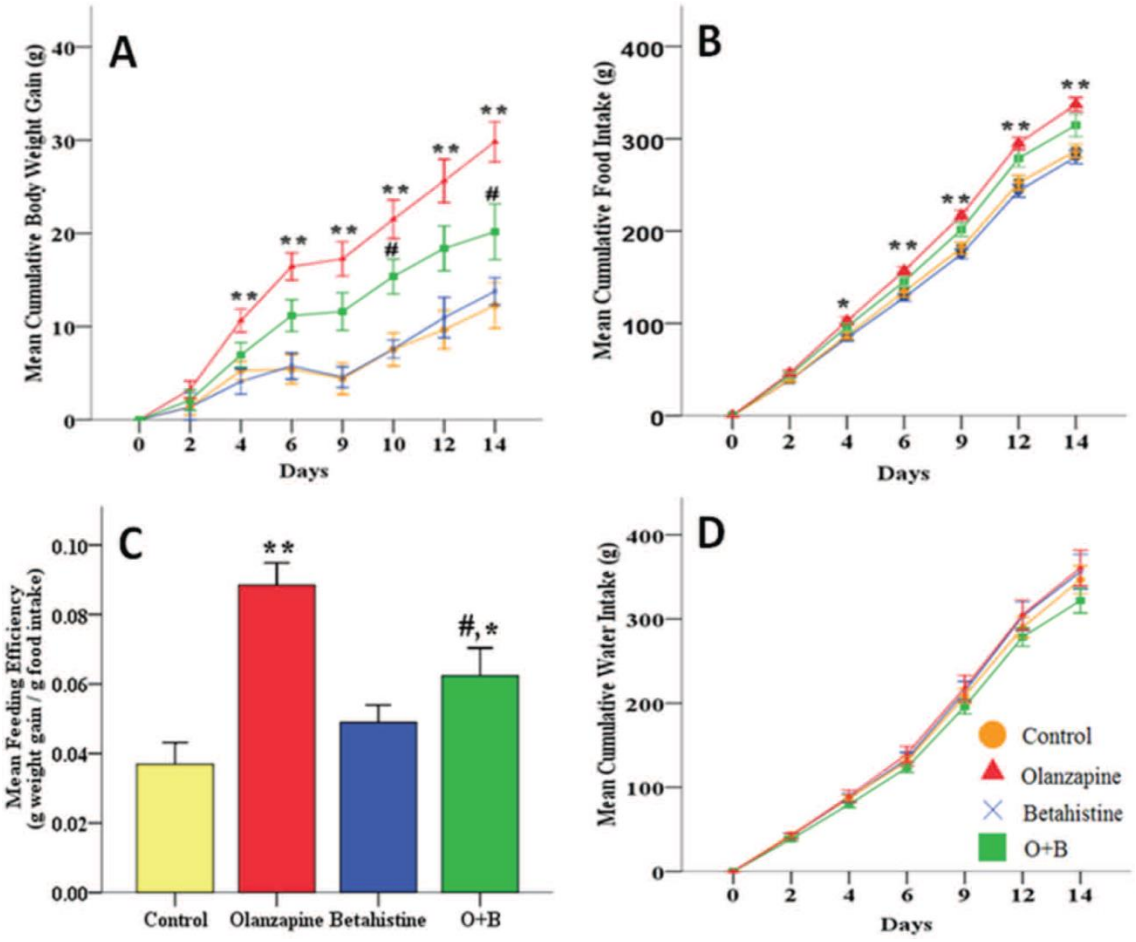


Figure 2.5 (A): Cumulative Body Weight Gain (g), (B): Cumulative Food Intake (g), (C): Feeding Efficiency (weight gain/food intake), (D): Cumulative Water Intake (g) of female Sprague Dawley rats treated with olanzapine (1 mg/kg, t.i.d.), betahistine (2.67 mg/kg, t.i.d.), co-treatment (O+B) or control (vehicle) for 14 days. **p<0.01 *vs.* control, #p<0.05 *vs.* olanzapine (From Deng et al., 2012, reprinted with permission of SAGE).

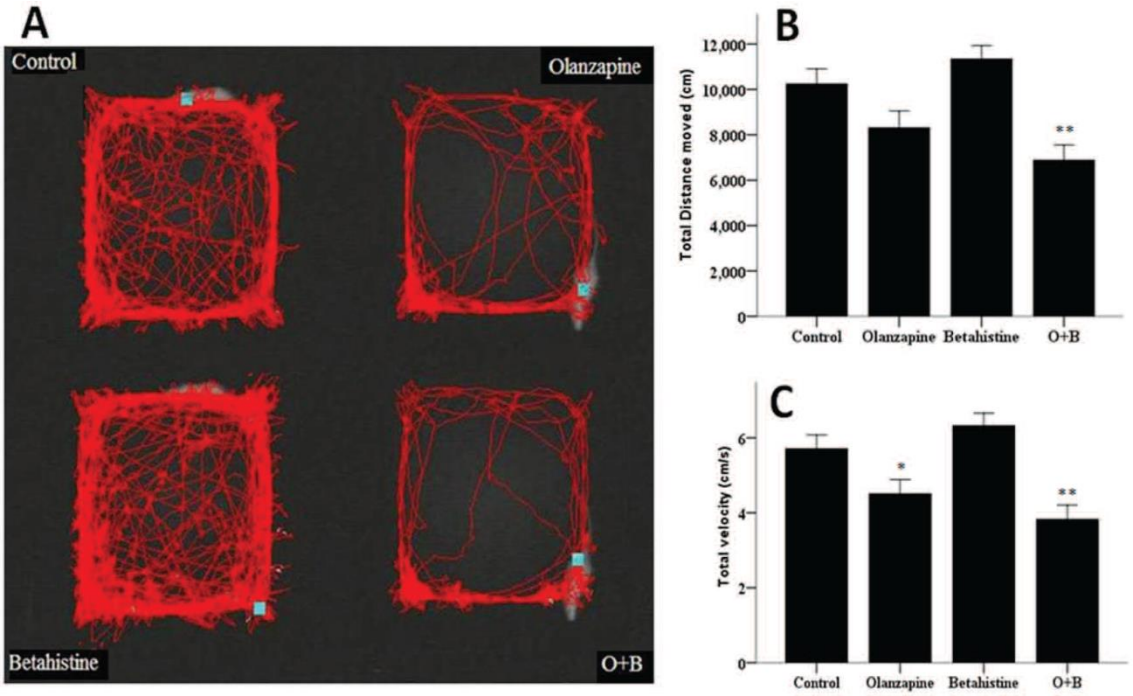


Figure 2.6 (A) Examples of locomotor activities from rats in the four treatment groups. The locomotor activities in the open field test were traced using the Ethovision software. (B) Total distance moved; (C) Velocity in the open field test. (O+B: co-treatment of olanzapine and betahistine). **p<0.05, **p<0.01 *vs.* control (From Deng et al., 2012, reprinted with permission of SAGE).

Overall, these findings further supported the important role of H_1R in the olanzapine-induced weight gain side-effects, and had important implications for clinical trials using betahistine to control olanzapine-induced obesity and its related metabolic disorders. However, the exact mechanism underlying betahistine's effect is not clear, which will be addressed in this thesis.

2.3 Rationales, aims and hypotheses

2.3.1 Rationales of this thesis

As reviewed above, a critical issue for schizophrenia patients is that control of their symptoms, often requires a life-time treatment of antipsychotic drugs. SGAs such as olanzapine are effective in treating the multiple domains of schizophrenia and are well-tolerated by patients, with a lower propensity to induce EPS side-effects. It is generally agreed that the pharmacological mechanisms of olanzapine therapeutics to treat schizophrenia can mainly be attributed to its relatively higher antagonistic affinity to 5-$HT_{2A}R$ and D_2R.

Unfortunately, many SGAs such as olanzapine cause serious side-effects such as weight gain, obesity, and other metabolic disorders. The management of these side-effects is possibly even more expensive and disruptive for families and society than the antipsychotic treatment itself (van Os and Kapur, 2009). Over the past decades, studies have revealed that hypothalamic H_1R and its downstream AMPK signalling, hypothalamic neuropeptides NPY and POMC, as well as BAT UCP_1 and PGC-1α signalling, may play key roles in the regulation of the olanzapine-induced weight gain side-effects.

Since H_1R antagonistic property is a main indicator for SGA-induced weight gain side-effects, it is important to investigate the potential to control SGA-induced weight gain by targeting H_1R. Our previous study has shown that a short-term (2 weeks) co-treatment with betahistine (a histamine H_1R agonist/H_3R antagonist) was effective in

preventing olanzapine-induced body weight gain in drug-naïve rats (Deng et al., 2012). However the underlying mechanism is still waiting to be revealed.

Therefore, it is critical to determine, using our established rat model, whether co-treatment with betahistine ameliorates olanzapine-induced weight gain and hyperphagia *via* modulating the hypothalamic H_1R, AMPK, as well as NPY, AgRP, POMC and CART (Han et al., 2008; Weston-Green et al., 2011; Deng et al., 2012). Furthermore, under clinical conditions, many chronic schizophrenic patients may have already been exposed to olanzapine treatment. Thus, it is also important to test whether betahistine would be able to prevent weight gain in subjects with chronic repeated olanzapine treatment. On the other hand, another key issue is to examine whether the co-treatment with betahistine affects the therapeutic effects of olanzapine. One way to address this issue is to examine the effects of co-treatment of olanzapine and betahistine on the key receptors such as 5-$HT_{2A}R$ and D_2R on the therapeutic effects of SGAs.

2.3.2 Aims

The **general aim** of this study is to investigate the effects and molecular mechanisms of betahistine on reducing olanzapine-induced weight gain in rat models.

The **specific aims** of this research were to:

1. Investigate whether co-treatment with betahistine is effective in reducing weight gain-associated with olanzapine in both drug-naïve subjects and rats with chronic and repeated exposure to olanzapine.

2. Reveal the molecular mechanisms underlying the effects of betahistine co-treatment in reducing olanzapine-induced weight gain side-effects in short-term treatment/drug-naïve subjects and chronic treatment/drug-naïve rats.

3. Examine whether betahistine co-treatment affects the key neurotransmission binding sites for antipsychotic efficacy (e.g. dopaminergic and serotonergic receptors) in the key brain regions, including the prefrontal cortex (PFC), cingulate cortex (Cg), nucleus accumbens (NAc), and caudate putamen (CPu).

2.3.3 Hypotheses

1. Co-treatment with betahistine will attenuate olanzapine-induced weight gain side-effects by activating hypothalamic H_1R-AMPK signalling, as well as activating NPY, AgRP, POMC and CART neuropeptides in drug-naïve subjects. (Chapter 3)

2. Betahistine co-treatment is effective to ameliorate olanzapine-induced body weight gain, in subjects with chronic and repeated exposure to olanzapine through modulating of H_1R-pAMPK signalling, hypothalamic NPY, POMC, and BAT $UCP_{1,}$ PGC-1α activities. (Chapter 4)

3. A short-term co-treatment with betahistine will not affect the key binding sites of therapeutic effects of olanzapine in the dopamine and 5-HT transmissions in a study of drug-naïve female rats. (Chapter 5)

4. Chronic co-treatment with betahistine will not affect the key binding sites for therapeutic effects of olanzapine in dopamine and 5-HT transmission in rats with repeated exposure of olanzapine. (Chapter 6)

Overall, in the thesis, all the above hypotheses have been tested in the established animal model for olanzapine-induced weight gain (Han et al., 2008; Weston-Green et al., 2011; Deng et al., 2012).

