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The Gut Microbiota as a Contributing Factor to Antipsychotic-Induced Weight Gain and Metabolic Dysfunction

Thesis presented by

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under the supervision of

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for the degree of

Doctor of Philosophy

APC
Alimentary Pharmabiotic Centre
Mining Microbes for Mankind

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Declaration

This thesis comprises original work carried out by the author and has not been submitted for any other degree neither at University College Cork or elsewhere.

Author Contributions

All of the work carried out herein was performed independently by the author with the following exceptions.

Chapter 3

Dr Harriet Schellekens performed the qPCR carried out on brain tissue.

Chapters 3,4,5,6

Dr Fiona Crispie performed the final process of pyrosequencing, after samples were prepared by the author.

Dr Orla O'Sullivan performed the bioinformatic analysis of the data generated by pyrosequencing.

Chapter 5

Dr Rebecca Wall performed the measurement of short chain fatty acids.

Signed

Kieran J. Davey

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Publications and presentations

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Drug Induced Weight Gain in the Rat: Metabolic, inflammatory, microbiota parameters and the role of gender.
Obese Species, Sicily 2011-Travel award

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Probiotics are pro-biotech
Round table review, 2012-Finalist in roundtable review writing competition

List of Abbreviations

5-HT	**5-hydroxytryptamine (serotonin)**
AAPD	Atypical antipsychotic drug
ABX	Antibiotic cocktail
ACC	Acetyl CoA carboxylase
AgRP	Agouti related peptide
AMP	Adenosine monophophate
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
BID	*Bis in die* (Twice a day)
Camp	cyclic adenosine monophosphate
CART	Cocaine amphetamine regulated transcript
CB	Cannabinoid
cDNA	Circular deoxyribonucleic acid
ChREBP	Carbohydrate regulatory element binding protein
CNS	Central nervous system
D	Dopamine
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
eCB	Endocannabinoid
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority

ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
EPS	Extra pyramidal symptoms
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
FDA	Food and Drug Administration
FFA	Free fatty acids
FiaF	Fasting-induced adipose factor
FMT	Faecal microbiota transplant
GABA	Gamma amino butyric acid
GLP-1	Glucagon like peptide 1
GPCR	G protein coupled receptor
GPR	G protein receptor
H	Histamine
HAL	Haloperidol
HDL	High density lipoprotein
HMP	Human Microbiome Project
HPA	Hypothalamus-pituitary-adrenal
HPT	Hypothalamus-pituitary-thyroid
IL	Interleukin
IP	Intraperitoneal
IR	Insulin receptor
IRS	Insulin receptor substrate
LDL	Low density lipoprotein

LHA	Lateral hypothalamus
LPL	Lipoprotein lipase
LPS	Lipopolysaccaride
LSD	Lysergic acid diethylamide
LSD (Fisher's)	Least significant difference
M	Muscarinic
MC	Melanocortin
MCH	Melanocyte concentrating hormone
MetS	Metabolic syndrome
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSH	Melanocyte stimulating hormone
NaOH	Sodium hydroxide
NOD	Nucleotide-binding oligomerisation domain
NPY	Neuropeptide Y
OLZ	Olanzapine
PCoA	Principal coordinate analysis
PFA	Perifornical area
POMC	Pro-opiomelanaocortin
PPAR	Proliferator-activated receptor
PVN	Paraventricular nucleus
PYY	Peptide YY
QUICKI	Qualitative Insulin Check Index
RIF	Rifaximin

RISP	Risperidone
RNA	Ribonucleic acid
rRN	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids
SNP	Single nucleotide polymorphism
SREBP	Sterol regulatory element binding protein
TLR	Toll like receptor
TNF	Tumour necrosis factor
VAN	Vancomycin
VEH	Vehicle
VLDL	Very low density lipoprotein

Abstract

Schizophrenia represents one of the world's most devastating illnesses due to its often life-long course and debilitating nature. The treatment of schizophrenia has vastly improved over recent decades with the discovery of several antipsychotic compounds; however these drugs are not without adverse effects that must be addressed to maximize their therapeutic value.

Newer, atypical, antipsychotics are associated with a compilation of serious metabolic side effects including weight gain, insulin resistance, fat deposition, glucose dysregulation and ensuing co-morbidities such as type II diabetes mellitus. The mechanisms underlying these side effects remain to be fully elucidated and adequate interventions are lacking. Further understanding of the factors that contribute these side effects is therefore required in order to develop effective adjunctive therapies and to potentially design antipsychotic drugs in the future with reduced impact on the metabolic health of patients.

We investigated if the gut microbiota represented a novel mechanism contributing to the metabolic dysfunction associated with atypical antipsychotics. The gut microbiota comprises the bacteria that exist symbiotically within the gastrointestinal tract, and has been shown in recent years to be involved in several aspects of energy balance and metabolism

We have demonstrated that administration of certain antipsychotics in the rat results in an altered microbiota profile and, moreover, that the microbiota is required for the full scale of metabolic dysfunction to occur.

We have further shown that specific antibiotics can attenuate certain aspects of olanzapine and risperidone–induced metabolic dysfunction, in particular fat deposition and adipose tissue inflammation.

Mechanisms underlying this novel link appear to involve energy utilization via expression of lipogenic genes as well as reduced inflammatory tone.

Taken together, these data indicate that the gut microbiota is an important factor involved in the myriad of metabolic complications associated with antipsychotic therapy. Furthermore, these data support the future investigation of microbial-based therapeutics for not only antipsychotic-induced weight gain but also for tackling the global obesity epidemic.

Chapter 1

General Introduction

1.1 Schizophrenia

Schizophrenia is a devastating disease which impacts drastically on an individual's mental and physical well being. Attempts to define and understand schizophrenia began in the mid-late 19th century with the emerging definitions of dementia praecox. These early characterisations of the mental disorder based on 'mental weakness' were further developed by Bleuler, who in the early 20th century replaced the term dementia praecox with schizophrenia and defined schizophrenia as a group of psychoses characterised by alterations in thinking, feeling and relations with the external world. Bleuler also conceived the diagnostic concept of the four A's: loosening of associations, inappropriate affect, ambivalence, and autism. This construct was extensively used for the diagnosis of schizophrenia until being widely replaced by Schneider's first-rank symptoms: audible thoughts, voices arguing, voices discussing, voices commenting, thought withdrawal, experiences of influenced thought, thought broadcasting, delusional perceptions and other experiences involving made volition or impulse (Schneider 1957). The current nosological status of schizophrenia is based on the Diagnostic and Statistical Manual (DSM-IV) of the American Psychiatry Association and does not rely on either of the aforementioned criteria, though the major themes are retained, with an emphasis on the presence of both positive and negative symptoms (Table 1.1).

Positive	*Negative*
Delusions	Affective flattening
Hallucinations	Alogia
Disorganised speech	Avoltion
Disorganised or catatonic behaviour	

Table 1.1 Symptoms of schizophrenia. *The positive and negative symptoms of schizophrenia currently included in the DSM-IV diagnostic criteria.*

Schizophrenia is a disabling group of brain disorders and has been described as the worst disease affecting mankind (Editorial 1988; Saha et al. 2005). This view point is based on the onset age (young adulthood), pervasiveness of the associated deficits and the often life-long duration of the disease (Tandon et al. 2008b). The incidence of schizophrenia is relatively low in the context of all disease, with annual incidence rates of 8-40/100,000 people and a lifetime risk of approximately 0.7% (McGrath et al. 2004; Saha et al. 2005; Saha et al. 2006). Despite this relatively low incidence, schizophrenia remains one of the major contributors to the global health burden and is a leading cause of disease-related disability worldwide (Lopez and Murray 1998).

Thus, there is a clear impetus for the development and advancement of pharmacotherapies used in the treatment of schizophrenia. This involves both the generation of newer, more efficacious drugs based on increased understanding of the neurobiology of schizophrenia, as well as the better use of already available therapies.

1.1.1 Neurochemistry of schizophrenia

Dopamine brain circuitry has long been viewed as the key factor in the pathogenesis of schizophrenia. This is based largely on the observation that compounds blocking dopamine receptors are useful in the treatment of psychosis (van Rossum, 1966), first recognized in the 1950's following the discovery of chlorpromazine (see section 1.2).

In the 1970s and 1980's, the development of receptor binding assays and the identification of receptor subtypes built on earlier evidence implicating dopamine in the mechanism of action of available antipsychotics (Carlsson and Lindqvist 1963). Indeed, this work led to the confirmation that antipsychotics block dopamine receptors (Creese et al. 1975; Creese et al. 1976) and the establishment of the dopamine hypothesis of schizophrenia (Snyder 1976). Later, positron emission tomography confirmed that 60-80% blockade of the dopamine D_2 receptor produces an optimal therapeutic antipsychotic effect and indeed seemed essential for antipsychotic activity (Creese et al. 1996; Kapur 1998).

Dopamine neurotransmission in the brain comprises four pathways. The mesolimbic pathway arises from the ventral tegmental area and projects to the nucleus accumbens as well as the amygdala and hippocampus. Hyperactivity of this pathway is believed to be the primary cause of the positive symptoms of schizophrenia (delusions, hallucinations) (Davis et al. 1991; Remington et al. 2011).

The mesocortical pathway also arises from the ventral tegmental area but projects to the frontal lobe of the pre-frontal cortex.

Hypoactivity of this pathway is believed to propagate the negative symptoms of schizophrenia (Davis et al. 1991; Remington et al. 2011).

The nigrostriatal pathway projects from the substantia nigra to the dorsal striatum and disruption of this pathway results in extrapyramidal symptoms (EPS) associated with antipsychotics (Dayalu and Chou 2008) (see section 1.2.2).

A distinct tuberoinfundibular pathway projects from the hypothalamus to the pituitary gland and this pathway regulates prolactin secretion. Thus, effects of antipsychotics on this pathways are responsible for antipsychotic-associated hyper-prolactinemia (Riecher-Rossler et al. 2009).

Dopamine receptors consist of two families, D_1 and D_5 comprise the D_1-like family which acts via G_s to activate adenylatecyclase and in turn increase cAMP (cyclic-adenosine monophosphate). D_2, D_3 and D_4, comprise the D_2-like family and act via G_i to inhibit adenylatecyclase and hence decrease cAMP. Thus, D_2 antagonism, the common denominator of all antipsychotics, reduces the hyperactivity of the mesolimbic dopaminergic pathway ameliorating the positive symptoms of schizophrenia.

The dopamine hypothesis of schizophrenia as an over-activity of dopamine neurons has been built upon and modified in recent times such that schizophrenia may be seen as a profound dysregulation of mesocorticolimbic dopamine circuitry with both hypo (mesocortical) and hyper (mesolimbic) functioning aspects in respective brain regions (Svensson, 2003).

Moreover, schizophrenia is highly heterogeneous disorder and the pathophysiology and neurobiology is far from fully understood. Neurotransmitter systems including serotonin, GABA (gamma-amino butyric acid) and glutamate have also been implicated and the role of these systems and their interactions is yet to be fully elucidated (Keshavan et al. 2008).

1.2 Treatment of schizophrenia

The earliest pharmacological treatments of schizophrenia involved cocaine or even castor oil. However, these treatments were ineffectual meaning the main stay of treatment from the 1930's to 1950's consisted of insulin-coma therapy (Ban 2004).

In the 1950's, serendipity provided the first widely accepted drug for the treatment of schizophrenia; chlorpromazine. Chlorpromazine was first synthesized in 1951 (Charpentier et al. 1952) before being introduced into clinical practice in 1953 (Delay et al. 1952) where it revolutionised the treatment of psychotic disorders (Turner 2007). Phenothiazines such as chlorpromazine were originally developed for use in the German dye industry before being used in medicine as antiseptics, anti-malarials and later as antihistamines. Indeed, it was while being utilised as an anti-shock therapy that the broader psychiatric potential of chlorpromazine was realised (Sigwald and Bouttier 1953; Shen and Giesler 1998; Lopez-Munoz et al. 2005).

1.2.1 Typical antipsychotics

Following the discovery of chlorpromazine, other antipsychotic compounds quickly followed including reserpine and haloperidol (Muller et al. 1952; Divry et al. 1959). Haloperidol is one of the few early antipsychotics that remains in relatively common use today (Verdoux et al. 2010).

1.2.2 Side effects of typical antipsychotics

The introduction of first-generation compounds revolutionised the treatment of psychiatric illness; however, it was quickly realised that they produce EPS, a range of motor related side effects (Ayd 1961; Shen and Giesler 1998; Arana 2000).