CHAPTER 3

BETAHISTINE AMELIORATES OLANZAPINE-INDUCED WEIGHT GAIN THROUGH MODULATION OF HISTAMINERGIC, NPY AND AMPK PATHWAYS

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Betahistine ameliorates olanzapine-induced weight gain through modulation of histaminergic, NPY and AMPK pathways

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KEYWORDS
Olanzapine;
Betahistine;
Histamine H_1 receptors;
AMP-activated protein kinase;
Neuropeptide Y;
Body weight gain;
Food intake

Summary Olanzapine is widely used to treat schizophrenia and other disorders, but causes adverse obesity and other metabolic side-effects. Both animal and clinical studies have shown that co-treatment with betahistine (a histaminergic H_1 receptor agonist and H_3 receptor antagonist) is effective for ameliorating olanzapine-induced weight gain/obesity. To reveal the mechanisms underlying these effects, this study investigated the effects of co-treatment of olanzapine and betahistine (O+B) on expressions of histaminergic H_1 receptor (H_1R), AMP-activated protein kinase (AMPK), neuropeptide Y (NPY), and proopiomelanocortin (POMC) in the hypothalamus associated with reducing olanzapine-induced weight gain. Olanzapine significantly upregulated the mRNA and protein expressions of H_1R, while O+B co-treatment significantly downregulated the H_1R levels, compared to the olanzapine-only treatment group. The NPY mRNA expression was significantly enhanced by olanzapine, but it was significantly reversed by O+B co-treatment. The hypothalamic H_1R expression was positively correlated with total food intake, and NPY expression. Olanzapine also increased AMPKα activation measured by the AMPKα phosphorylation (pAMPKα)/AMPKα ratio compared with controls, whereas O+B co-treatment decreased the pAMPKα/AMPKα ratio, compared with olanzapine only treatment. The pAMPKα/AMPKα ratio was positively correlated with total food intake and H_1R expression. Although olanzapine administration decreased the POMC mRNA level, this level was not affected by O+B co-treatment. Therefore, these results suggested that co-treatment with betahistine may reverse olanzapine-induced body weight gain *via* the H_1R-NPY and H_1R-pAMPKα pathways.

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CHAPTER 4

PREVENTING OLANZAPINE-INDUCED WEIGHT GAIN USING BETAHISTINE: A STUDY IN A RAT MODEL WITH CHRONIC OLANZAPINE TREATMENT

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Preventing Olanzapine-Induced Weight Gain Using Betahistine: A Study in a Rat Model with Chronic Olanzapine Treatment

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Abstract

Olanzapine is the one of first line antipsychotic drug for schizophrenia and other serious mental illness. However, it is associated with troublesome metabolic side-effects, particularly body weight gain and obesity. The antagonistic affinity to histamine H_1 receptors (H_1R) of antipsychotic drugs has been identified as one of the main contributors to weight gain/obesity side-effects. Our previous study showed that a short term (2 weeks) combination treatment of betahistine (an H_1R agonist and H_3R antagonist) and olanzapine (O+B) reduced (−45%) body weight gain induced by olanzapine in drug-naïve rats. A key issue is that clinical patients suffering with schizophrenia, bipolar disease and other mental disorders often face chronic, even life-time, antipsychotic treatment, in which they have often had previous antipsychotic exposure. Therefore, we investigated the effects of chronic O+B co-treatment in controlling body weight in female rats with chronic and repeated exposure of olanzapine. The results showed that co-administration of olanzapine (3 mg/kg, t.i.d.) and betahistine (9.6 mg/kg, t.i.d.) significantly reduced (−51.4%) weight gain induced by olanzapine. Co-treatment of O+B also led to a decrease in feeding efficiency, liver and fat mass. Consistently, the olanzapine-only treatment increased hypothalamic H_1R protein levels, as well as hypothalamic pAMPKα, AMPKα and NPY protein levels, while reducing the hypothalamic POMC, and UCP_1 and PGC-1α protein levels in brown adipose tissue (BAT). The olanzapine induced changes in hypothalamic H_1R, pAMPKα, BAT UCP_1 and PGC-1α could be reversed by co-treatment of O+B. These results supported further clinical trials to test the effectiveness of co-treatment of O+B for controlling weight gain/obesity side-effects in schizophrenia with chronic antipsychotic treatment.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

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Introduction

Second generation antipsychotic drugs have surpassed first-generation agents as the first line of treatment for schizophrenia. Among them, olanzapine is one of the most widely prescribed antipsychotic drugs to treat schizophrenia and other serious mental disorders such as bipolar disorder, dementia, major depression, and Tourette's syndrome due to its enhanced tolerability [1–6]. Unfortunately, olanzapine, along with clozapine, have the highest risk for substantial weight gain, obesity and other serious metabolic disorders including type II diabetes mellitus, with increased risk for cardiovascular disease and premature death [6–13].

Olanzapine has high binding affinities with multiple neurotransmitter receptors including dopamine D_2, serotonin 5-HT_{2A} and 5-HT_{2C}, histamine H_1 receptors, and muscarinic M_1 and M_3 receptors [8,14]. While D_2 and 5-HT_{2A} receptors play a critical role in the therapeutic effects of olanzapine [15,16], evidence indicates that the H_1, 5-HT_{2C}, and M_3 receptors are involved in antipsychotic-induced metabolic side-effects [8,9,17–24]. Strong evidence suggests that H_1 receptor antagonism is the key factor contributing to olanzapine/clozapine-induced weight gain and obesity [9,18,19,24–26]. In fact, a significant association of interaction between the genetic variants of H_1 receptors (rs346074-rs346070) and BMI/obesity has been identified recently in non-affective psychotic disorder patients treated with the high-H_1 receptor affinity antipsychotics olanzapine, clozapine and quetiapine [27].

Several animal studies have found that olanzapine could modulate histaminergic neurotransmission for the regulation of food intake and weight gain in rats [28,29]. Further evidence showed that weight gain and obesity associated with olanzapine and clozapine are mediated by activation of the hypothalamic AMP-activated protein kinase (AMPK) pathway via blockade of

H_1 receptors [25,30–32]. In fact, a recent study revealed an association between polymorphisms in the AMPK gene and weight gain induced by olanzapine and clozapine [33]. Additionally, it was reported that olanzapine down-regulates the anorexigenic neuropeptide proopiomelanocortin (POMC), but up-regulates the orexigenic neuropeptide Y (NPY), in the arcuate nuclei of the hypothalamus (Arc) [34–36]. Furthermore, reduced activation of the brown adipose tissue (BAT) is associated with obesity and diabetes in humans [37]. The BAT is enriched for uncoupling protein 1 (UCP_1) [38], which is involved in olanzapine-induced weight gain observed in rat models [39–41]. The peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and PGC-1β control mitochondrial biogenesis, which plays a critical role in the BAT thermogenesis [42], and is related with olanzapine-induced weight gain [39,41,43]. There is evidence that activation of BAT UCP_1 and PGC-1α are also modulated by the hypothalamic H_1R-AMPK pathways [44,45]. Therefore, it may be possible to control the antipsychotic-induced weight gain by modulating hypothalamic H_1 receptors and related pathways.

Recently we found that a short-term (2 weeks) co-treatment with betahistine (an H_1R agonist/H_3R antagonist) and olanzapine resulted in a −45% reduction of weight gain in drug-naïve rats compared to those treated solely with olanzapine [46]. This finding was confirmed by a recent short-term (6-week) clinical trial in which first episode schizophrenia patients with a combination treatment of olanzapine, betahistine and reboxetine (a selective norepinephrine reuptake inhibitor) had significantly less weight gain than those treated with olanzapine only [47], while betahistine+reboxetine combination treatment produced a two-fold larger weight-attenuating effect than reboxetine treatment alone [47,48].

These animal and clinical results from short-term trials supported the effects of betahistine in attenuating olanzapine-induced weight gain in drug naïve subjects [46]. It is worth noting that clinical patients suffering with schizophrenia, bipolar disease and other mental disorders often face chronic, even life-time, treatment with antipsychotic drugs [5]. Since betahistine has a very high safety profile with extremely low (1:100,000) adverse drug reaction [49], it has a huge potential for chronic management of antipsychotic-induced weight gain and obesity in schizophrenia and other mental disorders. It is important to note that antipsychotics cause a significant body weight gain not only in drug-naïve patients, but also in chronic patients who usually have already had previous antipsychotic exposure [5,13,26]. However it was not clear whether chronic co-treatment of betahistine and olanzapine would have similar weight-attenuating effects, so this was addressed in this chronic animal study. Furthermore, the effects of chronic co-treatment of olanzapine and/or betahistine on the protein levels of H_1 receptors, AMPKα, pAMPKα, NPY and POMC in the hypothalamus, as well as UCP_1, PGC-1α and PGC-1β levels in the BAT were also investigated.

Materials and Methods

Animals housing and measurements

Forty-eight female Sprague–Dawley rats (201–225 g) were obtained from the Animal Resources Centre (Perth, WA, Australia). In order to reduce potential stress caused by transportation, rats were housed in pairs for 1 week prior to the start of the experiment. They were allowed *ad-libitum* access to water and standard laboratory chow diet (3.9 kcal/g; 10% fat, 74% carbohydrate and 16% protein) throughout the whole experiment. During the experiment, they were housed in individual cages under environmentally controlled conditions (22°C, light cycle from 07:00 to 19:00 and dark cycle from 19:00 to 07:00). Body weight, food intake and water intake were measured twice per week. All experimental procedures have been approved by the Animal Ethics Committee, University of Wollongong, Australia (AE11/10); and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004).

Drug preparation and treatment

Prior to drug treatment, rats were trained for oral treatment procedures by feeding cookie-dough without drugs (0.3 g) for one week. In brief, the pellets with drugs were made prior by mixing droplets of water with cookie dough powder (containing 30.9% cornstarch, 30.9% sucrose, 6.3% gelatine, 15.5% casein, 6.4% fibre, 8.4% minerals, and 1.6% vitamins) [46,50,51]. Controls received an equivalent pellet without drug. Rats were observed during treatment administration to ensure complete consumption of the medication pellet. Water bottles were carefully monitored for leakage, and cages were checked for uneaten food.

Rats were administered the treatments in 3 phases (Figure 1A). In Phase 1, 48 rats were divided into two groups during the first 3.5 weeks (Day 0–23); one half of them (n = 24) were treated with olanzapine (1 mg/kg, t.i.d.), and the other half treated with vehicle. In Phase 2, from Day 23, olanzapine was withdrawn for 19 days; all rats did not receive any treatment during this period. In Phase 3, from week 6, the two groups were divided into 4 subgroups (n = 12) for further treatment of 5 weeks (Figure 1A): (1) olanzapine (1 mg/kg, t.i.d.), (2) co-treatment of olanzapine and betahistine, (3) betahistine (9.6 mg/kg, t.i.d.), and (4) control (vehicle). Drugs were administrated at the dosages mentioned above 3 times per day (07:00h, 14:00h, and 23:00h; with 8±1 hour interval).

After completing treatment, all rats were sacrificed (without fasting) by carbon dioxide asphyxiation. Post-mortem white adipose tissue including perirenal, periovary, inguinal and mesentery fat, sub-scapular brown adipose tissue, as well as the liver, were dissected and individually weighed [46,52]. Body length and femur length were also measured and recorded to ascertain the effect of body growth on the body weight of rats.

Liver histology

The liver lipid accumulation was examined using haematoxylin and eosin stains (HE; Sigma, St Louise, USA) [53,54]. In brief, frozen livers of rats were sectioned 10 μm thick using a cryostat (LEICA, Wetzlar, Germany) and the slides were air dried at room temperature for 60 minutes. Then they were fixed with ice cold 10% formalin for 5 minutes, followed by air drying for another 60 minutes and rinsed immediately in 3 changes of distilled water. For HE staining, after drying the slides for 30 seconds at room temperature, they were placed in xylene for 1 minute, followed by 100%, 95%, 80% and 70% ethanol for 1 minute, respectively. After dipping in distilled water for 30 seconds, haematoxylin staining was performed for 5 minutes, dipping into dH_2O again, and then placing the slides in Eosin solution for 2 minutes. The dehydration procedures were performed as follows: after the slides were dipped in dH_2O, 70%, 80%, 95% and 100% ethanol were conducted for 30 seconds or 1 minute.

Western blotting

Brain samples were taken 2 hours after the final drug treatment. Using the micro-dissection procedures established in our laboratory [32,36], the hypothalamic nuclei were dissected. The dissection targeted the Arc in an overlapping pattern over the third ventricle [55]. Since the Arc is small, the punched tissue

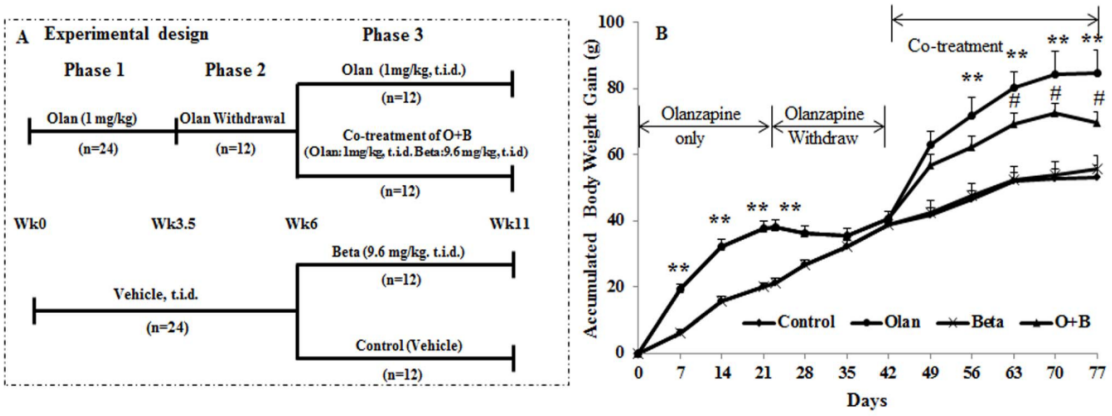

Figure 1. Effects of olanzapine and/or betahistine treatment on body weight gain. A: Outline of the experimental design. B: The trend of three phases of drug administration on the accumulated body weight side-effect. Olanzapine (1 mg/kg, t.i.d.; n = 12), betahistine (9.6 mg/kg, t.i.d.; n = 12), co-treatment (O+B; n = 12) or control (vehicle; n = 12) for 11 weeks. (◆: control ●: olanzapine, x: betahistine, ▲: O+B co-treatment). * $p<0.05$, ** $p<0.01$ *vs.* control, # $p<0.05$ *vs.* olanzapine.
doi:10.1371/journal.pone.0104160.g001

contained Arc and adjacent ventromedial nucleus (VMH); therefore the punched tissue was labelled as the mediobasal hypothalamus. The dissected brain tissue was placed into 0.5 mL Precellys Homogenising tubes and homogenised in ice-cold homogenising buffer [9.8 ml NP40 cell lysis buffer (Invitrogen, Camarillo, CA, USA), 100 µl β-Glycerophosphate (50 mM; Invitrogen), 33.3 µl PMSF (0.3M; Sigma-Aldrich, St Louis, MO, USA), and 100 µl Protease Inhibitor Cocktail (Sigma-Aldrich)]. The total protein concentrations of the tissue lysate were determined by the Bio-Rad DC Protein Assay (500-0116, Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard. The samples were centrifuged, and the supernatants were collected and stored at −80°C until required.

Homogenised brain samples containing 10 µg of protein were first heated at 95°C using a digital dry bath (Labnet International, USA) for 15 minutes in loading buffer containing 950 µl laemmli buffer (Bio-Rad) and 50 µl β-mercaptoethanol (Sigma-Aldrich) to denature the protein of the samples. Then, the samples were loaded into CRTGEL4–12% Bis-Tris Polyacrylamide Gels (Bio-Rad) including one channel of Precision plus Dual Colour protein Standards (Bio-Rad). The samples were subjected to electrophoresis in 1× XT-MOPS running buffer [50 ml 20× XT-MOPS running buffer (Bio-Rad) and 950 ml distilled water] at 100 V for 15 minutes followed by 200 V for 55 minutes. The separated proteins were then transferred electrophoretically onto a non-specific protein binding polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in the ice cold transfer buffer (150 ml 10× Tris/Glycine Buffer (Bio-Rad), 300 ml cold methanol and 1050 ml distilled water) at 100 V for one hour. The PVDF membranes were incubated in the Tris-Buffered Saline-Tween (TBST) (Sigma-Aldrich) solution containing 5% BSA for one hour at room temperature for blocking the remaining non-specific protein binding pores on the PVDF membrane. Each membrane was then incubated in the primary antibodies including anti-AMPKα (1:1000; Cell Signaling Technology, Beverly, MA, USA, #2532), anti-phospho-AMPKα (1:1000; Cell Signaling, #2535) and anti-histamine H_1 (1:1000; Santa Cruz Biotechnology, Dallas, USA, #SC-20633), anti-POMC (1:1000, Santa Cruz, # SC-20148), anti-UCP_1 (1:1000; Santa Cruz Biotechnology, #SC-6529), anti-PCG-1α (1:1000; Santa Cruz Biotechnology, #SC-13067) and anti-PGC-1β (1:1000; Abcam, #AB130741), which were diluted in TBST and 1% BSA buffer overnight at 4°C. Each membrane was washed 3×5 minutes in TBST buffer, followed by incubation for 1 hour at room temperature (RT) with horseradish peroxidise (HRP)-conjugated goat anti-rabbit (1:2000; Millipore, Billerica, MA, USA) or donkey anti-goat (1:2000, Santa Cruz Biotechnology) as secondary antibodies. The membranes were then each washed 3×5 minutes in TBST buffer at RT. The proteins of interest were visualised by reacting the membranes with Luminata Crescendo Western HRP Substrate (Millipore) via incubation, and exposing them to Amersham Hyperfilm ECL (GE Healthcare Life Science). Membranes were then re-probed with mouse anti-actin primary polyclonal antibody (1:10000; Millipore, Temecula, CA) and HRP-conjugated rabbit anti-mouse secondary antibody (1:3000; Millipore, Temecula, CA). The immunoreactive signals were quantified by densitometry and the values were corrected based on their corresponding actin levels. All results were normalised by taking the value of the vehicle group as 100%. Experiments were performed in duplicate.

Enzyme immunoassay (EIA)

The NPY EIA Kit (Phoenix Pharmaceutical, USA) was performed to determine the hypothalamic NPY level using the homogenised hypothalamic Arc tissue, which was prepared for the above western blot experiments.

Statistical analysis

Statistical analysis was performed using SPSS (version 19.0, IBM SPSS Statistics, USA). The Kolmogorov-Smirnov test was used to examine the distribution of data from all experiments. Body weight gain, food intake and water intake data from Phase 1 and 2 were analysed by two-way ANOVAs (DRUG TREATMENT×TIME as repeated measures). The Phase 3 data on body weight gain, food intake and water intake were analysed by three-way repeated ANOVAs (OLANZAPINE×BETAHISTINE×TIME as repeated measures). Two-way ANOVAs was used to compare the levels of NPY, H_1R, AMPKα, pAMPKα, POMC, UCP_1, PGC-1α and PGC-1β. Multiple comparisons were performed using a *post-hoc* Dunnett-T test. Pearson's or Spearman correlation tests were used to assess the relationships among these

measurements. For the data without abnormal distribution, a Mann-Whitney U test was applied. All data were presented as mean ± SEM, and statistical significance was accepted when $p<0.05$.

Results

Effects of olanzapine and/or betahistine on weight gain, food intake and feeding efficiency

Phase 1. Effects of olanzapine treatment. Figure 1B presents the accumulated body weight gain over the experimental period. In Phase 1, olanzapine treatment significantly increased body weight gain compared to vehicle through the treatment period of 3 weeks (all $p<0.001$) (Figure 1B). Consistent with weight gain changes, olanzapine significantly increased food intake through the treatment period (all $p<0.05$; Figure 2A). Furthermore, feeding efficiency (grams of body weight gain/grams of food intake) was significantly elevated by olanzapine treatment compared with the vehicle ($p<0.001$) (Figure 2B). However, there was no significant change of water intake in this phase ($p>0.05$).

Phase 2. Effect of olanzapine withdrawal. Following olanzapine withdrawal, the weight difference between the olanzapine-treated rats and vehicle were gradually narrowed: initially, olanzapine-treated rats had a significantly higher weight gain than the vehicle group ($p<0.001$), the weight loss of rats was detected following olanzapine withdrawal (Figure 1B). The weight of rats in the olanzapine group then reduced gradually to a level similar to the rats in the vehicle group after 12 days of olanzapine withdrawal ($p>0.05$), and remained at the same level as the control for the rest of the period of olanzapine withdrawal ($p>0.05$). Consistent with the changes in weight loss, olanzapine withdrawal led to a sharp decrease in food intake and remained at a lower level for 1.5 weeks compared to the vehicle group (Figure 2C), then gradually returned to a level similar to the vehicle group (Figure 2C). In contrast to olanzapine treatment, olanzapine withdrawal caused a significant decrease in feeding efficiency compared to the vehicle group ($p<0.001$) (Figure 2D). Similar to the first phase, no water intake difference between the groups was identified ($p>0.05$).

Phase 3. Effect of chronic betahistine co-treatment in reducing olanzapine-induced weight gain. As shown in Figure 1A, from week 7 to week 11, the rats were divided into four groups: olanzapine-only, olanzapine+betahistine (O+B) co-treatment, betahistine-only, and control (vehicle). In the olanzapine-only group, resumed olanzapine treatment significantly increased body weight gain compared to the control through the 5 weeks treatment period (all $p<0.01$; Figure 1B). On the other hand, although the O+B co-treatment group had a higher weight gain than the control and betahistine-only groups (all $p<0.01$; Figure 1B), it appeared to have a significantly lower body weight gain than the olanzapine-only group after 3 weeks' co-treatment ($p<0.05$; Figure 1B) and in total reduced −51.4% following 5 weeks' co-treatment ($p<0.05$). However, the betahistine-only treatment had no significant difference in weight gain compared to the control group (all $p>0.05$; Figure 1B). Therefore, co-treatment of betahistine and olanzapine can partly reduce/prevent weight gain induced by chronic olanzapine treatment (Figure 1B). There were no significant differences in the body or femur length among all treatment groups and the controls (Table 1), which suggested that none of the treatments affect animal growth.

The olanzapine-only treatment significantly increased food intake compared to the control for 3 weeks' of treatment ($p<0.01$; Figure 2E), then gradually reduced to a level similar to other groups. The O+B co-treatment significantly increased food intake compared with the control for the first 2 weeks' of co-treatment ($p<0.01$; Figure 2E), then it gradually declined similar to the control. Although no significant difference in food intake was detected between the O+B co-treatment group and olanzapine-only group (Figure 2E), the O+B co-treatment had a significantly lower feeding efficiency than the olanzapine-only treatment group ($p<0.05$). Therefore, O+B co-treatment was effective in decreasing feeding efficiency compared to the olanzapine-only treatment.

Fat deposits

Compared to the control, rats with olanzapine-only treatment had a significantly higher inguinal fat mass ($p<0.01$), periovary fat ($p<0.05$), and mesentery fat ($p=0.01$; Table 1). The olanzapine-only treatment group also had significantly higher inguinal fat ($p<0.01$), perirenal fat ($p<0.05$), periovary fat ($p<0.01$), and mesentery fat ($p<0.01$) than betahistine-only treatment. It is important that the rats with O+B co-treatment had significantly less inguinal fat mass than those with olanzapine-only treatment ($p=0.015$) and tended to have less periovary fat ($p=0.094$) and mesentery fat ($p=0.074$) than olanzapine-only treatment group (Table 1). However, there was no significant difference in subscapula brown fat mass among all treatment groups and controls (Table 1).

Liver weight and morphological changes

The rats with olanzapine-only treatment had significantly higher liver weight than controls ($p<0.01$) and those with betahistine-only treatment ($p<0.01$, Table 1). In contrast, the rats with O+B co-treatment had significantly lower liver weight than those with olanzapine-only treatment ($p<0.05$). Consistently, the HE stain showed that there was a significantly higher fat cell count in the olanzapine-only treatment group than controls, while there was a significantly lower fat cell count in the O+B co-treatment group than the olanzapine-only group ($p<0.001$; Figure 3E). In addition, the olanzapine-only group tended to have larger total fat cell areas than the control ($p=0.073$) and the O+B co-treatment group ($p=0.086$; Figure 3F).