As already mentioned, the efficacy of these antipsychotics was shown to be produced by antagonism of dopamine D_2 receptors. Antagonism of this same receptor is also responsible for the development of EPS. Binding studies suggest that while D_2 receptor occupancy of 65% seems to be required for antipsychotic activity, occupancy levels greater than 80% induce EPS (Kapur et al. 2000).

EPS may be acute, occurring within hours or days, or tardive, occurring after prolonged exposure. Acute syndromes comprise primarily: dystonia, akathisia and parkinsonism. Dystonia results in sustained muscle contractions and is characterised by repetitive movements, twisting or abnormal posture (Fahn 1988). Akathisia is a syndrome characterised by a sensation of inner restlessness and often results in an inability to sit still or remain motionless with affected patients displaying multiple complex movements in an attempt to control their urge to move (Braude et al. 1983).

Parkinsonism, or Parkinson's syndrome, is a compilation of motor deficits resembling those seen in Parkinson's disease, which may present together or individually; common components include rigidity, tremor and postural instability (Shin and Chung 2012).

Tardive syndromes are those that appear only after sustained treatment, typically >3 months (American Psychiatric 2000).

These may include tardive dyskinesia, tardive akathisia or tardive dystonia. Tardive dyskinesia is characterised by repetitive, involuntary movements, commonly of the face such as lip smacking, tongue protrusion or grimacing (Schooler and Kane 1982). Tardive dystonia, though less common, often presents in conjunction with tardive dyskinesia.

Thus, EPS are a major reason for non-adherence to treatment by patients (Tandon and Jibson 2002). This is especially problematic with the treatment of schizophrenia which has an extremely high rate of non-adherence in general (Mitchell and Selmes 2007).

Typical antipsychotics are also associated with other side effects including prolactinemia which results in endocrine dysfunction and can lead to sexual dysfunction or infertility (Riecher-Roessler et al. 2009; Roke et al. 2009). As mentioned, these effects are due to dopamine receptor antagonism in the tuberofundibular pathway.

1.2.3 Atypical antipsychotics

The occurrence of EPS in conjunction with an unsatisfactory treatment response rate experienced with typical antipsychotics meant there existed a need for the development of more efficacious and safer antipsychotics. Clozapine, discovered in 1958, was developed by Sandoz and following its introduction to clinical practice in the 1970's was noted for its lower incidence of EPS (Hippius 1989; Hippius 1999).

This finding caused much incredulity regarding the utility of clozapine, as the dogma of the day was that EPS reflected efficacy of antipsychotic drugs (Van Rossum et al. 1970; Hippius 1989; Hippius 1999).

Thus, clozapine represents the first atypical antipsychotic (see section 1.2.4). Clozapine's initial success was short-lived as the drug was associated with the life-threatening side effect agranulocytosis, the suppression of white blood cell production (Gerson and Meltzer 1992). This serious consequence of clozapine was especially brought to light during an epidemic in Finland (Idanpaanheikkila et al. 1975).

The clozapine programme was subsequently halted, though it remained available to a limited degree in some countries. Subsequently, with growing anecdotal evidence of clozapine's efficacy, coupled with advancements in the management of agranulocytosis, the clozapine programme was restarted in 1982, and the drug was approved by the FDA in 1989. Clozapine re-entered the market in 1990, primarily for treatment-refractory patients and with stringent patient monitoring rules attached (Crilly 2007). In addition to a lack of EPS, clozapine is also noted for it superior efficacy in preventing suicide in schizophrenia patients (Meltzer and Okayli 1995; Meltzer et al. 2003a).

With the realisation that clozapine's efficacy – without a propensity toward EPS was genuine, several other atypical antipsychotics were eventually developed some 30 years after the discovery of clozapine (Meyer and Simpson 1997). The first of these was risperidone, followed by others such as: quetiapine, ziprasidone, risperidone and olanzapine.

At the time of its discovery, clozapine was noted for a lack of effect on amphetamine-induced psychomotor agitation in rodents – the accepted model of the time for identifying potential antipsychotics that was known to depend on potent D_2 antagonism (Meyer and Simpson 1997).

This had important connotations, as researchers searched for alternative models to discover antipsychotic compounds. LSD (lysergic acid diethylamide), a compound known to produce certain schizophrenia-like effects in humans (Lake et al. 1981) had not been used in experimental models as it did not produce any measurable behaviour in rodents that could be translated to humans.

However, Janssen developed a drug-discriminating tool that allowed for screening of drugs with LSD-antagonising properties and hence potentially antipsychotic properties (Colpaert 2003).

This led Janssen to developing risperidone in the 1980's (Colpaert 2003). Crucially, LSD was known to produce its effect via both dopamine and serotonin receptors, and risperidone was found be a potent 5-HT_2 antagonist. Thus, the way was paved for the mixed pharmacology of subsequent atypical antipsychotics that now dominate the psychopharmacology landscape of schizophrenia therapy (Table 1.2).

Generic name	Brand names
Typical antipsychotics	
Chlorpromazine	Largactil, Thorazine
Thioridazine	Mellaril, Melleril, Novoridazine, Thioril
Mesoridazine	Serentil
Levomepromazine	Nosinan, Nozinan, Levoprome
Loxapine	Loxapac, Loxitane
Molindone	Moban
Perphenazine	Trilafon

Thiothixene	Navane
Trifluoperazine	Stelazine
Haloperidol	Haldol
Fluphenazine	Prolixin
Droperidol	Droleptan, Dridol, Inapsine, Xomolix, Innovar
Zuclopenthixol	Clopixol
Prochlorperazine	Compazine, Stemzine, Buccastem, Stemetil, Phenotil
Atypical antipsychotics	
Amisulpride	Solian
Asenapine	Saphris
Blonanserin	Lonasen
Clotiapine	Entumine
Clozapine	Clozaril
Iloperidone	Fanapt
Lurasidone	Latuda
Olanzapine	Zyprexa, Ozace
Paliperidone	Invega
Perospirone	Lullan
Quetiapine	Seroquel
Remoxipride	Roxiam
Risperidone	Risperdal, Zepidone
Sertindole	Serdolect
Sulpiride	Sulpirid, Eglonyl

Ziprasidone	Geodon, Zeldox
Zotepine	Nipolept
Third generation antipsychotics	
Aripiprazole	Abilify
In Phase II/Phase III trials	
Bifeprunox	DU-127,090
Pimavanserin	ACP-103
Vabicaserin	SCA-136
Zicronapine	LLU 31-130
ITI-007	-
LY 2140023	-
RP 5063	-
AZD 2624	-
OPC-34712	

Table 1.2 Drugs used in the treatment of schizophrenia. *Currently used drugs are classified as typical (first generation), second generation (atypical) and third generation. Several compounds are also currently in development 1(Tandon et al. 2008a; http://www.clinicaltrials.gov/ 2013).*

1.2.4 Typicality versus atypicality

The distinction between the first generation typical antipsychotics and second generation atypical was established based on their liability to induce EPS. Of note, early antipsychotics were only termed typical, or first-generation, following the introduction of the newer, atypical or second-generation drugs.

It must also be noted that both classes of antipsychotic are extremely heterogeneous in terms of pharmacology. A number of prevailing theories exist to explain what indeed is atypicality and separates the newer compounds from the older ones. This is an important point, as understanding why the two classes differ in EPS liability will allow for the better design of future antipsychotics, minimising or eliminating the occurrence of EPS. Indeed, the heterogeneity of both groups have led some to question whether atypicality really exists at all (Tyrer and Kendall 2009). Although, differences in potency, as well as efficacy in treating certain symptoms, suggests that the newer drugs are worthy of differentiation from older compounds(Meltzer 2009)

One prominent theory of atypicality relates to the ratio of serotonergic to dopaminergic receptor blockade (Meltzer et al. 1989). Atypical antipsychotics have a more diverse pharmacological profile than typical antipsychotics that act almost exclusively as dopamine antagonists. In particular, atypical antipsychotics are noted for their high affinity for serotonin receptors, especially the 5-HT_{2A} receptor (Miyamoto et al. 2004).

As serotonin can inhibit dopamine transmission (Kapur and Remington 1996), the blocking of serotonin receptor may augment dopamine release thereby lessening the effects of the antipsychotic and preventing EPS.

However, the 5-HT_{2A}:D_2 ratio of atypical antipsychotics does not predict their EPS liability (or lack thereof) (Kapur and Mamo 2003). Moreover, as already mentioned, the identification of a 'therapeutic window' whereby greater than 65% D_2 receptor occupancy confers therapeutic benefit while greater than 80% leads to EPS (Kapur et al. 2000) indicates that 5-HT_{2A} antagonism may be unnecessary for atypicality.

The second major hypothesis to explain the mechanism of reduced EPS with the atypical antipsychotics is commonly referred to as the 'fast-off' theory. This theory suggests that the faster dissociation rates of atypical compounds (i.e. lower affinity) compared to the typical drugs leads to adequate occupancy for therapeutic response but prevents occupancy levels reaching those required for EPS (Kapur and Seeman 2001).

It must also be noted that atypical antipsychotics do cause EPS, further greying the area between typical and atypical drugs. However, EPS as a side-effect of atypical compounds occurs much more rarely in clinical practice due mainly to the fact that they achieve a therapeutic response at lower doses, as increasing doses lead to increased incidence of EPS with atypical antipsychotics such as risperidone and olanzapine (Kapur et al. 1999; Tarsy et al. 2002).

1.3 Body weight regulation

The regulation of energy homeostasis is a critical biological process. Through evolution, humans have developed several physiological strategies in order to maintain both short and long-term energy balance. In the simplest terms, energy balance is a paradigm controlled under the first law of thermodynamics (energy can neither be created nor destroyed) and as such consists of energy intake, energy expenditure and stored energy (Goran 2000; Hall 2012) (Fig 1.1). However, the control of energy balance and body weight is a highly complex and multifactorial process and numerous factors contribute to the regulation of energy intake, energy storage and energy expenditure (Galgani and Ravussin 2008).

Energy Intake = Energy Burned + Energy Stored

Fig. 1.1 The energy balance equation.

It is clear therefore, that differences between each side of this equation results in a net energy balance which may be: positive; associated with weight gain, negative; associated with weight loss, or neutral; associated with weight maintenance (Fig 1.2). The goal of the regulatory system is to drive energy balance in the direction necessary to maintain adequate adiposity and body weight to ensure survival and several mechanisms are in place that can affect both sides of the equation in order to achieve this goal (Galgani and Ravussin 2008; Williams and Elmquist 2012)

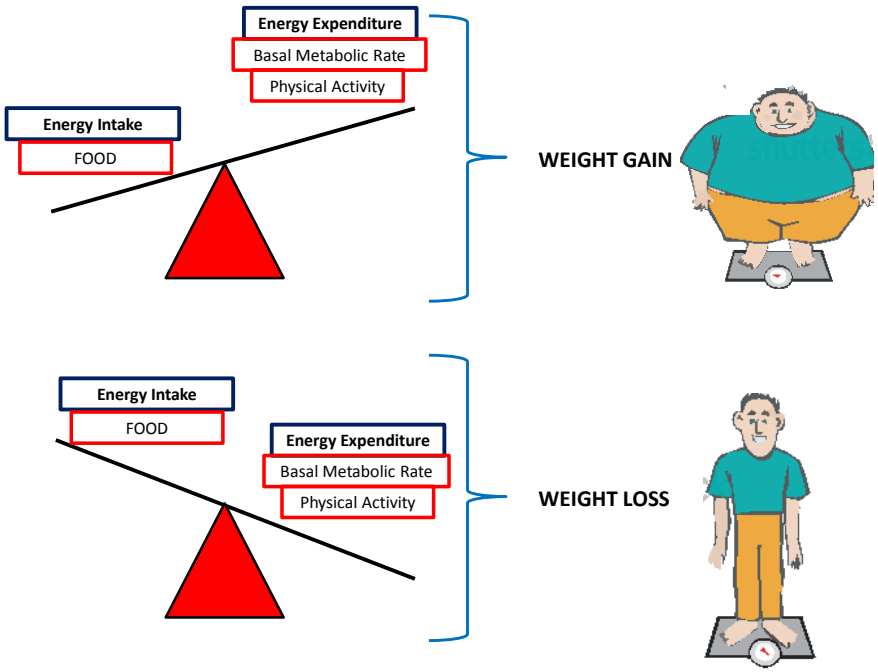


Fig. 1.2 Body weight regulation. *The main contributors to both sides of the energy balance equation and the effect of shifting the energy balance on body weight.*

To ensure an adequate energy balance, a central neural regulatory system receives information regarding the nutritional status and energy stores of the body, and subsequently generates appropriate signals to drive food intake, energy storage and energy expenditure in the direction necessary to maintain a steady state of adiposity and body weight. As we will see below however, this system consists of numerous inter-related factors, and moreover, the mechanisms of body weight regulation are complex and not yet fully understood (Gale et al. 2004).