Effects of olanzapine and/or betahistine treatment on the protein expression of hypothalamic H_1R, AMPKα, pAMPKα, NPY and POMC

Compared to the control, olanzapine treatment significantly increased the protein levels of H_1R (+37%, $p=0.003$; Figure 4A and B). The O+B co-treatment significantly decreased H_1R expression compared with the olanzapine-only treatment (−26%, $p=0.009$; Figure 4A and B). In terms of the protein expression of AMPKα, both olanzapine-only and co-treatment of O+B significantly enhanced the AMPKα level compared to the control (olanzapine only *vs.* control, +22%, $p=0.015$; co-treatment of O+B *vs.* control, +20%, $p=0.025$; Figure 4A and C). Both olanzapine-only treatment and co-treatment of O+B significantly enhanced the protein expression of pAMPKα compared with the control (olanzapine only *vs.* control, +51%, $p=0.001$; co-treatment of O+B *vs.* control, +29%, $p=0.047$; Figure 4A and D). However, the O+B co-treatment reduced the pAMPKα protein level compared with olanzapine-only treatment at a borderline significance (−22%, $p=0.054$; Figure 4A and D). Additionally, the NPY peptide was significantly up-regulated by olanzapine-only treatment ($p=0.047$), and co-treatment of O+B tended to elevate the NPY level compared to controls ($p=0.055$) (Figure 4F). On the other hand, compared with the control, olanzapine-only treatment had a significant effect in decreasing hypothalamic POMC protein levels (−52%, $p=0.016$), while co-

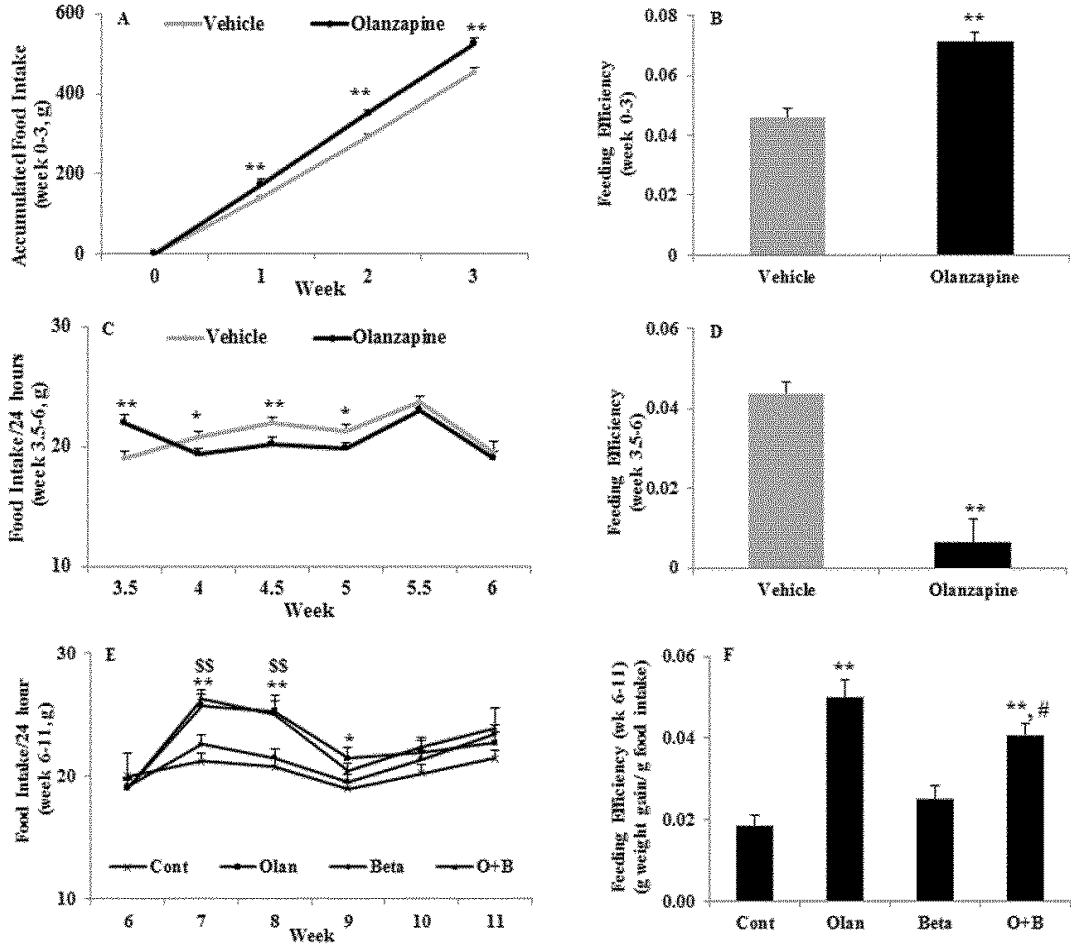

Figure 2. Effects of olanzapine and/or betahistine treatment on food intake and feeding efficiency. A–B: Accumulated food intake (A) and feeding efficiency (B) in the first phase of olanzapine treatment (1 mg/kg, t.i.d.; n = 12) compared with vehicles. C–D: Food intake (C) and Feeding efficiency (D) following olanzapine withdrawal. E–F: Food intake (E) and feeding efficiency (F) following chronic treatment of olanzapine (1 mg/kg, t.i.d.; n = 12), betahistine (9.6 mg/kg, t.i.d.; n = 12), co-treatment (O+B; n = 12) or vehicle (control; n = 12) for 5 weeks. (♦: control ●: olanzapine, x: betahistine, ▲: O+B co-treatment). * $p<0.05$, ** $p<0.01$, olanzapine *vs.* control; \$ $p<0.05$, \$\$ $p<0.01$, co-treatment of O+B *vs.* control; # $p<0.05$ O+B *vs.* olanzapine.
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treatment of O+B had no effect on POMC levels ($p>0.05$; Figure 4A and E).

Hypothalamic H_1R protein expression was positively correlated with total body weight gain ($r = 0.403$, $p = 0.028$), total food intake ($r = 0.486$, $p = 0.009$) and tended to correlate with feeding efficiency ($r = 0.207$, $p = 0.085$). In addition, the hypothalamic AMPKα expression also positively correlated with body weight gain ($r = 0.750$, $p = 0.000$), total food intake ($r = 0.553$, $p = 0.003$) and feeding efficiency ($r = 0.617$, $p = 0.001$). The protein expression of hypothalamic pAMPKα was positively correlated with total body weight gain ($r = 0.668$, $p = 0.000$), total food intake ($r = 0.515$, $p = 0.006$), as well as feeding efficiency ($r = 0.555$, $p = 0.003$). There were positive correlations between hypothalamic H_1R and AMPKα ($r = 0.518$, $p = 0.006$) and pAMPKα ($r = 0.444$, $p = 0.017$), and, there were negative correlations among hypothalamic POMC protein expression and body weight gain ($r = -0.456$, $p = 0.014$) and feeding efficiency ($r = -0.435$, $p = 0.019$). The hypothalamic NPY peptide level was positively correlated with body weight gain ($r = 0.382$, $p = 0.036$), and feeding efficiency ($r = 0.392$, $p = 0.032$).

Effects of olanzapine and/or betahistine treatment on the protein expression of UCP_1, PGC-1α, and PGC-1β in brown adipose tissue

Olanzapine significantly down-regulated BAT UCP_1 protein expression by 44% ($p = 0.024$), compared with the control, while co-treatment of O+B significantly reversed the decreased UCP_1 protein level by 43% caused by the olanzapine only treatment ($p = 0.037$) (Figure 5A and B). Similarly, BAT PGC-1α protein expression was downregulated by 21% ($p = 0.037$) under olanzapine-only treatment, whilst, it was reversed significantly by co-treatment of O+B ($p = 0.023$, Figure 5A and C). However, for PGC-1β protein expression, no any significant change was observed among the treatment groups (all $p>0.05$). Additionally,

Table 1. Mean fat mass, liver weight, and body length (mean ± SEM) in female Sprague Dawley rats treated with olanzapine (1 mg/kg, t.i.d.) and/or betahistine (9.6 mg/kg, t.i.d.) or control (vehicle).

	Control	Olanzapine	Betahistine	O+B
Fat pad mass (g) Inguinal	3.69±0.26	5.17±0.33**	2.85±0.20*,##	4.25±0.23#
Perirenal	4.95±0.45	5.54±0.40	3.75±0.37#	5.58±0.44
Periovary	6.81±0.54	8.82±0.65*	5.17±0.51##	8.30±0.64
Mesentery	2.54±0.18	3.41±0.30*	2.23±0.16##	3.03±0.20
Brown Fat	0.39±0.03	0.44±0.04	0.37±0.03	0.39±0.02
Liver (g)	12.09±0.26	13.76±0.44**	12.35±0.28##	12.78±0.39#
Body Length (cm)	22.30±0.21	22.54±0.13	22.71±0.15	22.41±0.10
Femur Length (cm)	5.26±0.04	5.19±0.02	5.24±0.03	5.20±0.02

*, $p<0.05$;
**, $p<0.01$ *vs.* control;
#, $p<0.05$;
##, $p<0.01$ *vs.* olanzapine.
doi:10.1371/journal.pone.0104160.t001

BAT UCP_1 and PGC1-α expressions in the BAT were negative correlated with hypothalamic pAMPKα levels ($r=-0.246$, $p=0.051$; $r=-0.374$, $p=0.040$).

Discussion

Long term antipsychotic use remains mainstay treatment in patients with schizophrenia. Clinical trials in the past two decades have proven that, whether in first episode/antipsychotic-naïve patients or in chronic schizophrenia patients with previous antipsychotic exposure, antipsychotic administration (particularly olanzapine and clozapine) can cause significant weight gain [5,7,13,56]. Similar to a previous report [57], the present study showed a withdrawal of oral olanzapine treatment also resulted in weight loss that was largely due to the decrease of food intake and feeding efficiency. Similar to the clinical findings, our results illustrated that, after drug withdrawal for over 2.5 weeks, the resumed olanzapine treatment significantly increased body weight gain [5,15,58,59]. Therefore, this study provided an animal model which mimicked closely the body weight changes caused by olanzapine in drug-naïve and re-administered chronic treatment patients.

The present study was the first in a chronic animal model to detect the effect of chronic O+B co-treatment on reducing the body weight gain side-effect in subjects with chronic olanzapine exposure. The results showed that chronic O+B co-treatment produces a significant weight-attenuating effect appearing after 1 week and being statistically significant after 3-week co-treatment, with about ~50% weight gain decrease compared to olanzapine-only treatment. Previously, a short-term study in drug-naïve rats found that 2-week O+B co-treatment significantly reduced (~45%) body weight gain [46]. Consistently with our short-term experiment, betahistine-only treatment showed no effect on weight gain and feeding efficiency [46]. A recent clinical trial reported that antipsychotic drug-naïve schizophrenia patients with a six-week combination treatment of olanzapine (10 mg, once daily), betahistine (48 mg, t.i.d.) and reboxetine (4 mg, b.i.d.) (a selective norepinephrine reuptake inhibitor) had significantly less weight gain than those on olanzapine only [47]. In addition, a six-week trial with 3 first episode schizophrenic patients also found that betahistine (48 mg, t.i.d.) was able to prevent weight gain related to olanzapine treatment (10 mg, once daily) [60]. It is of note that both the clinical and animal studies have indicated a time-dependent effect of antipsychotic (including olanzapine)-induced weight gain. There are three stages of development of weight gain/obesity; an early acceleration stage with a rapid increase in body weight, a middle stage with continuing body weight increase following at a steadier rate, followed by a "plateau" stage maintaining a heavier weight with ongoing antipsychotic treatment [58,61]. It is interesting that O+B co-treatment had a stronger weight gain reducing effects on the "plateau" stage (Figure 1B). Further studies are worth to investigate the effects if olanzapine dose was increased at this point, and the effects on the antipsychotics with less pronounced weight gain side-effects (as a negative control). The betahistine dosage (9.6 mg/kg rat body weight) used in this study is equivalent to ~93 mg/kg in humans (60 kg body weight) according to dosage translation between species based on body surface area following the FDA guideline [62]. Betahistine has 3–4 hours of plasma half-life in humans with one day of urine excretion, but no data showed the half-life of betahistine in rats [63]. Although there is no data available for the half-life of betahistine in rats, it is reasonable to suppose that betahistine is most likely to have a shorter half-life in rats than in humans. Therefore, the betahistine dosage (9.6 mg/kg rat body weight) used in this study should be relevant to the human dosage (48 mg, t.i.d.) used in clinical trials [47,60]. Taken together, results from the animal model and schizophrenia patients support the theory that both short-term and chronic co-treatment with betahistine should be effective to control olanzapine-induced weight gain in both drug-naïve subjects and those with previous antipsychotic exposure.