This adaptable regulatory system is also a vulnerable one. As such, numerous physiological and psychological factors can exert influences on the system, which, in the context of the modern world, may not ultimately be beneficial to the individual.

Thus, understanding the various components which act both centrally and peripherally to affect either side of the energy balance equation is critical to both understanding body weight regulation as well as tackling associated medical problems.

1.3.1 Energy intake

Our entire energy intake comes from one source - the food we eat. Thus, one might think controlling this side of energy balance is a relatively simple task. However, food intake is one of the most complex human behaviours and regulating it is a considerable physiological challenge. The complexity and importance of regulating energy balance and food intake is highlighted by the fact they are controlled by the intricate interaction between neural systems, hormonal and nutrient signals involving thousands of genes.

Appetite is regulated by a complex network of central and peripheral hormones, peptides and receptors (Stanley et al. 2005). Interactions between these components help maintain the homeostatic balance between energy intake and expenditure. Food intake and ingested nutrients modulate the peripheral release of a plethora of hormones and gut peptides, which all act through the brain to co-ordinate appetite and subsequent food intake. This bidirectional communication between the gut and the brain represents the brain gut axis and is important in body weight and obesity as well as potentially many other disorders (Konturek et al. 2004).

1.3.1.1 Hypothalamic regulation of food intake

The hypothalamus, situated at the base of the brain, is the major regulator of food intake. Studies carried out as long as seventy years ago demonstrated the critical role played by the hypothalamus in food intake (Hetherington and Ranson 1940; Elmquist et al. 1999). Lesions of the ventromedial hypothalamus led to hyperphagia and obesity while lesions of the lateral hypothalamus caused reduced food intake and leanness. Furthermore, electrical stimulation (i.e. activation) of the respective areas produced the opposite effects (Hetherington and Ranson 1940; Anand and Brobeck 1951; Delgado and Anand 1953; Stellar 1954). Subsequent work has revealed that the role hypothalamus plays in food intake is complex, and involves connections with other brain regions. Moreover, the role of the mediobasal hypothalamus and in particular the arcuate nucleus (ARC) has come to the fore.

The ARC, as the name suggests, is an arc-shaped collection of neuronal cell bodies. It is ideally located to sample the peripheral circulation through semi-permeable capillaries in the underlying median eminence, allowing it to integrate the peripheral signals with central regulation (Neary et al. 2004). The ARC contains two distinct neuron populations which exert potent and opposite effects on food intake. Agouti-related peptide (AgRP)/ Neuropepetide Y (NPY) co-expressing neurons are orexigenic, promoting food intake (and inhibiting energy expenditure) (Hahn et al. 1998) while pro-opiomelanocortin (POMC) and cocaine-amphetamine regulated transcript (CART) co-expressing neurons are anorexigenic, reducing food intake (and increasing energy expenditure) (Elias et al. 1998; Elias et al. 2001).

1.3.1.2 Neuropeptide Y

NPY is potently orexigenic and acts primarily via its Y1 and Y5 receptors in exerting this function (Stanley et al. 1986; Stanley et al. 1992; Zarjevski et al. 1993; Gerald et al. 1996; Michel et al. 1998).

It is unsurprising therefore, that NPY expression is increased in genetically obese, leptin deficient, (ob/ob) mice (Wilding et al. 1993) and that secretion of NPY in the hypothalamus is increased when fat stores are depleted, but levels are decreased by expanding fat mass (White and Kershaw 1990; Kalra et al. 1991; Wilding et al. 1993).

1.3.1.3 Agouti-related peptide

AgRP is also a potent orexigenic peptide (Rossi et al. 1998). Like NPY, AgRP expression is localised to the ARC (Shutter et al. 1997) and is up-regulated by fasting (Hahn et al. 1998).

AgRP acts as a competitive antagonist of the melanocortin MC_3 and MC_4 receptors (Ollmann et al. 1997; Haskell-Luevano and Monck 2001) and though less potent than NPY, AgRP appears to have longer lasting effects on food intake (Seeley et al. 2000).

The MC_3 and MC_4 receptors are highly unusual in human physiology as they have an endogenous agonist (melanocortins) and endogenous antagonist (AgRP). The melanocortins are anorexigenic and act by binding primarily to the MC_3 MC_4 receptors (Mountjoy et al. 1992; Huszar et al. 1997; Irani et al. 2011). AgRP therefore inhibits this anorexic effect, indirectly promoting feeding.

1.3.1.4 Pro-opiomelanocortin

POMC is a precursor of several biologically active peptides and is cleaved in a site-specific manner to produce a number of products including α- melanocyte stimulating hormone (α-MSH) and β-MSH. These melanocortins act on the melanocortin receptors MC_3 and in particular MC_4 to illicit anorexic responses (Cone et al. 1996; Cone 2005). AgRP neurons can also release GABA, the main inhibitory neurotransmitter in the CNS, and thus can also inhibit nearby POMC neurons via this GABA release (Cowley et al. 2001).

The transduction of neuronal signals to increase/decrease food intake is far from fully understood, however a number of integrated pathways which are involved have been identified (Valassi et al. 2008; Harrold et al. 2012). Neurons from the ARC signal to other discrete hypothalamic nuclei including the paraventricular nucleus (PVN), lateral hypothalamus (LHA) and perifornical area (PFA) (Sainsbury and Zhang 2010; Rediger et al. 2012)(Fig 1.3). Secondary neurons from the ARC innervate these regions and cause the release of further catabolic/anabolic neuropeptides.

1.3.1.5 Hypothalamus-pituitary-adrenal axis

Neuropepetides synthesised in the PVN include coritcotropin-releasing hormone and thyrotropin-releasing hormone, both of which are anorectic (Kow and Pfaff 1991; Grill et al. 2000). These hormones are also crucially involved in regulating the hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-thyroid (HPT) axes.

Both these axes can affect peripheral energy regulation through the release of hormones such as glucocorticoids in the case of the HPA (Dallman et al. 1993; Dallman et al. 1995), and oxytocin by the thyroid gland (Olson et al. 1991). Thus, the PVN plays a crucial role in regulating energy intake and energy balance by integrating signals from the ARC with these hormone systems (Schwartz et al. 2000).

1.3.1.6 Melanocyte-concentrating hormone

Melanocyte-concentrating hormone (MCH) is an orexigenic hormone synthesised in the LHA/PFA hypothalamic regions and is important for stimulating a feeding response (Qu et al. 1996; Shimada et al. 1998). Other hormones in this region, orexin A and orexin B, as their name suggests, are also orexigenic (Sakurai et al. 1998).

1.3.1.7 Orexins

The orexins are involved in the regulation of sleep (Chemelli et al. 1999; Lin et al. 1999) and thus may play an integrative function within the hypothalamus, helping to coordinate its various homeostatic functions including feeding, sleep, arousal and stress responses (Yamanaka et al. 2003; Spinazzi et al. 2006)

The bidirectional contact between these various nuclei and other brain regions remains to be fully elucidated, but it is clear that the complex interaction between them is central to the proper regulation of energy balance.

1.3.1.8 Dopamine

Dopamine also plays an important role in the hypothalamic control of food intake (Schwartz et al. 2000).

There is evidence that decreased dopaminergic tone in hypothalamic nuclei contributes to the obesity/insulin resistant phenotype as seen in seasonally obese (hibernating) animals in which dopaminergic neuronal activities change profoundly with the changing seasons (Luo et al. 1999). Antagonism of hypothalamic D_2 receptors has been shown to affect food intake, inducing hyperphagia in rats (Parada et al. 1988).

However, dopamine's role in feeding behaviour is highly complex and far from fully understood. Indeed, projections of the nigrostriatal pathway have recently been implicated in feeding behaviour and evidence suggests the dorsal striatum may be particularly important to this function of dopamine (Palmiter 2007; Narayanan et al. 2010).

1.3.1.9 Serotonin

Serotonin (5-HT) is potently anorexigenic and is keenly involved in the regulation of appetite and energy balance. The 5-HT_{2C} receptor is particularly implicated in this role (Lam et al. 2008) though several other 5-HT receptors including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_7 seem to be involved in possibly complementary mechanisms (Nonogaki, 2008).

Serotonin's satiety effects are mainly hypothalamic driven, though the wide expression of receptors suggests its influence on eating behavior may not be restricted to the hypothalamus (Leibowitz and Alexander 1998; Lucki 1998).

The 5-HT_{2C} receptor shares a high degree of homology with 5-HT_{2A} and 5-HT_{2B} receptors and all three are coupled to $G_{\alpha q}$. Their activation typically invokes activation of phospholipase C, the generation of the second messenger inositol triphosphate (IP3) and influx of extracellular calcium resulting in activation of protein kinase C (Leysen 2004).

These receptors are found in several brain regions including the hypothalamus, striatum, frontal cortex and several others where they modulate both excitatory and inhibitory neurons allowing for normal functioning of feeding, locomotor and anxiogenic processes.

5-HT_{2c} knockout mice have been shown to be hyperphagic and obese (Tecott et al. 1995). Furthermore, 5-HT_{2c} receptors have been shown to be co-expressed with POMC neurons (Xu et al. 2008) and re-expression of 5-HT_{2c} receptors on POMC neurons was shown to rescue the obese phenotype in 5-HT_{2c} null mice (Xu et al. 2008). Thus, 5-HT_{2C} blockade or deletion can result in decreased POMC expression and subsequent hyperphagia (Nonogaki et al. 2008).

5-HT_{2c} agonist have been shown to reduce weight in humans (Sargent et al. 1997) and some agonists such as the sibutramine and fenfluramine have been used clinically to treat obesity (though these are no longer used due to adverse effects).

1.3.1.10 Histamine

Histamine is also anorexigenic and central histamine originates in the tuberomammilary body of the posterior hypothalamus and arising histaminergic neurons project to several brain areas with the highest density found in the hypothalamus (JÃ¸rgensen et al. 2007). Histamine's hypothalamic effects are mediated by the H_1 receptor which is a post-synaptic G_α protein coupled receptor and activation results in increase mobilisation of intracellular calcium and thus activation of phospholipase C as well other intracellular mechanisms. H_1 receptor knockout mice have increased food intake and body weight gain (Masaki et al. 2004) and histamine H_1 antagonists dose dependently increase feeding in rats (Sakata et al. 1988).

Histamine may not only have a direct effect on appetite but also plays an important role in the regulation of leptin's central effects. Leptin administration has no effect in H_1 knockout mice (Morimoto et al. 1999) and ob/ob mice show decreased levels of histamine in the hypothalamus. This demonstrates the important role played by histamine in the mechanisms of action of leptin (Machidori et al. 1992). Hence, histamine H_1 receptor antagonism seems to disrupt the action of leptin (Masaki et al. 2001) preventing leptin from exerting its satiety effects, causing or at least exacerbating leptin resistance (Starrenburg and Bogers 2009). Histamine can also effect peripheral metabolism by activating the sympathetic nervous system promoting energy expenditure by accelerating lipolysis in white adipose tissue and hence reduce fat deposition in adipose tissue (Tsuda et al. 2002).

1.3.1.11 Noradrenaline

Noradrenaline can affect food intake with α_1 and α_2-adrenoceptor agonists having opposing effects on food intake (McCabe et al. 1984; Wellman and Davies 1992).

The incorporation of these many monoamine systems in to an already complex regulatory system that controls food intake highlights the incredibly multi faceted nature of energy regulation, and the scale of the task in trying to understand and combat metabolic disorders such as obesity.