Consistent with the body weight changes in this study, the olanzapine-only group had more white fat mass and higher liver weight than the control and betahistine-only groups, which also corresponded with previous reports [31,46,51,64,65]. On the other hand, compared to the olanzapine-only treatment, chronic O+B co-treatment decreased inguinal fat mass and liver weight in this study. Further HE staining confirmed that olanzapine-only treatment significantly increased fat accumulation in the liver; however O+B co-treatment reduced liver fat accumulation. These results suggested that weight gain decrease in rats treated with O+B was at least partially from reduced fat accumulation. Further study is needed to investigate changes in lipid metabolism. There was no difference in body and femur length among these groups, which indicated that none of the treatments affected animal growth.

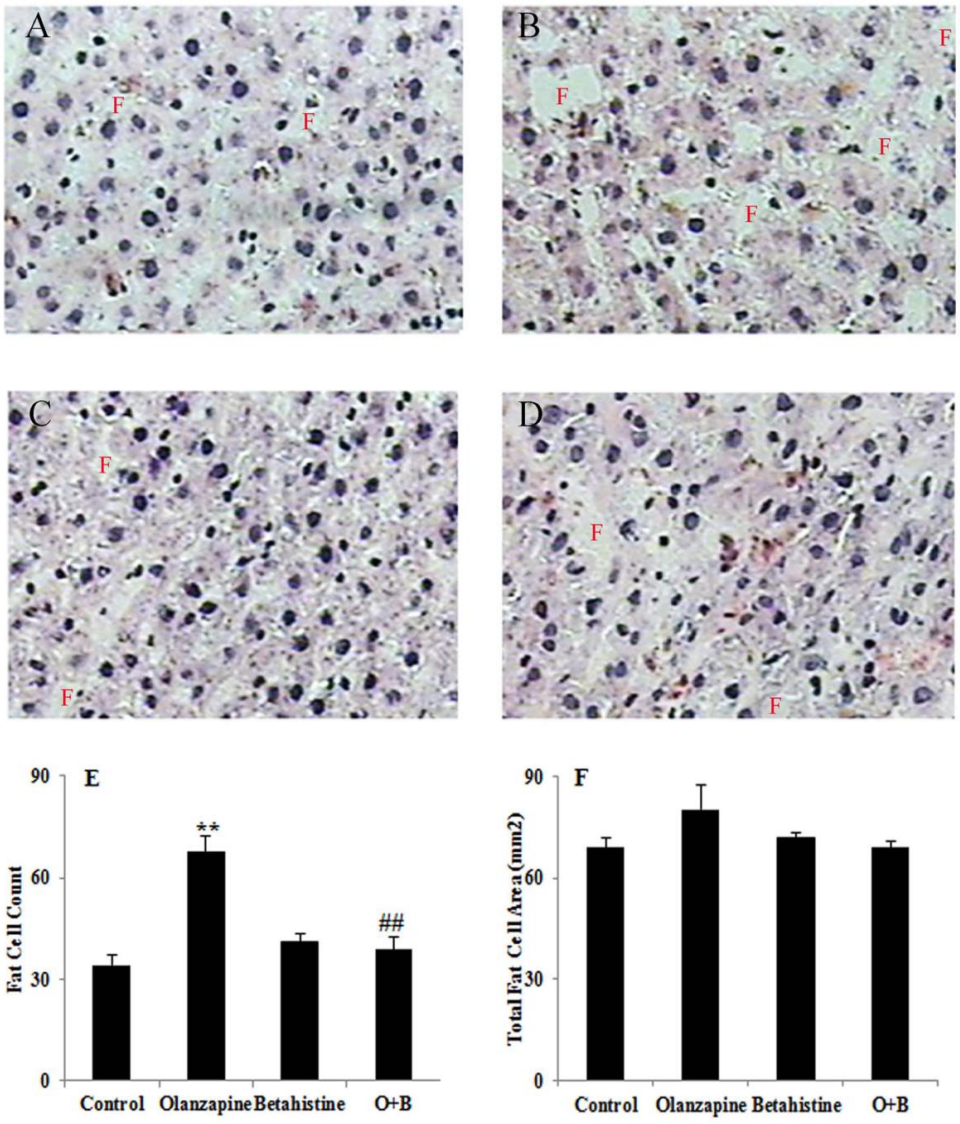

Figure 3. Effects of olanzapine and/or betahistine treatment (n = 12) on lipid droplet deposition of hepatic tissue. A–C: HE staining of hepatic tissue from rats treated with Vehicle (A), Olanzapine-only (B), Betahistine-only (C), and O+B co-treatment (D). E: Fat cell counts on the liver sections of different treatment groups. F: Total fat cell area measured on the liver sections of different treatment groups. ** $p<0.01$ *vs.* control; ## $p<0.01$ *vs.* olanzapine.
doi:10.1371/journal.pone.0104160.g003

The hypothalamic nuclei, particularly the arcuate nucleus (Arc) and ventromedial hypothalamus (VMH) play crucial roles in the regulation of energy homeostasis [20,66,67]. Histamine H_1R antagonists are well documented to increase appetite and obesity development [18,68]. Several meta-analyses examined the potency of the antagonistic properties of antipsychotics for H_1R, and the potential to utilise them to predict the likelihood of the obesity side-effect [19,69,70]. H_1R antagonist properties have been identified as the main predictor for the development of antipsychotic-induced body weight gain/obesity side-effects (approximately Clozapine>Olanzapine>Risperidone>Haloperidol>Ziprasidone>Aripiprazole) [19,20,71,72]. Consistent with these reports, the present study revealed that olanzapine-only treatment up-regulated the hypothalamic H_1R levels in line with increased body weight gain and feeding efficiency/hyperphagia induced by this treatment. To our knowledge, this is the first long term animal study to investigate the effects of chronic olanzapine and betahistine co-treatment on hypothalamic H_1R expression in the rat brain. Consistently, a recent study from our group reported that acute intracerebroventricular (ICV) injection of 2-(3-trifluoromethylphenyl histamine (FMPH; an H_1R agonist) attenuated olanzapine induced hyperphagia [32]. It has been noted that betahistine (as a H_3R antagonist) may increase histamine release

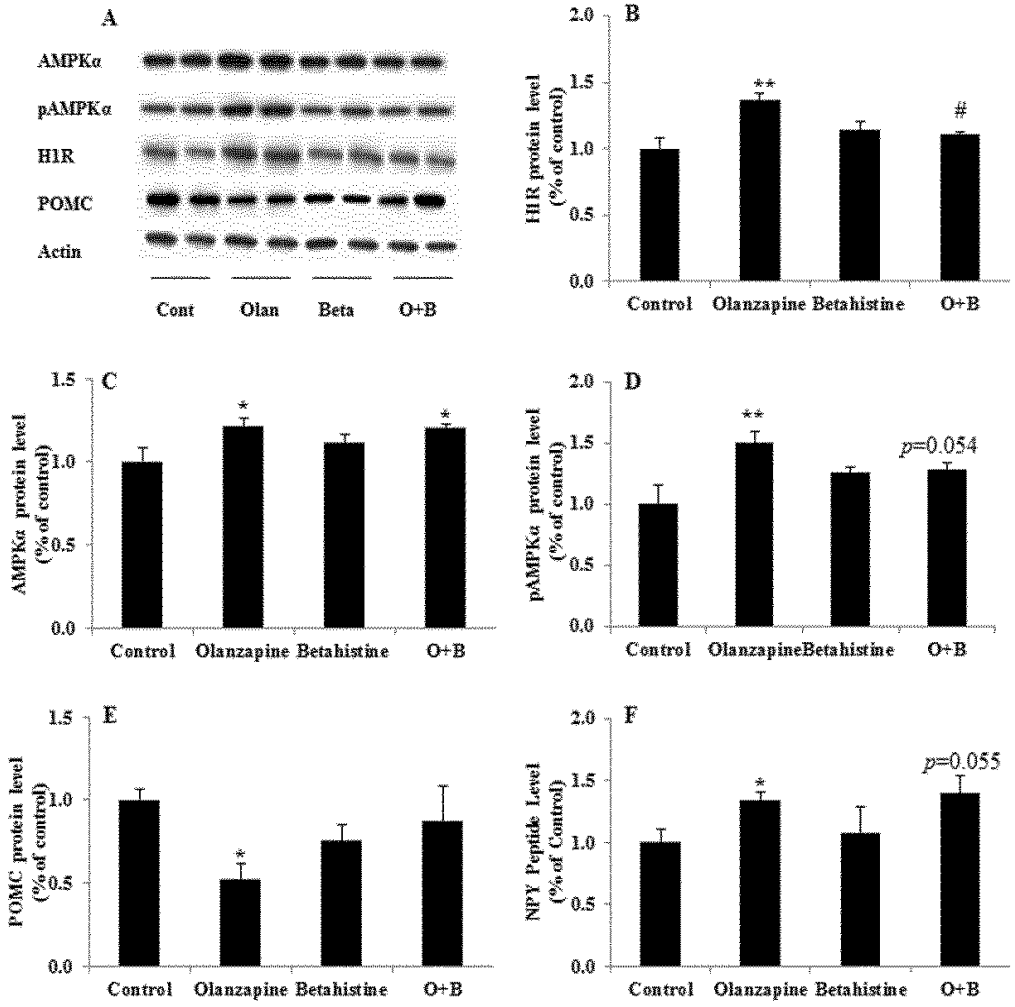

Figure 4. Effects of olanzapine and/or betahistine treatment on the hypothalamic protein levels of histamine H_1R, AMPKα, pAMPKα, and POMC. A: Examples of the images of the western blot experiment showing the protein expressions of histamine H_1R, AMPKα, pAMPKα, POMC and β-actin (n = 6). B–F: Effects of olanzapine and/or betahistine treatment on protein expressions of (B) hypothalamic H_1R, (C) AMPKα, (D) pAMPKα, (E) POMC, (F) neuropeptide Y (NPY). Abbreviations: H_1R: H_1 receptor, AMPKα: AMPK-activated protein kinase α, pAMPKα: the AMPK phosphorylation α and POMC: proopiomelanocortin. * $p<0.05$, ** $p<0.01$ *vs.* control; # $p<0.05$, # $p<0.05$ *vs.* olanzapine.
doi:10.1371/journal.pone.0104160.g004

via blocking presynaptic H_3 autoreceptors, which could augment its direct agonistic effects on H_1R receptors [46].

There is strong evidence that hypothalamic H_1R and its linked AMPK signalling pathways play a crucial role in the antipsychotic-induced weight gain side-effect [18,24,25]. In fact, several studies have reported that olanzapine-elevated hypothalamic pAMPK was linked to its weight gain/metabolic side-effect [25,30,32,73]. In this study, we found that olanzapine only increased pAMPKα and AMPKα levels in the mediobasal hypothalamus (including the Arc and VMH) compared with the control. However, the O+B co-treatment reduced pAMPKα expression compared with olanzapine-only treatment. Importantly, there were positive correlations between pAMPKα and body weight gain, food intake, feeding efficiency, as well as between AMPKα and body weight gain. Our findings were confirmed by a recent report by [31] that AMPK inhibition in the Arc reduced the olanzapine-induced weight gain side-effects in female rats by means of functional inhibition of AMPK using adenoviruses carrying dominant negative forms AMPK (DN-AMPK). This result is also in line with another study from our group that the acute ICV injection of FMPH (an H_1R agonist) significantly attenuated olanzapine-induced AMPK levels and food intake [32]. Further investigations is needed to examine whether O+B co-treatment has different effects on AMPKα isoforms, and its downstream targets such as acetyl-CoA carboxylase (ACC) and pACC compared with olanzapine-only treatment.

The present study showed that olanzapine downregulated the protein levels of UCP_1 and PGC-1α (biomarkers for thermogenesis), but not PGC-1β in the BAT; however these decrease were reversed by co-treatment with betahistine. The results are consistent with previous reports that the expression of BAT UCP_1 and PGC-1α protein are decreased by chronic olanzapine

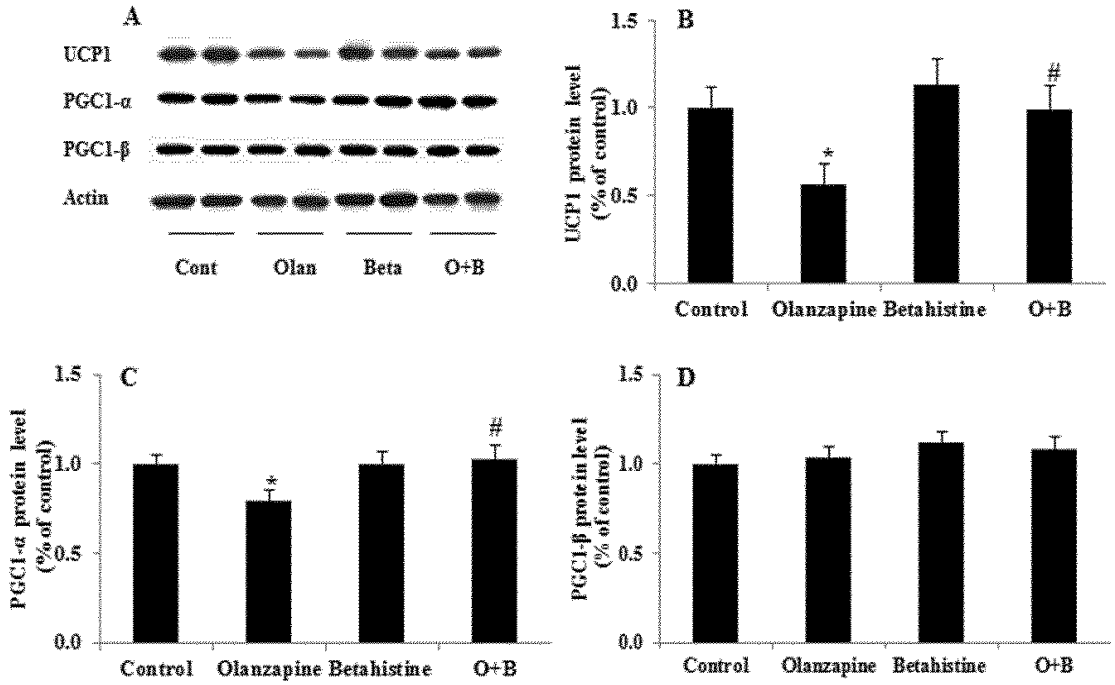

Figure 5. Effects of olanzapine and/or betahistine treatment on the protein levels of UCP_1, PGC-1α, and PGC-1β in brown adipose tissue. A: Examples of the images of the western blot experiment showing the protein expressions of UCP_1, PGC-1α, PGC-1β and β-actin (n = 6). B–D: Effects of olanzapine and/or betahistine treatment on protein expression of (B) UCP_1, (C) PGC-1α, and (D) PGC-1β. Abbreviations: UCP_1: uncoupling protein 1, PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC-1β: Peroxisome proliferator-activated receptor gamma coactivator 1-beta. * $p<0.05$, ** $p<0.01$ *vs.* control; # $p<0.05$, # $p<0.05$ *vs.* olanzapine.
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treatment, which is associated with decreased energy expenditure and increased feeding efficacy/weight gain induced by chronic olanzapine treatment [39,40]. Further studies have shown that the rapid weight gain in the early stage of antipsychotic treatment is due to a significant increase in food intake (leading to an increase in feeding efficiency), while weight gain/maintaining heavier weight following chronic treatment is largely due to decrease in energy expenditure (such as less activity and reduced thermogenesis; also leading to an increase in feeding efficiency) [39,40,58]. In this study, this time course was confirmed in the rats with repeated and chronic olanzapine treatment. In the chronic model, we found that chronic O+B co-treatment reduced feeding efficiency and increased BAT UCP_1 and PGC-1α expressions (suggesting an increase of thermogenesis in BAT), but did not change food intake. Consistently, we found that chronic co-treatment with betahistine did not change the expression of hypothalamic NPY and POMC induced by olanzapine treatment. In consideration of our previous findings that the O+B co-treatment did not affect locomotor activity [46], the BAT UCP_1 and PGC-1α changes in this study suggest that betahistine co-treatment may regulate energy expenditure by upregulating thermogenesis. Furthermore, this experiment also revealed that the BAT UCP_1 and PGC-1α levels were negatively correlated with pAMPKα protein levels in the mediobasal hypothalamus (including the Arc and VMH). Previous studies reported that AMPK modulated BAT thermogenesis and UCP_1 and PGC-1α expressions [36,45,74,75]. As a result, it is suggested that betahistine co-treatment may regulate BAT UCP_1 and PGC-1α through the hypothalamic H_1R-pAMPK pathway. Therefore, these results suggest that activation of hypothalamic AMPK contributes to olanzapine-induced weight gain; however O+B co-treatment may reduce olanzapine-induced weight gain at least partly through attenuating the H_1R-pAMPK activation, which modulates BAT UCP_1 and PGC-1α expression and upregulates thermogenesis. Since the fasting or food intake conditions may influence the hypothalamic neuropeptides and appetite signalling pathways, the hypothalamic changes observed should considered in the context of rats sacrificed without fasting in this study.

One of limitation of this study was that plasma olanzapine levels were not monitored through the experimental periods. According to dosage translations between the species based on the body surface area following the FDA guidelines for clinical trials [51,62,76], the olanzapine dosage used in this project is equivalent to the recommended dosage for treating schizophrenia patients. Olanzapine has a shorter half-life in rats compared with humans. In humans, the half-life of olanzapine in plasma is 24.2 hours, compared with 72 hours in the brain [15]. However, in the rat, the half-lives of olanzapine are 2.5 hours and 5.1 hours in the plasma and brain, respectively, and the high level is retained for 8 hours after a single dose treatment trough gavage [77]. Therefore, in the present study, rats were administered with olanzapine three times/day with 8 hours intervals to ensure a consistently high concentration for better mirroring the human scenario of oral administration once per day. This treatment protocol has been proven to mimic the development of olanzapine-induced body weight in female rats [36,46,51,58]. In view of the possibility that betahistine may affect olanzapine metabolism, further studies are

also important to detect whether betahistine could affect plasma olanzapine levels during the O+B co-treatment period.

In this study, compared to olanzapine-only treatment group, the O+B co-treatment group showed less inguinal fat, and tended to have less periovary and mesentery fat mass, which suggests an effect of O+B co-treatment on reducing white fat mass. One technical limitation in the present study was that the white fat mass was dissected and weighed from post-mortem rat bodies. The advanced NMR (nuclear magnetic resonance) analysis may provide more detailed information about fat mass changes. Additionally, as olanzapine treatment may cause severe dyslipidemia side-effect in patients, therefore it is valuable to investigate whether O+B co-treatment could reverse olanzapine caused dyslipidemia in the future studies.

Conclusions

To sum up, this study provides evidence in a rat model that significant body weight gain induced by olanzapine treatment could be reversed following drug withdrawal, however unfortunately weight gain resumed after re-introducing olanzapine treatment. Since patients suffering from schizophrenia and other mental disorders often require long lasting and repeated antipsychotic treatment, it is very important to control weight gain/obesity side-effects caused by chronic antipsychotic treatment. In this study, we found that co-treatment with betahistine is effective in significantly reducing weight gain induced by olanzapine through the chronic treatment course. This study further demonstrated that the mechanisms of betahistine in reducing olanzapine-induced body weight gain are through the modulation of the hypothalamic H_1R-AMPK-BAT UCP_1-PGC-1α pathway. Extending previous successful trials in drug-naïve subjects in both animal and first episode schizophrenia patients [46,47,60], this study provides further evidence to support a clinical trial to test the effectiveness of co-treatment of olanzapine and betahistine for controlling the weight gain/obesity side-effect in schizophrenia with chronic and repeated antipsychotic treatment.

Author Contributions

Conceived and designed the experiments: JL XH NP CD. Performed the experiments: JL. Analyzed the data: JL XH CD. Contributed reagents/materials/analysis tools: JL XH NP CD. Contributed to the writing of the manuscript: JL XH NP CD.

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CHAPTER 5

EFFECTS OF OLANZAPINE AND BETAHISTINE CO-TREATMENT ON SEROTONIN TRANSPORTER, 5-HT_{2A} AND DOPAMINE D_2 RECEPTOR BINDING DENSITY

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Effects of olanzapine and betahistine co-treatment on serotonin transporter, 5-HT_{2A} and dopamine D_2 receptor binding density☆

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ABSTRACT

Olanzapine is widely used in treating multiple domains of schizophrenia symptoms but induces serious metabolic side-effects. Recent evidence has showed that co-treatment of betahistine (a histaminergic H_1 receptor agonist and H_3 receptor antagonist) is effective for preventing olanzapine-induced weight gain/obesity, however it is not clear whether this co-treatment affects on the primary therapeutic receptor binding sites of olanzapine such as serotonergic 5-HT_{2A} receptors (5-HT_{2A}R) and dopaminergic D_2 receptors (D_2R). Therefore, this study investigated the effects of this co-treatment on 5-HT_{2A}R, 5-HT transporter (5-HTT) and D_2R bindings in various brain regions involved in antipsychotic efficacy. Female Sprague Dawley rats were administered orally (t.i.d.) with either olanzapine (1 mg/kg), betahistine (2.7 mg/kg), olanzapine plus betahistine (O + B), or vehicle (control) for 2 weeks. Quantitative autoradiography was used to detect the density of [^{3}H]ketanserin, [^{3}H]paroxetine and [^{3}H]raclopride binding site to 5-HT_{2A}R, 5-HTT and D_2R. Compared to the controls, olanzapine significantly decreased [^{3}H]ketanserin bindings to 5-HT_{2A}R in the prefrontal cortex, cingulate cortex, and nucleus accumbens. Similar changes in 5-HT_{2A}R bindings in these nuclei were also observed in the O + B co-treatment group. Olanzapine also significantly decreased [^{3}H]paroxetine binding to 5-HTT in the ventral tegmental area and substantia nigra, however, both olanzapine only and O + B co-treatment did not affect [^{3}H]raclopride binding to D_2R. The results confirmed the important role of 5-HT_{2A}R in the efficacy of olanzapine, which is not influenced by the O + B co-treatment. Therefore, betahistine co-treatment would be an effective combination therapy to reduce olanzapine-induced weight gain side-effects without affecting olanzapine's actions on 5-HT_{2A}R transmissions.

CHAPTER 6

CHRONIC BETAHISTINE CO-TREATMENT REVERSES OLANZAPINE'S EFFECTS ON DOPAMINE D_2 BUT NOT 5-$HT_{2A/2C}$ BINDINGS IN RAT BRAINS

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Chronic betahistine co-treatment reverses olanzapine's effects on dopamine D_2 but not 5-$HT_{2A/2C}$ bindings in rat brains

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ABSTRACT

Olanzapine is widely prescribed for treating schizophrenia and other mental disorders, although it leads to severe body weight gain/obesity. Chronic co-treatment with betahistine has been found to significantly decrease olanzapine-induced weight gain; however, it is not clear whether this co-treatment affects the therapeutic effects of olanzapine. This study investigated the effects of chronic treatment of olanzapine and/or betahistine on the binding density of the serotonergic 5-HT_{2A} (5-HT_{2A}R) and 5-HT_{2C} (5-HT_{2C}R) receptors, 5-HT transporter (5-HTT), and dopaminergic D_2 receptors (D_2R) in the brain regions involved in antipsychotic efficacy, including the prefrontal cortex (PFC), cingulate cortex (Cg), nucleus accumbens (NAc), and caudate putamen (CPu). Rats were treated with olanzapine (1 mg/kg, t.i.d.) or vehicle for 3.5 weeks, and then olanzapine treatment was withdrawn for 19 days. From week 6, the two groups were divided into 4 groups ($n = 6$) for 5 weeks' treatment: (1) olanzapine-only (1 mg/kg, t.i.d.), (2) betahistine-only (9.6 mg/kg, t.i.d.), (3) olanzapine and betahistine co-treatment (O + B), and (4) vehicle. Compared to the control, the olanzapine-only treatment significantly decreased the bindings of 5-HT_{2A}R, 5-HT_{2C}R, and 5-HTT in the PFC, Cg, and NAc. Similar changes were observed in the rats receiving the O + B co-treatment. The olanzapine-only treatment significantly increased the D_2R binding in the Cg, NAc, and CPu, while the betahistine-only treatment reduced D_2R binding. The co-treatment of betahistine reversed the D_2R bindings in the NAc and CPu that were increased by olanzapine. Therefore, chronic O + B co-treatment has similar effects on serotonin transmission as the olanzapine-only treatment, but reverses the D_2R that is up-regulated by chronic olanzapine treatment. The co-treatment maintains the therapeutic effects of olanzapine but decreases/prevents the excess weight gain.