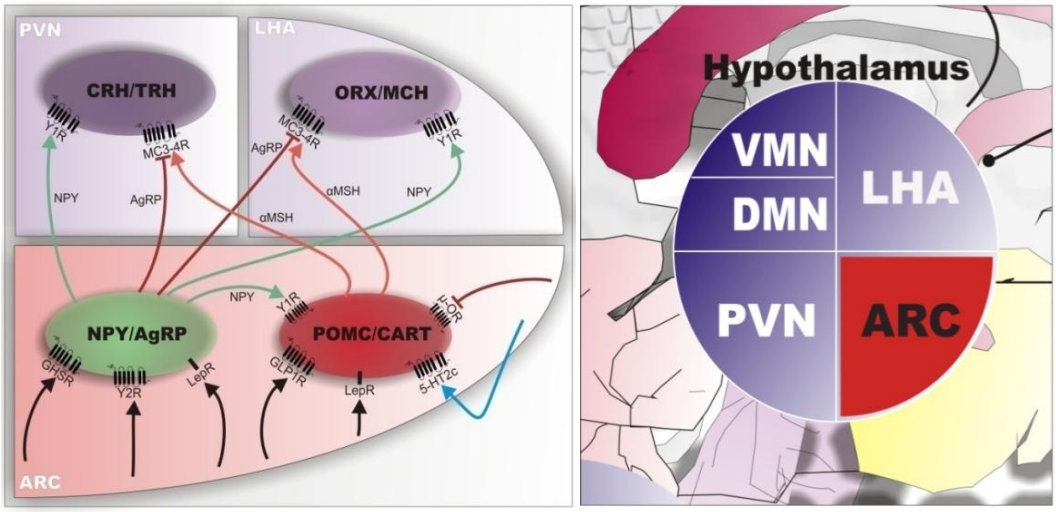


Fig. 1.3 Hypothalamic regulation of energy intake. *The major nuclei of the hypothalamus: VMN; ventromedial nucleus, PVN; paraventricual nucleus, DMN; dorsomedial nucleus, LHA; lateral hypothalamus, and ARC; arcuate nucleus. The ARC contains both orexigenic neurons coexpressing NPY/AgRP and anorexigenic POMC/CART neurons which receive peripheral signals including ghrelin, leptin, insulin, GLP-1 and PYY as well as central signals such as monoamines.. The ARC (arcuate nucleus) communicates with other hypothalamic regions via melanocortin (MC_3-R and MC_4-R) and neuropeptide Y (NPY) receptors. (Figure courtesy of H. Schellekens).*

1.3.2 Peripheral signals in the regulation of energy balance

The communication to stimulate feeding is dependent on energy stores, i.e. adiposity. Hormones termed adiposity signals relate information regarding the body's energy status to the hypothalamus allowing for an appropriate neuronal and endocrine response. Arguably the most important of these signals is the hormone leptin. Leptin is released directly from adipocytes, and plasma leptin levels correlate to adipose mass (Friedman and Halaas 1998). Moreover, brain leptin levels are proportional to plasma levels and hence adiposity (Schwartz et al. 1996).

Over forty peripheral hormones are known to affect food intake. Many of these signals are released from peripheral tissues and act centrally as feedback signals in order to influence appetitive drive. These include adiponectin, resistin, PYY, leptin, ghrelin and insulin which all also have important peripheral metabolic effects (Fig 1.4).

Cholecystokinin, amylin and pancreatic glucagons are peptides released rapidly from the intestine and pancreas with the onset of feeding and have short durations of action. These peptides all act to propagate meal termination. The postprandially released cholecystokinin was the first gut hormone demonstrated to have an effect on food intake (Gibbs et al. 1973). Amylin, secreted from the pancreatic α cell secretory vesicles in response to food, has a glucose-regulatory and an anorexigenic action, reducing food intake via actions on the hindbrain area postrema and central nucleus of the amygdala (Lutz 2006).

PYY, glucagon-like peptide 1 (GLP-1) and oxyntomodulin are peptides for which longer-term feeding inhibitory actions have been identified (Stanley et al. 1985; Cohen et al. 2003; Dakin et al. 2004; Baggio and Drucker 2007). These are released from intestinal L cells in the distal intestine and levels rise slowly during a meal, peaking after meal termination and remaining elevated for several hours. Pancreatic polypeptide is also released postprandially into the circulation in proportion to calories ingested to reduce food intake (Batterham et al. 2002).

In addition, adipokines including leptin, adiponectin and resistin are secreted by the adipose tissue in proportion to fat mass.

These adipokines mediate their central effects via the hypothalamus to affect food intake as well as energy expenditure (Zhang et al. 1994; Tovar et al. 2005; Ahima and Lazar 2008; Kadowaki et al. 2008). Leptin counteracts the effect of NPY and promotes the synthesis of α-MSH. Leptin mediated inhibition of food intake is long-term, in contrast to the rapid inhibition by cholecystokinin and the slower suppression of hunger between meals mediated by PYY.

Insulin is secreted by the pancreas upon increase in glucose load and, like leptin, has profound anorexigenic effects on appetite (Woods et al. 1979; Hallschmid et al. 2004). Insulin accesses the brain via receptor-mediated transport across the blood-brain barrier where it acts on insulin receptors that are highly expressed in the ARC and are also co-expressed with POMC, CART and NPY, meaning insulin is in prime position to carry this weight-reducing function. Insulin also of course exerts crucial functions in peripheral tissues such as liver, muscle and adipose tissue to maintain glucose homeostasis.

Ghrelin acts as a growth hormone-releasing peptide and is secreted from the stomach. Ghrelin is the only peripheral hormone identified as being orexigenic. Therefore, ghrelin has been aptly coined the 'hunger hormone' due to its strong appetite enhancing action (Tschop et al. 2000; Nakazato et al. 2001; Kojima et al. 2004). Moreover, ghrelin secretion is enhanced in between meals and under conditions of negative energy balance, such as starvation and anorexia (Wren et al. 2001; Lawrence et al. 2002; Sun et al. 2004). In contrast, food intake leads to a decrease in plasma ghrelin levels. Considerable evidence supports the role for the hormone ghrelin in mealtime hunger and meal initiation, increasing food intake and body weight (Tschop et al. 2000; Cummings et al. 2001a).

Ghrelin exerts its effect centrally via the growth hormone secretagogue receptor, a G-protein coupled receptor.

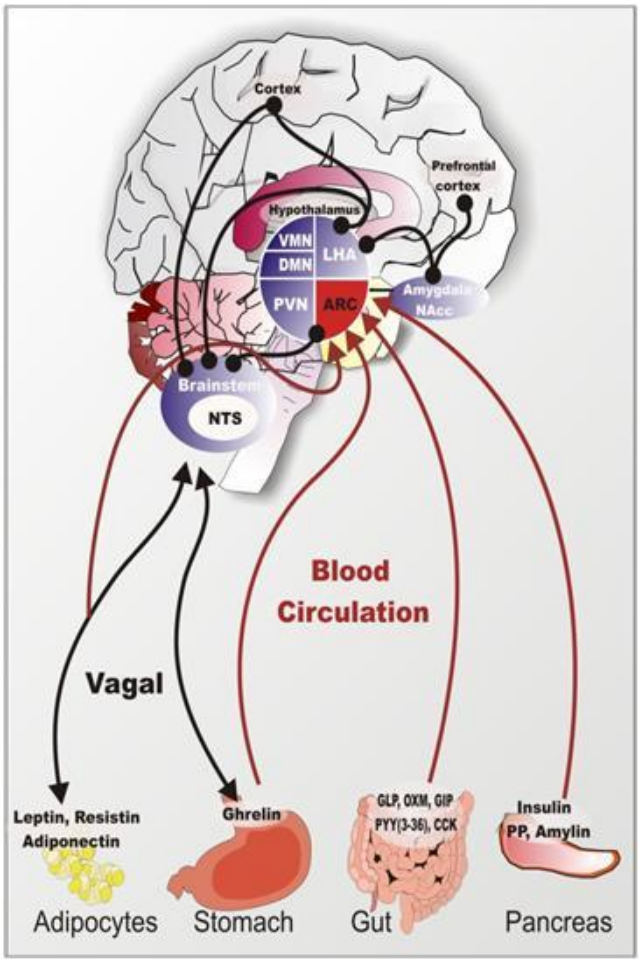


Fig. 1.4 Bi-directional regulation of energy balance. *Signal from the periphery including the gut, adipocytes, stomach and pancreas are integrated centrally producing outputs that in turn signal to these organs in order to ensure proper energy balance is maintained (Figure courtesy of H. Schellekens).*

1.3.3 Energy storage

The body is able to store excess energy if intake exceeds the body's energy needs (i.e. expenditure). This is an extremely valuable asset in terms of long term survival and short-term homeostasis as it ensures that the body has access to required energy stores even in times when energy intake is severely reduced.

This is an obvious advantage in times such as famine of course but also perhaps more relevantly today in circumstances such as strenuous exercise.

Short-term energy requirements can be met by the storage of energy as carbohydrate, i.e. glycogen, in the liver and muscle tissue. In the fed-state (postprandial) carbohydrates (mainly glucose) are assimilated into the hepatic portal blood stream and transported to tissues for use as an energy source. The remainder of the absorbed glucose is transported directly to the liver where some of the excess glucose is converted to glycogen by the process of glycogenesis and stored. As the option for glycogen stores is relatively limited, further glucose is converted to fat, also within the liver, via the process of lipogenesis (Fig.1.5).

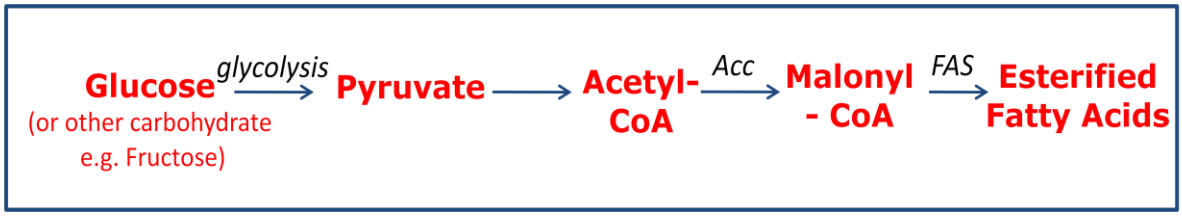


Fig.1.5 Biochemical process of lipogenesis. *Carbohydrate is converted to acetyl-CoA via glycolysis and acetyl-CoA is converted to malonyl-CoA catalysed by acetyl-CoA carboxylase (Acc) which is in turn catalysed by fatty acid synthase (FAS) to form fatty acids.*

The storage of large amounts of energy is achieved by storing it as fat. Fat is energy dense, 1g containing 9 kilocalories (kcal), twice as much as carbohydrate or protein. Dietary fat is absorbed primarily as triglycerides in the form of chylomicrons. Chylomicrons bypass the liver as they reach the venous circulation by way of the lymphatic system. Lipoprotein lipase (LPL) is the key enzyme involved in the uptake of triglycerides into adipocytes (Wang and Eckel 2012). LPL is bound to capillary endothelium and converts circulating triglycerides to free fatty acids and monoglycerides.

These molecules can then diffuse into the cell where they are reassembled into triglycerides and stored, mainly as triacylglycerol.

Fatty acids produced in the liver via de novo lipogenesis are packaged as triglycerides, typically as very low density lipoprotein (VLDL), and released into the bloodstream before being transported to adipose tissue and stored as outlined above.

1.3.3 Energy expenditure

Three processes account for energy expenditure, namely: resting metabolic rate, diet-induced thermogenesis and physical activity. Resting metabolic rate is the energy expended to survive i.e. maintain heat rate, breathing etc. and is the main contributor to an individuals daily energy expenditure (Donahoo et al. 2004). Basal metabolic rate is metabolic rate during sleep and is generally around 3% lower than resting metabolic rate due to the energy required for arousal (Donahoo et al. 2004). These terms are therefore commonly used interchangeably. Inter-individual variations in daily resting metabolic rate can reach 20-25% though an individuals' tends to be stable. The typical adult resting metabolic rate is approximately 1 kcal/min (Goran 2000).

Diet-induced thermogenesis constitutes the energy cost of digesting, metabolising and storing nutrients. Also known as the thermic effect of food, diet-induced thermogenesis is approximately proportional to the caloric density of the meal, with the energy cost of a meal being roughly 10% of the energy intake (Schutz 2004).

Physical activity or exercise is the most variable aspect of energy expenditure (van Baak 1999) and comprises all movement carried out during a day.

An Individual's occupation, environment, age and gender all have a significant impact on one's daily activity and thus on energy expenditure (Goran 2000).

Thus, energy expenditure coupled with food intake is clearly an important factor in the regulation of energy homeostasis and for the development of related disorders such as obesity. Moreover, due to the voluntary nature of energy expenditure (i.e. exercise), energy expenditure is also an important target in tackling obesity and related diseases (Poehlman et al. 1991) (see section 1.4.3.).

1.4 Obesity

The prevalence of obesity is rapidly increasing in both developed and developing countries and is a considerable burden upon health care systems worldwide (Bloom et al. 2008; Swinburn et al. 2011). In the U.S., the prevalence of obesity now exceeds 30% (Flegal et al. 2010) with European figures trending towards the same astonishing rate (Berghoefer et al. 2008). Perhaps even more worrying is the increasingly high rates of childhood and adolescent obesity Worldwide, the prevalence of childhood overweight now stands at approximately 60% with 43 million pre-school children were overweight in 2010 (de Onis et al. 2010). Thus, with obesity now accepted as a global epidemic, there has never been a greater need for research into the aetiology of the disease and ways to combat it.