CHAPTER 7

GENERAL DISCUSSION

7.1 Overall discussion

As discussed in earlier chapters, olanzapine, as the first line of SGAs, is widely prescribed to treat schizophrenia and other mental disorders. However it is associated with troublesome weight gain/obesity side-effects. Previous clinical trials have revealed that, whether in first episode/antipsychotic-naïve patients or in chronic schizophrenia patients with previous antipsychotic exposure, SGAs can cause significant weight gain side-effects (Lieberman et al., 2005; Deng, 2013). In addition, obesity and schizophrenic metabolic disorders associated with SGA treatments are the leading causes of premature death. Therefore, it is extremely important to prevent and treat weight gain/obesity induced by SGAs. This thesis investigated the effects of betahistine to prevent and treat olanzapine-induced obesity and related mechanisms in a female rat model. The following key outcomes have been achieved.

7.1.1 Further validated the animal model for olanzapine-induced weight gain

Previously, all studies in the animal model for olanzapine-induced weight gain used the drug-naïve animals (Panariello et al., 2011; Van Der Zwaal et al., 2014). This thesis is the first study showing that administration of olanzapine resulted in substantial weight gain in both drug-naïve rats and those with chronically repeated treatment. In particular, olanzapine treatment was observed to induce weight gain from week 1 and that weight gain was maintained by continuing drug treatment (Chapter 4); this corresponded with

the results of our and other laboratories using drug-naïve rats (Han et al., 2008; Fernø et al., 2011; Deng et al., 2012). Interestingly, the weight loss effect was observed after a withdrawal of olanzapine treatment in the current study (Chapter 4). Similarly, a previous study demonstrated that withdrawal of oral olanzapine treatment caused weight loss (Goudie et al., 2002). The weight loss during the drug withdrawal period is largely due to the reduction of food intake and feeding efficiency. This is the first study to reveal that, importantly, re-induction of olanzapine treatment caused significant weight gain in rats (Chapter 4). Thus, these findings mimic the clinical observation that olanzapine also caused significant weight gain in chronic schizophrenia patients with repeated exposure to SGAs (Lieberman et al., 2005; Maayan et al., 2010). Therefore, this thesis further validated the animal models mimicked olanzapine-induced body weight gain side-effects in drug-naïve and chronic patients.

7.1.2 Proved the efficacy of co-treatment with betahistine in reducing olanzapine-induced weight gain in both drug-naïve rats and those with chronic repeated olanzapine administration

The present study demonstrated that short-term (2 weeks) co-treatment with betahistine partially reduced olanzapine-induced weight gain side-effects in drug-naïve rats (Chapter 3). The result corresponded with short-term clinical trials in which co-treatment (6 weeks) with betahistine can cause less body weight gain compared to olanzapine only in drug-naïve patients (Poyurovsky et al., 2005; Poyurovsky et al., 2013). More importantly, the current chronic animal study (Chapter 4) revealed the efficacy of chronic (5 weeks) co-treatment with betahistine in reducing the body weight gain side-effects in rats with previous olanzapine exposure.

In particular, the 2 weeks co-treatment with betahistine significantly prevented (-45%) olanzapine-induced weight gain side-effects and reduced feeding efficiency in drug-naïve rats. Betahistine treatment alone had no effect on weight gain and food intake (Deng et al., 2012) (Chapter 3). Similarly, chronic (5 weeks) co-administration with betahistine significantly reduced (-51.4%) olanzapine-induced weight gain and feeding efficiency in rats with previous olanzapine exposure (Chapter 4). Therefore, co-treatment with betahistine is effective to control olanzapine-induced weight gain in both drug-naïve and chronic/repeat treatment rats. These results provided strong supports for further clinical trials to improve olanzapine-induced obesity side-effects using betahistine co-treatment in both drug-naïve and repeatedly treated subjects.

Although the dosage of betahistine used in the chronic animal study was higher than in the short-term drug-naïve study (9.6 mg/kg *vs.* 2.67 mg/kg), both of them led to similar effects on body weight gain reduction (about 50%), which suggested that betahistine has no dosage dependent effects in reducing olanzapine-induced weight gain. Further study is needed to examine whether the lower dosage (2.67 mg/kg) is also effective in reducing weight gain in chronic/repeated olanzapine treatment conditions.

7.1.3 Revealed the mechanisms underlying effects of betahistine co-treatment on reducing weight gain/obesity side-effects induced by olanzapine

The current studies (Chapters 3 and 4) provide evidence of the mechanisms underlying the effects of co-treatment with betahistine in ameliorating olanzapine-induced weight gain in female rat models. Corresponding with the previous findings in our and other

laboratories, in which both the short term/drug-naïve and chronic/repeated studies demonstrated that the regulation of olanzapine-induced weight gain may occur through the upregulation of hypothalamic H_1R-AMPKα signalling, and NPY levels, accompanied by downregulation of POMC mRNA expression (Kim et al., 2007; Meltzer, 2007; Deng et al., 2010; Fernø et al., 2011; Sezlev et al., 2013; He et al., 2014; Skrede et al., 2014; Zhang et al., 2014a). In addition, this study revealed that the expression of BAT UCP_1 and PGC-1α protein levels (biomarkers of thermogenesis) are reduced by chronic olanzapine treatment (Chapter 4); this confirmed their roles in the decreased energy expenditure and increased weight gain and feeding efficiency induced by chronic treatment with olanzapine (Stefanidis et al., 2008; Zhang et al., 2014b) (Chapters 3 and 4, Figure 2.3).

On the other hand, it was proven by the present short-term/drug-naïve and chronic/repeated animal studies that co-treatment with betahistine attenuates hypothalamic H_1R and associated pathways in controlling olanzapine-induced weight-gain. To our knowledge, this thesis is the first study to systematically investigate the effects of olanzapine and betahistine co-treatment on hypothalamic H_1R expression in the rat brain using both drug-naïve and chronic animal models. This finding was confirmed by a recent report from our laboratory that acute ICV injection of FMPH (an H_1R agonist) reduced olanzapine-induced hyperphagia (He et al., 2014). Give that betahistine (also an H_3R antagonist) can increase histamine release by blocking presynaptic H_3 autoreceptors, betahistine enhances its direct agonistic effects on H_1R receptors (Deng et al., 2012) (Chapter 2, Figure 2.4).

Both the short-term and chronic studies have proven that the H_1R-AMPKα signalling pathway is elevated by olanzapine, which is reversed by co-treatment with betahistine (Chapters 3 and 4). Furthermore, it is important the AMPKα/pAMPKα changes are correlated with the changes in body weight gain, hyperphagia and feeding efficiency. Since acute ICV injection of FMPH significantly reduced the olanzapine-induced enhanced AMPKα levels (He et al., 2014), the effect of co-treatment with betahistine in reversing AMPKα/pAMPKα signalling is through its activating H_1Rs.

Moreover, AMPK phosphorylation could be promoted by stimulating the orexigenic hormones such as NPY and AgRP (Lopez et al., 2008). It was revealed in Chapter 3 (2 weeks animal study) that co-treatment with betahistine attenuates the elevated hypothalamic NPY mRNA expression induced by olanzapine. Since there are synaptic interactions between NPY afferents and histaminergic neurons, and the H_3R antagonist suppresses NPY induced feeding (Itoh et al., 1999), it is possible that co-treatment with betahistine may activate histamine H_3R to inhibit olanzapine-induced hyperphagia by suppressing NPY.

Although the current studies reported that olanzapine decreases the POMC mRNA expression, it was not reversed by co-treatment with betahistine, which indicates that betahistine reduction of olanzapine-induced weight gain was not *via* the POMC pathway (Chapters 3 and 4). This result was consistent with previous reports that hypothalamic H_1R is independent of the POMC-melanocortin 4 receptor pathway in regulation of food intake and body weight (Yoshimatsu, 2006); instead hypothalamic POMC neurons, are regulated by 5-HT_{2C}R as discussed in Chapter 2.

The present chronic study also showed that BAT UCP_1 and PGC-1α levels are reduced by chronic olanzapine treatment, and this reduction is reversed by chronic co-treatment of O+B (Chapter 4). The results illustrated that the two biomarkers of thermogenesis involved in the decreased energy expenditure and increased weight gain induced by chronic olanzapine treatment, are reversed by co-treatment with betahistine. Furthermore, this study also observed that negative correlation between hypothalamic pAMPKα, and BAT UCP_1 and PGC-1α levels, which is in line with the previous report about hypothalamic AMPK modulating BAT thermogenesis, and UCP_1 and PGC-1α expressions (Lopez et al., 2010; Morrison et al., 2014; Wan et al., 2014; Zhang et al., 2014b). Therefore, co-treatment with betahistine may regulate BAT UCP_1 and PGC-1α *via* the hypothalamic H_1R and pAMPKα pathway.

To sum up, this study demonstrated the mechanisms of co-treatment with betahistine in reducing olanzapine-induced body weight gain *via* modulation of the hypothalamic H_1R-AMPKα, NPY, and BAT UCP_1-PGC-1α pathways. Understanding the mechanisms of betahistine in the prevention and treatment of olanzapine-induced obesity throughout these signalling pathways will potentially lead to a new treatment strategy for schizophrenia and highlight the need for the development of more effective antipsychotic drugs with fewer side-effects.

7.1.4 Provides evidence that co-treatment with betahistine does not affect therapeutic efficacy of olanzapine

As discussed in Chapter 2, another key issue was whether co-treatment with betahistine affects the therapeutic effects of olanzapine. The present studies investigated the effects of olanzapine and betahistine co-treatment on the 5-HT_{2A}R, 5-HT_{2C}R, 5-HTT, and D_2R

bindings in the brain regions associated with the therapeutic efficacy of olanzapine, including the PFC, NAcC, NAcS and Cg in both drug-naïve and chronic/repeated olanzapine-treated rats (Chapters 5 and 6).

Corresponding with previous reports, the present studies demonstrated a significant downregulation of 5-HT_{2A}R binding density by olanzapine treatment in various brain regions including the PFC, Cg, NAcC and NAcS (Tarazi et al., 2002; Kuroki et al., 2008; Meltzer and Massey, 2011), which was not affected by the treatment durations (2 weeks *vs.* 5 weeks), or drug exposure experience (drug-naïve *vs.* re-exposure). These results confirmed olanzapine's therapeutic efficacy *via* binding to serotonergic 5-HT_{2A}R in these brain regions. More importantly, the current studies revealed that co-treatment with O+B does not affect olanzapine's effect on 5-HT_{2A}R. Nor did betahistine-only treatment affect 5-HT_{2A}R binding.

In addition, 5-HT_{2C}R is another target for the therapeutic effects of antipsychotics (Reynolds et al., 2005). Consistent with the 5-HT_{2A}R, previous studies reported that 5-HT_{2C}R was also downregulated by SGA treatment including olanzapine (Tarazi et al., 2002). This present study revealed that both olanzapine-only and O+B co-treatment has similar effects on downregulation of 5-HT_{2C}R binding density in the PFC, Cg, NAcC and NAcS, while betahistine-only has no significant effect on 5-HT_{2C}R bindings. The effect of co-treatment with betahistine on 5-HT_{2C}R was only examined in the chronic study; it may have a similar effect in the short-term treatments, although further studies are necessary to confirm this.