The development of this obesity epidemic largely stems from alterations in gene x environment interactions. Genetic predisposition within individuals increases the likelihood of the development of obesity (Loos and Bouchard 2003; Dahlman and Arner 2007; Bouchard 2008).

These include variants of the fat mass and obesity associated gene (FTO) and the MC_4 receptor (Li and Loos 2008; Loos et al. 2008; Willer et al. 2009). However, the alarming rate at which obesity rates have increased in recent times indicates that lifestyle and environmental changes represent the major cause for a shift toward an obese society rather than genetic changes (Marti et al. 2004).

As can be seen from the energy balance equation earlier (Fig. 1.1), the root cause of obesity is energy imbalance i.e. more calories consumed than expended (Chakrabarti 2009). However, genetic and environmental interaction acts in concert with physiological processes to make obesity a highly complex disease. For obese individuals therefore, eating less and exercising more is not as straightforward a remedy as it may seem. From an evolutionary point of view our bodies have a primal nature that drives eating and storage of body fat (Wells, 2009). Moreover, in obese individuals, the homeostatic set point is changed and the obese state is perceived as the normal state (Harris 1990) meaning all the 'good intentions' of diets, can be undermined by the body's physiological adjustments in chronic obesity. Losing excess weight, it seems, is a very unnatural thing to do in non-hibernating mammals, making efforts to tackle the obesity epidemic extremely complex.

1.4.1 Obesity and energy intake

The recent boom in the prevalence of obesity worldwide clearly indicates that lifestyle factors are the major contributor to this epidemic. An increase in the availability of cheap, energy-dense foods has undoubtedly resulted in increases in caloric intake.

A reliance on processed foods, ready meals and fast food which all contain a high carbohydrate and fat content means that people are chronically over consuming these macro-nutrients leading to a prevalence of weight gain and obesity (Hill and Peters 1998; Hill 2006; Heitmann et al. 2012).

1.4.2 Obesity and energy storage

As discussed above, excess energy is primarily stored as triglyceride within adipocytes. However, a chronic state of positive energy balance brought about by sustained intake of calorie rich foods results in an accumulation of triglyceride and ultimately adipocyte dysfunction.

Excessive delivery of triglycerides to adipocytes leads to adipocyte hypertrophy and hyperplasia (Ferre 2003; de Ferranti and Mozaffarian 2008). Evidence suggests that the adipose tissue reaches a critical mass after which hypertrophy and hyperplasia result in cell dysfunction, leading to pro-inflammatory responses which contribute to the metabolic dysfunction associated with obesity (Langin 2006).

1.4.3 Obesity and energy expenditure

As with energy intake, lifestyle changes have contributed in a significant way to decreases in energy expenditure. Advances in transport and technology have led to a more sedentary lifestyle. A reduction in exercise and general physical activity levels combined with increased food intake are thought to be the major forces driving the ever growing numbers of overweight and obese individuals (Goran 2000; Heitmann et al. 2012).

The high rate of obesity has led to increases in complications associated with obesity, notably the metabolic Syndrome (MetS) (also known as Reaven's syndrome X and the insulin resistance syndrome). Metabolic syndrome describes a constellation of cardiovascular risk factors, specifically: insulin resistance (type 2 diabetes), glucose intolerance, dyslipidemia and hypertension (see section 1.5).

1.5 Metabolic syndrome

The metabolic syndrome is a constellation of obesity-related abnormalities (Eckel et al. 2005; Alberti et al. 2009; Bruce and Byrne 2009; Bruce and Hanson 2010). Definitions of the metabolic syndrome are varied and differ based on epidemiological parameters such as race and gender. However, general guidelines on what constitutes the metabolic syndrome have been compiled (Table 1.3). The main metabolic complications associated with the syndrome include: glucose dysregulation, increased adiposity, dyslipidemia and raised blood pressure.

Measurement	Diagnostic cut-off point
Waist Circumference	*≥ 102 cm (men); ≥88 cm (women)*
Triglycerides	≥ 150 mg/dl
HDL	*< 40 mg/dl (men); < 50 mg/dl (women)*
Blood Pressure	≥ 130 mm Hg (systolic); ≥ 85 mm Hg
Fasting Glucose	≥ 100 mg/dl

Table 1.3 Components and diagnostic criteria for the metabolic syndrome. *HDL; high density lipoprotein (adapted from (Grundy et al. 2005).*

Importantly, obesity *per se* is not a prerequisite of the metabolic syndrome. This can be an important distinction as 'healthy-obese' individuals may not be at risk of serious co-morbidities and equally, non-obese, metabolically-unhealthy individuals may be. This difference is particularly relevant in the context of visceral fat, as increased central adiposity (i.e. visceral fat) is a component of the metabolic syndrome and, as will be discussed below, plays a considerable role in the milieu of metabolic complications ensuing from the syndrome. Indeed, the co-occurrence of adiposity within the metabolic syndrome increases the relative morbidity risk for an individual (Cheng and Leiter 2006; Mikhail 2009).

Unsurprisingly given its close links to obesity, the prevalence of the metabolic syndrome is at an all time high with some 31% of Americans fulfilling the above diagnostic criteria in 2002 (Ford et al. 2002). Furthermore, as a major risk factor for the development of Type II diabetes, this increase in the prevalence of the metabolic syndrome undoubtedly contributes to the current and future predicted rise in cases Type II diabetes (Wild et al. 2004; Shaw et al. 2010).

1.5.1 Visceral adiposity

Visceral fat comprises the adipose tissue found in the abdominal cavity, where is acts to insulate and support several organs. Hence, adipose tissue is found in discrete locations associated with particular organs. These depots include mesenteric fat (associated with the intestines), perirenal fat (associated with the kidneys) and gonadal fat associated with uterus and ovaries in females and the epididymis and testes in males).

Adipose tissue, a common type of connective tissue, is comprised of circa 80% adipocytes with the remainder being made up by macrophages, fibroblasts and endothelial cells.

Visceral adiposity may be measured in humans directly by tomography or magnetic resonance imaging (MRI), or as a function of central obesity i.e. waist to hip ratio. The accurate assessment of visceral adiposity is becoming increasingly important clinically for proper assessment of patient metabolic disease risk (Shuster et al. 2012). Increased visceral fat is associated with increased risk of developing the metabolic syndrome (Nieves et al. '03). Some definitions of the metabolic syndrome do not distinguish between visceral fat and subcutaneous fat while some do, as the debate over the significance of such differences continues.

Visceral adiposity correlates with other metabolic derangements (Wisse '04) and recent imaging studies support a correlation between visceral abdominal adiposity and the metabolic syndrome versus abdominal subcutaneous adipose tissue (Demerath et al. '08).

As discussed earlier, adipose tissue plays a critical role in energy storage. However, the adipose tissue is no longer viewed as a simple storage depot but rather as an active endocrine organ (Fantuzzi 2005). The adipose tissue has the capacity to release a number of factors including adipokines and cytokines which can act in an autocrine, paracrine or endocrine fashion to modulate metabolic processes (Maury and Brichard 2010).

Adipose tissue secretes several immune-modulating factors such as cytokines; tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1β and hormones such as leptin.

The release of these molecules is triggered by macrophages which infiltrate the adipose tissue in obese individuals and together with adipocytes release these proteins.

As mentioned in section 1.3.2, leptin is a hormone which acts both centrally and peripherally to regulate energy balance by decreasing food intake and restricting triglyceride deposition (Gade et al. 2010). Leptin levels increase proportionally with increasing adiposity (Galic et al. 2010) and increasing leptin exerts a strong negative feedback on insulin sensitivity thereby enhancing insulin resistance.

TNF-α can directly evoke insulin resistance within adipose tissue as well as systemically via interruption of insulin signaling pathways (Bastard et al. 2006b). This involves the serine phosphorylation of both the insulin receptor and the insulin receptor substrate resulting in diminished activation of second messengers responsible for insulin's metabolic effects (Wisse 2004).

Adipose tissue also secretes adiponectin, an anti-inflammatory adipokine. Reduced circulating levels of adiponectin have been linked to development of the metabolic syndrome (Kadowaki et al. 2006) as well as sub-clinical coronary heart disease (Maahs et al. 2005).

Hence, these inflammatory-related molecules play a key role in the development of the metabolic syndrome and as such in the cardiovascular risk of the individual

1.5.2 Inflammation

Obesity is now recognised as a chronic low grade inflammatory state (Das 2001; Bastard et al. 2006a; Bastard et al. 2006b) The inflammation associated with this disease is tightly-linked to abnormalities that together constitute the metabolic syndrome. The link between inflammation and obesity was first made by Hotamisligil, who showed increased TNF-α release from white adipose tissue was associated with obesity, and that nullifying TNF-α improved insulin sensitivity (Hotamisligil et al. 1993).

The underlying cause of obesity-related inflammation is not altogether understood. Endoplasmic reticulum (ER) stress is one mechanism however that contributes to the induction of an inflammatory state observed in obesity (Zeyda and Stulnig 2009). ER stress in the liver and adipose tissue occurs in obesity due to excess lipid accumulation and disturbed energy metabolism brought about by over-nutrition (Ozcan et al. 2004). This activates a stress response known as the unfolded protein response which activates a number of inflammatory cascades via activation of Nf-KB and/or JNK pathways (Hummasti and Hotamisligil 2010).

The crucial step in obesity-induced inflammation is macrophage infiltration of adipose tissue (Weisberg et al. 2003; Xu et al. 2003). Though not fully elucidated, this appears to occur in response to a number of factors including monocyte chemotactic protein-1,IL-8 or leptin (Cancello and Clement 2006; Yu et al. 2006; Surmi and Hasty 2008).

These macrophages, in conjunction with adipocytes, produce and release a number of pro-inflammatory cytokines such as TNF-α and IL-6, promoting local and systemic inflammation.

As discussed in the previous section, the pro-inflammatory cytokines released by adipose tissue/macrophages can contribute to insulin resistance by impairing insulin sensitivity. In addition to the aforementioned effects of leptin and TNF-α, IL-1 can prevent insulin signalling via serine phosphorylation of IRS-1 following the activation of ERK (Jager et al. 2007). Also, IL-6 blocks IRS-mediated signalling in the liver via induction of SOCS-3 protein expression and in muscle via activation of protein kinase C-delta (Weigert et al. 2006).

Obesity leads to adipocyte hypertrophy and resulting cell death (Cinti et al. 2005; Murano et al. 2008). In obesity, adipose-tissue macrophages are found surrounding the necrotic cells in what are known as crown-like structures (Apovian et al. 2008). It is thought these dying cells, along with other factors such as the extracellular matrix protein osteopontin, attract the macrophages to adipose tissue adding to macrophage accumulation (Cinti et al. 2005; Nomiyama et al. 2007).

Thus, the inter-relationship between obesity, insulin resistance and inflammation is a complex and over-lapping one (Dandona et al. 2004). Inflammation is a key marker of metabolic disease and acts as a central player in a vicious spiral of metabolic derangements in association with obesity and/or insulin resistance which can ultimately lead to type 2 diabetes and/or cardiovascular disease (Xu et al. 2003; Dandona et al. 2004; Shoelson et al. 2006; Apovian et al. 2008).

1.5.3 Insulin resistance

The metabolic syndrome has also been known as the insulin syndrome, highlighting the pivotal role this hormone plays in energy regulation (Reaven 2005). Insulin is a hormone

secreted by the pancreas and plays a key role in glucose homeostasis as well as several other functions important for energy homeostasis.

Insulin acts primarily on muscle, liver and adipose tissue causing the uptake of glucose and reducing the plasma glucose concentration. Insulin resistance is the key factor in the pathogenesis of type II diabetes mellitus.

Obesity has long been recognised to be associated with increased risk of insulin resistance. The link between obesity and insulin resistance is not fully elucidated but seems to be mediated mainly through increased adiposity and the development of a pro-inflammatory state as outlined above (Everson et al. 1998; Dandona et al. 2004; Kahn et al. 2006).

1.5.4 Dyslipidemia

Abnormal lipid profile is a major factor in the metabolic syndrome and is both a cause and effect of insulin resistance. Moreover, obesity is highly co-morbid with dyslipidemia (Fabbrini et al. 2010). Dyslipidemia commonly presents as the atherogenic triad, namely, elevated plasma triglycerides, decreased high-density lipoprotein (HDL)-cholesterol and the presence of small dense low-density lipoproteins (LDLs) (Czyzewska et al. 2010). The ratio of HDL-cholesterol to LDL-cholesterol is a marker of a person's chance of developing atherosclerosis, with HDL the "good" cholesterol and LDL the "bad" cholesterol. This distinction is based on the fact that HDL delivers cholesterol to the liver to be metabolised or excreted while LDL delivers cholesterol to cells which can lead to atherosclerosis. Dyslipidemia resulting from the metabolic syndrome is therefore a major link between obesity, atherosclerosis and ultimately cardiovascular disease.

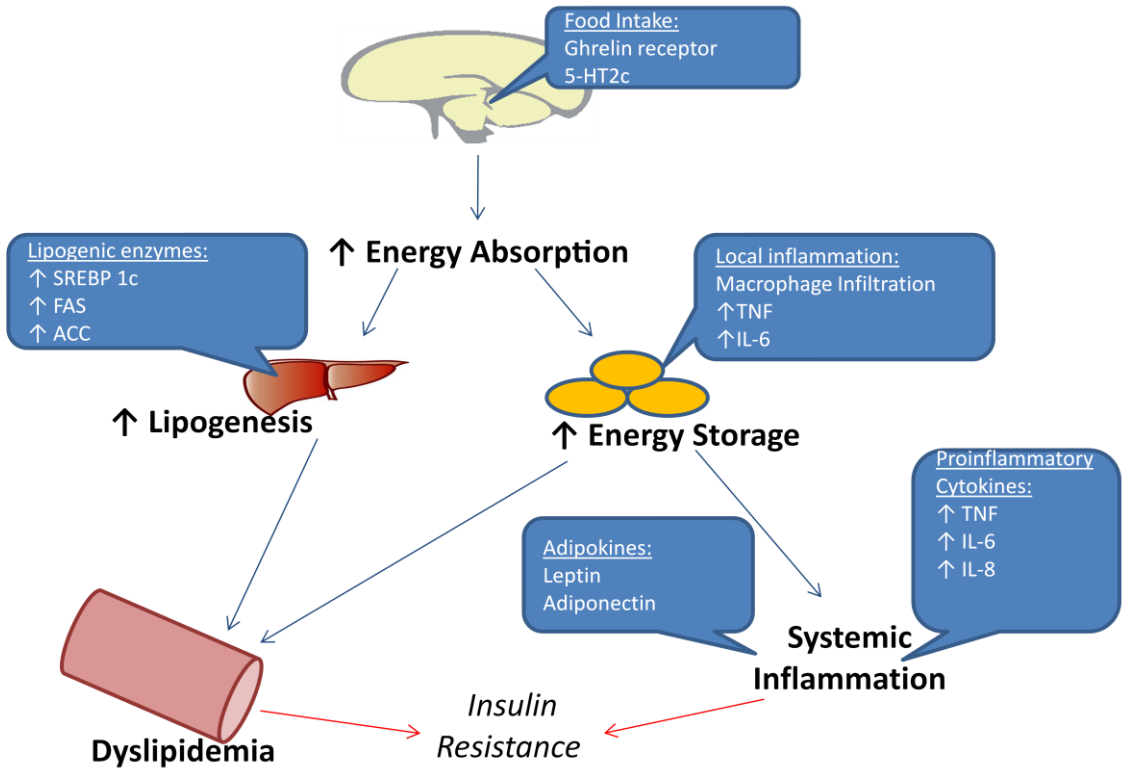


Fig 1.6 Overlapping factors that contribute to obesity and associated morbidities. *Numerous integrated physiological processes contribute to the development of obesity. Increased food intake leads to increased delivery of nutrients to the liver, subsequent increases in lipogenesis leads to increased energy storage and expansion of adipose tissue. Increased adipose mass leads to tissue inflammation and eventual release of proinflammatory factors as well as free-fatty acids leading to dyslipidemia and insulin resistance which further propagates metabolic dysfunction leading to a vicious cycle.*

1.6 Side effects of atypical antipsychotics

The emergence of atypical antipsychotic drugs and the subsequent reduction in the prevalence of EPS was a substantial step forward in improving patient outcomes and increasing adherence to treatment. However, these newer compounds brought with them their own constellation of side effects including weight gain and a number of associated metabolic abnormalities including; visceral fat deposition, glucose dysregulation, dyslipidemia and low grade inflammation.

These side effects are a considerable clinical problem as they too contribute to the huge problem of patient non-adherence to antipsychotic treatment (Baptista et al. 2004; Weiden et al. 2004). Thus, reduced compliance due to metabolic side effects can lead to patient relapse and poor patient outcomes (Nasrallah 2003).

As outlined earlier, obesity and metabolic disorders represent a major problem in modern healthcare due to their association with a number of serious co-morbidities including type II diabetes mellitus and cardiovascular disease. As such, the metabolic side effects of antipsychotics are of considerable concern as they too can lead to these detrimental diseases (Stahl et al. 2009; Starrenburg and Bogers 2009).

The primary metabolic side effect observed in patients is that of body weight gain. However, as one might expect given the aforementioned heterogeneity of this drug class, the various atypical antipsychotics show widely differing weight gain liabilities. Clozapine, the original atypical, is the worst offender in terms of inducing weight gain in patients, closely followed by the structurally similar olanzapine (Allison et al. 1999; Schwartz et al. 2004).

1.6.1 Antipsychotic-induced weight gain

There are currently several atypical antipsychotic drugs (AAPDs) in use or in development (Table 1.2) including: clozapine, olanzapine, quetiapine, risperidone, ziprasidone, ariprazole, paliperidone, lurasidone and amisulpride. In terms of weight gain liability, clozapine and olanzapine have long been recognised as having the highest incidence (Allison et al. 1999; Schwartz et al. 2004; Newcomer 2005).

Moreover, evidence suggests that olanzapine and clozapine are also the main culprits in causing metabolic dysregulation (Newcomer 2007).

Clozapine is the oldest of the atypical compounds and its clinical use is indicated only for treatment-resistant schizophrenia and requires weekly monitoring of patients. Olanzapine is also widely used with reported sales of $8b in 2008, making its propensity to induce metabolic changes of particular concern.

Quetiapine and risperidone have also been associated with weight gain and metabolic disturbances, albeit to a lesser degree (Lee et al. 2004; Brecher et al. 2007). In particular, a retrospective study found low dose quetiapine was associated with significant weight gain in patients (Williams et al. 2010).

Paliperidone is the active metabolite of risperidone and though information is limited, indications are it has a similar efficacy and side effect profile to its parent drug (Marino and Caballero 2008) with reported incidences of weight gain in patients of 6-8% (Harrington and English 2010; Gopal et al. 2011).

Ziprasidone and aripiprazole seem to be weight neutral and show little or no adverse metabolic effects, and have even been shown to attenuate some of olanzapine's negative effects (Snigdha et al. 2008). Amisulpride is an atypical drug used in Europe but is not licensed by the Food and Drug Administration (FDA) for use in the U.S.; it is associated with a low risk of both weight gain and metabolic symptoms in comparison to other atypical antipsychotics.

1.6.2 Antipsychotic-induced metabolic dysfunction

AAPD treatment has been associated with all aspects of the metabolic syndrome (Tables 1.4 and 1.5). The aetiology of these adverse effects however is not yet known. It is clear however that a number of central and peripheral mechanisms overlap to induce weight gain and increased fat mass which ultimately leads to insulin resistance and dyslipidemia culminating in the development of co-morbidities such as cardiovascular disease and/or diabetes mellitus.

1.6.2.1 Antipsychotic-induced visceral adiposity

Weight gain associated with AAPD use consistently involves increased visceral adiposity (Cheng et al. 2005; Coccurello et al. 2006; Raskind et al. 2007; Ferno et al. 2009). Mechanisms of AAPD induced visceral fat accumulation include not only hyperphagia-driven weight gain but also direct disruption of lipid handling.

A number of preclinical studies have demonstrated increased adipose mass independent of weight gain (Victoriano et al. 2009; Albaugh et al. 2010). Alterations in adipocyte size as well as number have been observed demonstrating that AAPDs alter adipose physiology in addition to merely increasing weight. Mechanisms underlying these effects may involve genetic pathways as evidence suggests that antipsychotics, especially olanzapine and clozapine, can directly impact on key genes involved in lipid storage including FAS and SREBP (Ferno et al. 2006; Ferno et al. 2009; Ferno et al. 2011).

1.6.2.2 Antipsychotic-induced insulin resistance

Decreased insulin sensitivity and disruption to glucose handling is commonly found in patients receiving AAPDs, particularly olanzapine and clozapine, (Dolder and Jeste 2003; Haupt et al. 2007; Newcomer et al. 2009; Smith et al. 2009). Importantly, AAPD treatment can cause the development of new onset diabetes (Farwell et al. 2004; Holt and Peveler 2006; Lambert et al. 2006) and in rare cases, even ketoacidosis (excess metabolism of fatty acids in the liver leading to excess ketone bodies) (Cohen and Correll 2009). Therefore, as an antipsychotic side effect, insulin resistance is a prime concern and accordingly all AAPDs now carry a black label warning for diabetes mellitus, indicating the scale of this problem.

Intriguingly, there is evidence that the progression to diabetes in cases of AAPD-induced diabetes is much quicker than is typically seen in non-psychiatric obese patients. This suggests mechanisms beyond weight gain are involved in AAPD–induced diabetes (Scheen and De Hert 2007). One hypothesis is that insulin resistance caused by AAPD-induced weight gain is compounded by inadequate compensation by β-cells due to direct AAPD effects, resulting in quicker progression to diabetes in some patients (Bergman and Ader 2000; Scheen and De Hert 2007). These direct effects of AAPD on insulin secretion are thought to involve antagonism of muscarinic (M) receptors with the M_2 (Johnson et al. 2005) and M_3 receptors (Stahl et al. 2009) implicated.

Furthermore, AAPD-induced insulin resistance has been found in patients independent of effects on weight, and the degree of insulin resistance was found to be above and beyond that normally found in association with obesity alone (Kim et al. 2010).

This supports the idea that AAPDs have specific actions resulting in insulin resistance as opposed to simply being a consequence of obesity. This is somewhat corroborated by findings of the FDA that 25% of new-onset Type II Diabetes were not associated with substantial weight gain or obesity (Newcomer 2004).

AAPDs may also directly induce insulin resistance via a number of potential mechanisms including a direct effect on β-cell function (which produce and secrete insulin) thereby impairing insulin secretion (Bergman and Ader 2005; Johnson et al. 2005). Preclinical models have shown that a single injection of olanzapine or clozapine can alter hepatic metabolism, supporting this hypothesis (Houseknecht et al. 2007; Chintoh et al. 2008a). As discussed above, evidence also suggests that AAPDs can cause increases in visceral fat mass without causing overt weight gain. Thus, AAPD induced dysfunction in adipose tissue physiology could indirectly lead to the development of insulin resistance via the release of pro-inflammaotry mediators (see section 1.5) independent of effects on body weight.

1.6.2.3 Antipsychotic-induced inflammation

Antipsychotic therapy has been associated with both local and systemic inflammation. Increased levels of circulating cytokines, including TNF, soluble TNF and IL-2 receptors have been observed in response to antipsychotics, in particular clozapine in (Schuld et al. 2000; Kluge et al. 2009). These increases in cytokines are likely secondary to other metabolic effects of these drugs, however they are still an important consequence and are important consideration when assessing the side effects of atypical antipsychotics (Baptista and Beaulieu 2002).

Moreover, the development of a pro-inflammatory state in adipose tissue was found following antipsychotic administration in rats (Victoriano et al. 2010).

1.6.2.4 Antipsychotic-induced dyslipidemia

Antipsychotic treatment, both clinical and pre-clinical, has been shown to result in at least some of form of lipid abnormality (Graham et al. 2005; Coccurello et al. 2008; Perez-Iglesias et al. 2009). Hence, dyslipidemia may in many cases be caused by antipsychotic treatment as a result of weight gain and spiraling metabolic dysfunction (Meyer and Koro 2004). However, a direct influence cannot be ruled out due to evidence of acute effects of some AAPDs on cholesterol and triglycerol levels in animal models, for example, clozapine increased circulating levels of triglycerides, phospholipids and cholesterol in rats (Ferno et al. 2009), though the mechanism underlying such a direct effect is not yet understood.

Furthermore, some clinical studies have not found a correlation between weight gain and observed dyslipidemia suggesting an alternative mechanism for AAPD induced dyslipidemia (Meyer and Koro '04).