The 5-HTT gene polymorphism studies have revealed an association with responses to the therapeutic effects of olanzapine and other SGAs (Bozina et al., 2007; Zhang and Malhotra, 2011). To date, no study has investigated the direct effects of SGAs on 5-HTT bindings. To my knowledge, this thesis is the first study to investigate the direct effects of olanzapine-only and co-treatment with betahistine on 5-HTT bindings. In terms of the 5-HTT bindings in the short-term treatment study (Chapter 5), both O+B co-treatment and olanzapine-only treatment significantly attenuated 5-HTT bindings in the SN and VTA of drug-naïve rats. Comparatively, the chronic study (Chapter 6) demonstrated that the reduced 5-HTT binding density was observed in the PFC, Cg, NAcC and NAcS in olanzapine-only treatment and in the Cg and NAcS by co-treatment with betahistine. It is interesting to note that co-treatment with betahistine had a similar effect on 5-HTT bindings compared with olanzapine-only treatment in both short term treatment in drug-naïve subjects and chronic treatment in drug re-exposure rats, although these changes occurred in different brain regions (Chapters 5 and 6). These difference could be due to different treatment dosage (2.67 mg/kg, *vs.* 9.6 mg/kg), treatment period (2 weeks *vs.* 5 weeks) or drug exposure experience (drug-naïve *vs.* repeated); further studies are necessary to identify the mechanism that caused these difference between the short-term/drug naïve and chronic/drug re-exposure treatment.

The different effects on D_2R bindings have been observed in the short and chronic treatment studies (Chapters 5 and 6). Specifically, both olanzapine and co-treatment of O+B did not cause any significant changes in D_2R bindings in all brain regions tested after 2 weeks treatment. However, our chronic olanzapine treatment upregulated the D_2R binding levels in the Cg, NAcC, NAcS, and CPu; and this is consistent with a previous report in which chronic treatment of olanzapine at a high dosage (5mg/kg/day

via osmotic minipumps) enhanced D_2R binding in the PFC, CPu, and NAc (Tarazi et al., 2001). This D_2R upregulation induced by antipsychotics may be the underlying mechanisms for "dopaminergic supersensitivity" observed in clinics (Samaha et al., 2007; Seeman, 2011); long-term/chronic treatment with antipsychotics caused enhanced vulnerability to psychosis frequently observed after drug withdrawal (Samaha et al., 2007). Furthermore, although there was lower risk of EPS associated with SGAs compared with FGAs, the elevated D_2R binding level in the CPu observed in the chronic olanzapine treatment may partially explain its risk for the development of EPS, which is in line with other reports (Tarazi et al., 2001). Furthermore, it is interesting that chronic betahistine-only treatment at the higher dosage (9.6 mg/kg) significantly decreased D_2R binding in the NAc and CPu, while no change was observed in short-term treatment at a lower dosage. This difference is possibly attributed to both the difference in dosage and treatment duration (2.67 mg/kg for 2 weeks study *vs.* 9.6 mg/kg for 5 weeks study). It is important to note that olanzapine-elevated D_2R binding in the NAc was reversed by co-treatment with betahistine, which suggests that co-treatment with betahistine may also reduce "dopaminergic supersensitivity" to some extent (see details in Chapter 6). This may provide clinical benefits for chronic olanzapine-treatment in schizophrenia patients.

Overall, the series of binding experiments in both short-term/drug-naïve and chronic/drug-repeated treatment subjects revealed the effects of olanzapine and/or betahistine administrations on the 5-$HT_{2A}R$, 5-$HT_{2C}R$, 5-HTT and D_2R bindings in the brain regions involved in the therapeutic effects of antipsychotics (Kuroki et al., 2008). Since both olanzapine-only and O+B co-treatment have similar effects in attenuating 5-$HT_{2A}R$, 5-$HT_{2C}R$ and 5-HTT levels, betahistine is a safe drug for co-administration with

olanzapine without influencing olanzapine's therapeutic action on 5-HT neurotransmission. Additionally, since chronic olanzapine co-treatment with betahistine can reverse the elevated D_2R binding caused by chronic olanzapine treatment, co-treatment with betahistine may improve therapeutic effects by preventing the "dopaminergic supersensitivity" caused by chronic antipsychotic treatment.

7.2 Recommendations for further research

Based on the findings of this thesis, recommendations for further research are presented as follows:

1) Olanzapine causes not only weight gain but also other metabolic side-effects including dyslipidemia. In this thesis olanzapine-only treatment has been found to increase white fat accumulation and liver fat accumulation (Chapters 3 and 4), while betahistine co-treatment decreased white fat and liver fat accumulation compared to the olanzapine only group (Chapter 4). Therefore, further study would be interesting to determine whether O+B co-treatment could prevent or reverse dyslipidemia caused by olanzapine treatment.

2) Although olanzapine is the first line of SGAs in the clinic, other SGAs with serious metabolic side-effects, such as clozapine and risperidone, are also widely prescribed to patients with schizophrenia and other mental disorders (Patel et al., 2009). Since these SGAs also have a high H_1R antagonist affinity, it would be valuable to investigate whether betahistine co-treatment is also effective to reverse the weight gain side-effects associated with these SGAs.

3) One limitation of this study is that only one dosage of olanzapine (1 mg/kg, t.i.d., equivalent to 10 mg in humans) was administered in this study. Since olanzapine is recommended to be prescribed at a range of 5-20 mg, further studies are also important to test whether betahistine has the same effects in the animal model when used as a co-treatment with various dosages of olanzapine.

4) Although, clinically, female patients have a much higher risk than males off SGA-induced weight gain side-effects (Gebhardt et al., 2009; Seeman, 2009; Weston-Green et al., 2010; Treuer et al., 2011), SGAs do cause significant weight gain in males. This thesis used a female rat model for olanzapine-induced weight gain, since it is well established and validated in our and other laboratories (Goudie et al., 2002; Choi et al., 2007; Weston-Green et al., 2011; Deng et al., 2012). It is important to extend this study to the male rat model. For example, several studies reported the male rat model for olanzapine-induced weight gain could be established under special feeding conditions with high carbohydrate/medium fat/low protein (45%/31%/14%) diets (Minet-Ringuet et al., 2006b; Shobo et al., 2011). Therefore, given that olanzapine-induced weight gain/obesity occurs in female and male patients, it is important to test in the male model whether using co-treatment of betahistine and olanzapine is effective to ameliorate olanzapine-induced body weight gain side-effects.

5) The results of this project (presented in Chapters 3 and 4) showed that co-treatment with betahistine partially ameliorates olanzapine-induced side-effects *via* regulation of the hypothalamic H_1R level and related signalling pathways. Since other brain regions such as the dorsal vague complex in the brain stem are also involved in

body weight regulation (Deng et al., 2007; Weston-Green et al., 2008), it is necessary to investigate whether co-treatment with betahistine has similar effects in these brain regions, and their contributions to control SGA-induced weight gain side-effects.

6) AMPK is composed of 3 subunits with various isoforms, including the catalytic AMPKα (α1, α2), and non-catalytic AMPKβ (β1, β2) and AMPKγ (γ1, γ2, γ3) (Moffat and Ellen Harper, 2010). Furthermore, a study revealed the crucial association between polymorphism in AMPK subunit genes and olanzapine-induced weight gain side-effects (Souza et al., 2012). Due to the limitation of small amount of samples (i.e. hypothalamic Arc), in this study, only AMPKα was investigated (Chapters 3 and 4). Therefore, it is also important to reveal whether co-treatment of O+B has different effects on the various AMPKα subunits and isoforms.

7) Olanzapine-only treatment had no effect on D_2R binding density after 2 weeks' of drug treatment (Chapter 5), however it led to enhanced D_2R binding density after chronic treatment (Chapter 6). It was demonstrated that the elevated D_2^{High} receptors were responsible for psychotic symptoms, which is reversed by antipsychotic treatments, while long term antipsychotics can further enhance dopamine supersensitivity in patients (Seeman, 2011). It has been reported that 9-day olanzapine treatment led to a 2 fold increase in the proportion of D_2^{High} receptors (Seeman, 2011). Therefore, it is important to investigate whether olanzapine-only and co-treatment with betahistine affect the expression of D_2^{High} receptors.

8) This PhD research (both short-term and chronic animal studies) has found that co-treatment of olanzapine and betahistine can significantly reduce olanzapine-induced weight gain, and revealed the underlying neuroendocrinological mechanisms in animal models. Therefore, this study provides solid evidence to support clinical trials to further investigate the effect of co-treatment with betahistine for ameliorating the body weight gain/obesity side-effects in schizophrenia patients with both acute/drug-naïve and chronic/repeated antipsychotic treatment.

7.3 Conclusion

To sum up, this PhD study has shown that co-treatment with betahistine can partially reverse the olanzapine-induced body weight gain side-effects *via* attenuating food intake and/or feeding efficiency in both short term/drug-naïve and chronic/drug-repeated female rat models. Furthermore, these studies revealed the mechanisms of co-treatment with betahistine on ameliorating olanzapine-induced weight gain side-effects, which are modulated through hypothalamic H_1R-AMPKα signalling and neuropeptides including the orexigenic NPY pathway, as well as the BAT UCP_1-PGC-1α pathways modulating thermogenesis. Therefore, these results not only confirm the importance of these pathways in SGA-induced weight gain/obesity side-effects, but also implicate the pathways as promising targets for pharmacological intervention to reduce the SGA-induced weight gain.

On the other hand, the present studies provide the first evidence to show that co-treatment with betahistine does not affect the key receptor binding sites for the

therapeutic efficacy of olanzapine at serotonergic 5-HT_{2A}R, 5-HT_{2C}R and 5-HTT; this indicates that co-treatment with betahistine does not affect olanzapine's action on 5-HT neurotransmissions in the relevant brain nuclei associated with antipsychotic therapeutic effects. In addition, chronic betahistine treatment significantly reversed D_2R biding density enhanced by olanzapine, which suggests further investigations into whether co-treatment with betahistine could improve the efficacy of olanzapine by preventing the "dopaminergic supersensitivity" caused by chronic antipsychotic treatment.

Last but not least, in conjunction with previous preclinical and clinical trials in drug-naïve subjects (Poyurovsky et al., 2005; Deng et al., 2012; Poyurovsky et al., 2013), the results from this study further supports a clinical trial to investigate the effects of co-treatment with betahistine on reducing olanzapine-induced weight gain side-effects in schizophrenia patients with chronic and repeated antipsychotic treatment.

APPENIDCES

Appendix A-Chapter 2 Supplementary

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The Figures 2.4, 2.5 and 2.6 in the Chapter 2 were reprinted from my previous publication "Deng C, Lian J, Pai N, Huang X-F (2012). Reducing olanzapine-induced weight gain side-effect by betahistine: a study in the rat model. *Journal of Psychopharmacology* 26 (9) 1271-1279". As one of the authors of this paper, I was permitted to re-use these figures in my thesis by the publisher SAGE (see page 107).

Appendix B-Chapter 3 Supplementary

Supplement 1 Statement from co-authors

This is to attest that the PhD candidate, Jiamei Lian, contributed significantly to the investigation (Lian J, Huang X-F, Pai N and Deng C (2014). Betahistine ameliorates olanzapine-Induced weight gain through modulation of histaminergic, NPY and AMPK Pathways. *Psychoneuroendocrinology*, 48, 77-86): designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. Three co-authors are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts.

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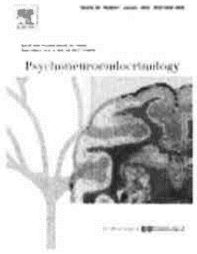

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Publication: Psychoneuroendocrinology

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Appendix D-Chapter 5 Supplementary

Supplement 1 Statement from co-authors

This is to attest that the PhD candidate, Jiamei Lian, contributed significantly to the investigation (Lian J, Huang X-F, Pai N and Deng C (2013). Effects of olanzapine and betahistine co-treatment on serotonin transporter, 5-HT_{2A} and dopamine D_2 receptor binding density. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 47, 62-68): designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. Three co-authors are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts.

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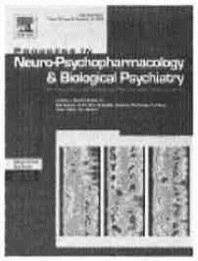

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Author: Jiamei Lian,Xu-Feng Huang,Nagesh Pai,Chao Deng

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Appendix E-Chapter 6 Supplementary

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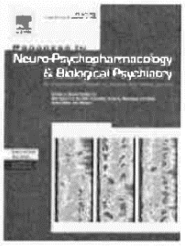

Title: Chronic betahistine co-treatment reverses olanzapine's effects on dopamine D2 but not 5-HT2A/2C bindings in rat brains

Author: Jiamei Lian,Xu-Feng Huang,Nagesh Pai,Chao Deng

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