DRUG	N	PERIOD	STUDY PARAMETERS	WEIGHT	MAIN FINDINGS	REF
Olanzapine	133		Patients with early psychosis	80% > 7% increase	22/133 patients developed MetS	(Patel et al. 2009)
Quetiapine	134	52w		50% > 7% increase	18/134 patients developed MetS	
Risperidone	133			57.6%> 7% increase	11/133 patients developed MetS	
Olanzapine	3				Average gain of 3 BMI points	(Oriot et al. 2008)
Quetiapine	12		Drug-naïve; lean		Insulin Resistance indicated by HOMA scores	
Risperidone	10	36w	patients with no MetS	↑body weight (all groups)	(for all groups)	
Ariprazole	11					
Olanzapine	573		open study	6.26 kg mean gain	statistically significant but sub-clinically relevant	(Kinon et al. 2001)
Haloperidol	103	58w		0.69 kg mean gain	increase in cholesterol.(olanzapine)	
Olanzapine	10	104w	open study	↑body weight	↑body fat ↑fasting glucose ↑fasting insulin ↑insulin resistance (HOMA)	(Ebenbichler et al. 2003)
Olanzapine	10	8w	consecutive inpatients	3.3 kg mean gain	↑body fat 9/10 ↑Leptin	(Eder et al. 2001)
Olanzapine	30	8w	First-episode schizophrenia patients	6 kg mean gain	60% > 7% increase in body weight	(Poyurovsky et al. 2002)

DRUG	N	PERIOD	STUDY PARAMETERS	WEIGHT	MAIN FINDINGS	REF
Olanzapine	438	52w	Retrospective (1st yr of treatment)	40% > 7% increase	8% developed new onset diabetes	(Farwell et al. 2004)
Risperidone	482		Retrospective (1st yr of treatment)	37% > 7% increase	3.5% developed new onset diabetes	
Olanzapine	54				↑Triglycerides ↑Cholesterol	(Perez-Iglesias et al. 2009)
Risperidone	58	52w	Drug-naive patients	10.5 kg mean gain	↑Insulin	
Haloperidol	52					
Olanzapine	43		Drug naive, first	7.5 kg mean gain	↑Cholesterol ↑Triglycerides	(Perez-Iglesias et al. 2007)
Risperidone	44	12w	episode patients	5.6 kg mean gain	↑Cholesterol	
Haloperidol	41			3.8 kg mean gain	↑Cholesterol	
Olanzapine	54		Drug naive, first	7.16 kg mean gain	weight gain rapid onset and the plateaued	(Perez-Iglesias et al. 2008)
Risperidone	58	52w	episode patients	5.85 kg mean gain	steady weight gain	
Haloperidol	52			4.28 kg mean gain	very gradual weight gain	

DRUG	N	PERIOD	STUDY PARAMETERS	WEIGHT	MAIN FINDINGS	REF
Olanzapine	228			4.6 kg mean gain	45.7% >7% increase	(Bobes et al. 2003b)
Risperidone	232	App. 52w	Retrospective	3.1 kg mean gain	30.6%>7% increase	
Haloperidol	130		outpatient study	2.9 kg mean gain	22.4%>7% increase	(Bobes et al. 2003b)
Quetiapine	43			no significant gain	0%>7% increase	
Olanzapine	30	12w	newly diagnosed	↑body weight and BMI	↑Blood sugar	(Ingole et al. 2009)
Risperidone	30		Schizophrenia pts.	↑body weight and BMI		
Olanzapine	7	1w	Healthy, normal weight men		blunted insulin effects on glucose disposal; FFA reduced	(Vidarsdottir et al. 2010)
Olanzapine	146		open-label	4.6 kg mean gain	↑glucose↑insulin ↓insulin sensitivity ↑T.G	(Newcomer et al. 2009)
Risperidone	134	24w	randomized trial	3.6 kg mean gain	↑glucose ↓insulin sensitivity	
Quetiapine	115			3.7 kg mean gain		
Olanzapine	24		non-diabetic		Waist circumference and BMI predicted presence of insulin resistance	(Haupt et al. 2007)
Risperidone	16	> 12w	patients	N/A		
Ziprasidone	11					
Olanzapine	23	21w	treatment comparison	↑BMI all groups	↑prolactin ↑insulin resistance↓insulin sensitivity	(Smith et al. 2009)
Risperidone	23				↓prolactin	

DRUG	N	PERIOD	STUDY PARAMETERS	WEIGHT	MAIN FINDINGS	REF
Olanzapine	12	taking medication	non-obese patients	N/A	↑insulin resistance ↓glucose effectiveness	(Henderson et al. 2005)
Clozapine	12				↑insulin resistance ↓glucose effectiveness	
Risperidone	12				no significant changes	
Olanzapine	8	104w	non-obese patients		↑insulin resistance↓glucose effectiveness ↑basal glucose	(Henderson et al. 2006)
Quetiapine	7				no change vs. Control	
Olanzapine	31	24w	Randomized	↑ weight	↑ adiposity @6w & 24w ↑visceral adiposity	(Ader et al. 2008)
Risperidone	28		prospective study	↑ weight	↑adiposity @ 24w ↑visceral adiposity	
Olanzapine	9	12w	Drug naive or<7w treatment	4.7 kg mean gain (↑ 7.3%)	↑waist:hip ratio ↑%body fat ↑fasting insulin ↑C-peptide ↑fasting triglyverides. No changes found in leptin/prolactin or HDL	(Graham et al. 2005)
Olanzapine	7	3d	Healthy men & women		↑fasting plasma ↑triglycerides	(Albaugh et al. 2011b)
Quetapine	17	8w	Drug naïve patients	11.8% > 7% increase	↑insulin resistance ands secretion	(Chen et al. 2011)
Risperidone	30	28d	Healthy men	50% > 7% increase Avg. 4.23 kg	Weight gain attenuated by a glucocorticoid receptor antagonist	(Gross et al. 2010)

DRUG	N	PERIOD	STUDY PARAMETERS	WEIGHT	MAIN FINDINGS	REF
Olanzapine	22	28d	Healthy men	Avg ↑ 3.2 kg	↑ waist circumference	(Gross et al. 2009)
Olanzapine	59	12w	Drug-free pts	Significant ↑	↓ adiponectin ↑prevalence of MetS	(Wampers et al. 2012)
Risperidone	54	12w		Significant ↑	↑adiponectin	
Olanzapine	30	16w	Newly diagnosed patients	Significant ↑ BMI	↑fasting glucose ↑cholesterol ↑triglycerides	(Fernandez-Egea et al. 2011)

Table 1.4 Clinical studies investigating the metabolic effects of antipsychotics. *↑; significant increase unless otherwise stated, MetS; metabolic syndrome, BMI; body mass index, HOMA; homeostasis mode assessment, FFA; free fatty acids, HHL; high density lipoprotein*

DRUG	DOSE	SPECIES/ STRAIN	Sex	N	Duration	Weight	Adiposity	Lipid Profile	Insulin	Glucose	OTHER	REF
Olanzapine	3mg/kg	Rat/ Sprague-Dawley	M	8	Acute	-	-	-	↓Production (To glucose Challenge)	↑H.G.P ↓Utilisation ↓G.I.R	↓C-Pepitide Levels	(Chintoh et al. 2009b)
Clozapine	3mg/kg	Rat/ Sprague-Dawley	M	8	Acute	-	-	-	↓Production (To glucose Challenge)	↑H.G.P ↓Utilisation ↓G.I.R	↓C-Pepitide Levels	
Clozapine	25/50 mg/kg	Rat/ Sprague Dawley	F/ M	6	Acute	-	-	↑TG ↑Phospho lipids			↑Cholesterol	(Ferno et al. 2009)
Olanzapine	2/7.5 mg/kg	Rat/ Sprague Dawley	F		Chronic 28d	non-significant ↑	↑Visc Fat	-	-	↑H.G.P ↓Utilisation	↓Locomotor Activity	(Chintoh et al. 2008b)
				20								
Olanzapine	1.75 mg/kg	Rat/Sprague-Dawley	F	10	Chronic 28d	Avg ↑ 3kg day 4 on	↑Visc Fat	-	-	Hyperglycemia	↑Cholesterol ↑Food Intake	(Lykkegaard et al. 2008)
Olanzapine	4-12 mg/kg	Rat/ Sprague-Dawley	M	10	Chronic 35d	no change	↑ % fat from week 1		↓ Insulin Sensitivity	↑Plasma Levels	↓ Leptin (acutely)	(Albaugh et al. 2010)
Olanzapine	2 mg/kg	Rat/Hooded Lister	F	10	Chronic 28d	Initial ↑ overall ↓	no change	-	-	-		(Fell et al. 2008)
Clozapine	4 mg/ kg	Rat/ Wistar	F	16	Chronic 21d	no change	↑Visc Fat	↑Adipone-ctin	no change	no change		(Cooper et al. 2008a)

DRUG	DOSE	SPECIES/ STRAIN	Sex	N	Duration	Weight	Adiposity	Lipid Profile	Insulin	Glucose	OTHER	REF
Clozapine	7.5 mg/ kg	Rat/ Sprague-Dawley	F	8	Sub-Chronic 14d	non-significant ↑		↑Triglycerides	↑Serum Levels	↑Serum Levels	↑Leptin Levels	(Tulipano et al. 2007)
Olanzapine	1/2 mg/kg	Rat/Hans-Wistar	M	14	Chronic 20d	↓overall	↑VisC Fat	↑Adiponectin	-	-	↑Prolactin ↓Testosterone	(Cooper et al. 2007)
Olanzapine	1/2 mg/kg	Rat/Hans-Wistar	F	6	Chronic 20d	↑	↑renal fat		↑Plasma Levels	no change found	↑Prolactin	(Cooper et al. 2005)
Olanzapine	3.2 mg/kg	Rat/Han-Wistar	F	6	Acute	-	-		Resistance Indicated	↑H.G.P ↓G.I.R		(Housekn echt et al. 2007)
Risperidone	0.4 mg/kg	Rat/Sprague-Dawley	M	12	Chronic 28d	no change	no change	-	non-significant ↑	no change	↑Glucagon	(Lin et al. 2006)
Olanzapine	1 mg/kg	Rat/Sprague-Dawley	M	10	Chronic 42d	↑	-	↑TG ↑HDL	no change	no change	↑Leptin Levels	(Minet-Ringuet et al. 2006)
Olanzapine	4-20 mg/kg	Rat/Sprague-Dawley	F/ M	10	Chronic 33d	↑(F Only)	↑ Parametrial Fat	-	↑Plasma Levels ↓Insulin Sensitvity	↑Plasma Levels		(Albaugh et al. 2006)
Olanzapine	2 mg/kg	Rat/Sprague-Dawley	M	6	Chronic 46d	non-sig. ↑	no change	-	no change	↑Fasting Levels	↑Meal Frequency	(Victoriano et al. 2009)

DRUG	DOSE	SPECIES/ STRAIN	Sex	N	Duration	Weight	Adiposity	Lipid Profile	Insulin	Glucose	OTHER	REF
Olanzapine	2 mg/kg	Rat/Sprague-Dawley	F	11	Chronic 56d	↑10% vs controls	↑Visceral Fat					(Raskind et al. 2007)
Olanzapine	2 mg/kg	Rat/Hooded-Lister	F	10	Chronic 21d	↑ from day 1	non-sig. ↑		no change	-		(Fell et al. 2007)
Olanzapine	1 mg/kg	Rat/Sprague-Dawley	F	8	Sub-Chronic	↑	-		-	-		(Davoodi et al. 2009)
Olanzapine	2 mg/kg	Rat/Sprague-Dawley	M	6	Chronic 42d	↑	↑Subcutaneous fat		-	-		(Minet-Ringuet et al. 2006)
Olanzapine	6/12 mg/kg	Mice/c57BL/6	F	12	Chronic 28d	↑	↑					(Cope et al. 2005)
Olanzapine	3 mg/kg	Mice CD-1	F	10	Chronic 36d	↑ d32 on	↑TG		↑Serum Levels	↑Serum Levels		(Coccurello et al. 2006)
Olanzapine	3 mg/kg	Mice/CD-1	F	10	Chronic 50d	↑ from d10 on	↑ uterine fat	↑Triglycerides ↑NEFA	Hyper-insulinemia	↑Serum Levels	↑Cholesterol nocturnal hypomotility	(Coccurello et al. 2008)
Olanzapine	2 mg/kg	Rat/Hanover-Wistar	F	8	Sub-chronic	↑d4 on	↑				↑energy expenditure	(Liebig et al. 2010)
Olanzapine	0.25-2 mg/kg	Rat/Sprague-Dawley	F	12	Sub-chronic 14d	↑d4 on	↑ all deposits		↓fasting plasma levels		↑ghrelin ↑CCK	(Weston-Green et al. 2011a)

Table 1.5 Preclinical studies of antipsychotics' metabolic effects. *↑; significant increase unless otherwise stated, HGP; hepatic glucose production, GIR; glucose infusion rate, SREBP; sterol regulatory-binding protein, CCK; cholecystokinin; TG, triglycerides*

1.7 Pharmacology of atypical antipsychotics: metabolic side effects

The major receptors that are targeted by AAPD are not only dopamine but also 5-HT, histamine, adrenergic and muscarinic receptor subtypes (Table 1.5). Centrally, antagonism of these receptors induces alterations in eating behavior, leading to increased body weight as discussed above.

DRUG	Association with weight gain/MetS	Receptor Affinity							
		D_2	5-HT_{1A}	5-HT_{2B}	5-HT_{2C}	H_1	M_3	α_1	α_2
Olanzapine	High	M	N	H	M	H	M	M	L
Clozapine	High	L	N	H	M	H	M	H	L
Risperidone	Moderate	H	N	H	M	N	N	H	M
Quetiapine	Moderate	L	N	H	M	M	L	H	N
Ziprasidone	Low	H	H	H	H	N	N	M	N
Aripiprazole	Low	H	M	H	L	L	L	M	L

Table 1.6 Major receptor affinities of certain atypical antisychotics *N=none, L=low, M=moderate,H=high (adapted from Miyamoto et al 2005 and Horacek et al,2006)*

1.7.1 Dopamine receptors

As already discussed in section 1.3.1, monoamines, including dopamine, play an important role in central energy regulation. However, as outlined, typical antipsychotics are noted for their higher potency at dopamine receptors, and yet it is atypical drugs which are associated with metabolic effects to a much greater degree. Hence, this undermines any dopamine-led hypothesis of AAPD induced weight gain. However, that is

not to say that dopamine circuitry is not still an import factor in AAPD-induced metabolic side-effects.

Evidence suggests that atypical compounds such as olanzapine may have discrete effects on the dopamine pathways. This selectivity could perceivably play a role in the side effect profile of these drugs and offer a potential mechanism of antipsychotic-induced increases in feeding and weight gain. This is due to the fact that dopamine transmission in the mesocorticolimbic region is crucial for motivation or "wanting" rewards (Wise 2004; Toh and Williams 2011). Thus, it has been postulated that increased burst firing of dopaminergic neurons in the nucleus accumbens and prefrontal cortex may promote feeding, and that low dopamine levels in the brain may therefore promote excessive behaviours such as eating to restore dopamine levels (Palmiter 2007). This hypothesis is supported by a study showing that obesity-prone rats have reduced extracellular midbrain dopamine levels as well as reduced dopamine release following electrical stimulation in the nucleus accumbens, prefrontal cortex and dorsal striatum (Geiger et al. 2008).

Atypical antipsychotics such as olanzapine and clozapine could therefore, by normalising dopamine transmission in mesocorticolimbic areas, invoke increased motivation towards eating, ultimately leading to weight gain. In line with this theory, a recent study investigating the eating behaviours of 153 schizophrenic patients found that those taking atypical antipsychotics were more responsive to external eating cues than those treated with typical antipsychotics (Sentissi et al. 2009). This is intriguing as reaction to contextual

cues in animal feeding paradigms has been particularly associated with the mesolimbic dopamine pathway (Narayanan et al. 2010).

It has also been suggested recently that chronic blockade of D_2 receptor in β cells of the pancreas may contribute to insufficient compensation by the pancreas in the setting of hyperglycemia (Hahn et al. 2012)

1.7.2 Serotonin Receptors

Several AAPDs, including clozapine and olanzapine, are high affinity antagonists for numerous 5-HTreceptors (Table 1.5). Thus, antagonism of 5-HT neurotransmission by these drugs could conceivably disrupt normal regulation of appetite, food preference and energy balance. However, the association between 5-HT_{2c} and AAPD induced weight gain is not cut and dry and is challenged by the fact that ziprasidone, also an AAPD with high affinity for 5-HT_{2c} receptors, is not associated with increased weight gain (Citrome 2009). It has been demonstrated however, that ziprasidone also acts at the 5-HT_{1A} receptor as a partial agonist (Ohlsen and Pilowsky 2005) which may confer protection against weight gain (Kirk et al. 2004).

A number of AAPDs have a higher affinity for 5-HT_{2A} than for D_2 receptors (which Meltzer and colleagues have proposed as a criteria for atypicality) (Meltzer et al. 1989). This may indirectly play a role in increased feeding and weight gain. This is due to the fact that 5-HT_{2A} antagonism in the prefrontal cortex results in increased dopamine output (Horacek et al. 2006) and may also lead to increased motivation and reward associated with eating, as mentioned above.

Pharmacogenetic evidence for the role of 5-HTreceptors in AAPD-induced weight gain/metabolic disturbance is beginning to emerge (Lencz and Malhotra 2009).

Three single nucleotide polymorphisms (SNP) in the promoter region of the 5-HT_{2c} receptor gene have been identified (Yuan et al. 2000) with one of the polymorphisms being associated with AAPD induced weight gain in drug-naive patients (Reynolds et al. 2002). However, the validity of these findings has been challenged by some on the grounds of lack of subject variance and lack of complete explanation for weight gain (Martyn 2005). A further polymorphism in the 5-HT_{2C} receptor gene has been identified, as patients with the 5-HTR_{2c}-759 C-allele showed a significantly higher risk of weight gain (Laika et al. 2010).

Further genetic components for AAPD-induced weight gain have been reported including variants of alleles for the 5-HT_{2A} receptor as well as for the adrenergic β-3 receptor and these genetic variants seem to have an additive effect (Basson et al. 2001a)**.**

Hence, serotonin antagonism, independent of and in conjunction with dopamine antagonism, may cause increased feeding/altered feeding patterns in patients receiving atypical antipsychotics, contributing to increased body weight.

1.7.3 Histamine Receptors

Some AAPDs, especially clozapine and olanzapine, exhibit strong antagonistic effects at the H_1 receptor supporting the view of this receptor as a key player in AAPD metabolic side effects. Indeed, two meta-analyses have implicated the histamine H_1 receptor as the primary receptor in AAPD-induced obesity (Kroeze et al. 2003; Matsui-Sakata et al. 2005).

As well as effects on appetite antagonism of H_1 receptors by AAPDs may also prevent histamine driven lipolysis, leading to a disproportionate accumulation of triglycerides and increased adipose mass as seen in AAPD treated patients (Tsuda et al. 2002; Masaki et al. 2004; Deng et al. 2010).

Further evidence for a histamine-driven mechanism in AAPD adverse side effects is the reduction in hypothalamic histamine H_1 receptor mRNA expression seen in olanzapine treated rats (Han et al. 2008).

There is also an implication that the histamine H_3 receptor contributes to AAPD side effects as antagonism of this pre-synaptic, inhibitory auto-receptor could slow the production and secretion of histamine, therefore compounding the enhancement on feeding behavior and energy storage of H_1 antagonism (Deng et al. 2010).

1.7.4 Muscarinic Receptors

Muscarinic acetylcholine receptors comprise five subtypes, M_1-M_5 with M_1, M_3 and M_5 being $G_{q/11}$ protein coupled receptors. M_2 and M_4 are $G_{i/o}$ protein coupled receptors which when activated inhibit adenylatecyclase decreasing cAMP. In the CNS, the M_1 is the most abundant receptor expressed primarily in the forebrain where it plays an important role in cognition (Bymaster and Felder 2002).

Hence, central muscarinic effects of AAPDs seems to related more to their effects on cognitive symptoms of schizophrenia rather than weight gain and this is supported by the work of Matsui-Sakata and colleagues who found that muscarinic receptor affinity was

correlated with H_1 receptor binding such that H_1 was the key receptor involved in weight gain in this study (Matsui-Sakata et al. 2005).

However, in the periphery, M_3 receptors are involved in the regulation of insulin release from the pancreas and glucose metabolism (Gautam et al. 2006) making them a potential target for direct metabolic effects of certain AAPDs.

Indeed, it has been suggested that M_3 affinity may be the best predictor of antipsychotic-induced diabetes (Silvestre and Prous 2005). This is supported by the fact olanzapine and clozapine have the highest affinities for the M_3 receptor (Roth et al. 2004).

As already mentioned, the M_2 and M_3 receptors have been implicated in the direct metabolic effects of AAPDs. Antagonism of these sites on pancreatic β-cells may diminish insulin secretion contributing to disrupted glucose homeostasis ultimately leading to diabetes mellitus (Johnson et al. 2005; Stahl et al. 2009).

1.7.5 Adrenergic Receptors

A recent study showed that the α2 receptor antagonist, yohimbine, prevented clozapine-induced hyperglycemia (Savoy et al. 2010). This suggests that AAPD adrenocepter antagonism could potentiate activation of the sympathetic nervous system disrupting glucose handling in the periphery. However, the contribution of α1/α2 receptors to AAPD metabolic effects is likely to be a minor one as evidence of a prominent role for adrenoceptors in AAPD-induced weight gain or metabolic disturbances is lacking. The effect of AAPD on adrenoceptors is more strongly implicated in sexual dysfunction side effects of antipsychotics such as priapism (Andersohn et al. 2010).

The disruption of these systems may also contribute to metabolic disturbances independent of weight changes, in particular altered glucose homeostasis by impacting on sympathetic outflow.

This is due to the fact that sphlanic sympathetic nerves directly innervate the liver and adipose tissue where they impact on glucose and free-fatty acid metabolism by increasing gluconeogenesis and increasing lipolysis respectively (Nonogaki 2000).

In line with this, an acute intravenous administration of olanzapine to rats induced hepatic insulin resistance and increased glucose production suggesting a direct central mechanism of AAPD induced effects on glucose regulation (Martins et al. 2010).

1.8 Predisposition to antipsychotic side effects

A number of potential factors have been proposed as predisposing a patient to adverse side affects of antipsychotics including sex, baseline weight and therapeutic response.

That weight gain is a predictor of drug efficacy (or vice-versa), has been suggested by a number of studies with leptin implicated as a mechanistic link (Meltzer et al. 2003b; Venkatasubramanian et al. 2010). However, such a correlation has not been observed in a number of studies (Citrome et al. 2009b; Hermes et al. 2011). Thus, it remains to be determined but it seems a correlation between weight gain and therapeutic outcome is at best weak.

Another factor commonly suggested as predictive of weight gain is a low baseline weight or BMI. This correlation has been found in some settings (Basson et al. 2001a; Gebhardt

et al. 2009; Verma et al. 2009). A number of studies, however, have not found any such connection and the validity of such correlations has subsequently been challenged (Allison et al. 2009).

Another reported difference in the epidemiology of antipsychotic side effects is sex. As with other variables, no clear cut distinction exists here. A review by Aichhorn et al, found the incidence of weight gain and the metabolic syndrome to be more frequent in females and that this correlated to higher drug plasma levels in women (Aichhorn et al. 2007). This strongly indicates a sex difference, although another study of olanzapine found that males had a higher frequency of weight gain (Basson et al. 2001a). While a number of clinical studies have found sex differences in the propensity to incur weight gain when taking antipsychotics, these have so far not been conclusive (Haack et al. 2009).

1.9. Schizophrenia and obesity

It is important to note that schizophrenia has been associated with obesity related abnormalities since before even the first antipsychotic was used.

Patients with schizophrenia have a higher prevalence of obesity and diabetes than the general public (Brown 1997; Bobes 2007). The reasons for this are multifactorial but stem mainly from socioeconomic reasons. Patients with schizophrenia tend to live a sedentary lifestyle, have poor diets and are more likely to smoke (Pack 2009).

Furthermore, schizophrenia itself is associated with a proinflammatory phenotype as well dysfunction in stress responses leading to high levels of cortisol (Thakore et al. 2002; Fan

et al. 2007; Richard and Brahm 2012). Hence, it is likely disease pathogenesis also contributes to poor metabolic health of patients with schizophrenia (Leonard et al. 2012).

It is therefore important that epidemiological data assessing the metabolic side effects of AAPDs is carefully scrutinized in order to ensure separation of drug from non-drug related events.

1.10 Animal models of metabolic side effects of antipsychotics

In order to investigate the mechanisms behind antipsychotic induced weight gain, rodents have typically been used to reproduce the clinically observed side effects. However, a number of confounding factors have been realised that means the task of developing reliable and valid animal models for antipsychotic induced weight gain has not been easy in this area of research.

Firstly, mice appear to be largely resistant to the metabolic effects of these drugs and a number of studies have failed in producing significant weight gain in treated mice following a number of different protocols (Arjona et al. 2004). However, some groups have reported weight gain in mice and other aspects of the metabolic syndrome following antipsychotic treatment (Cheng et al. 2005; Coccurello et al. 2008). However these required extended periods of treatment and in the case of clozapine actually caused weight loss, neither of which are the case in clinical practice.

Rats have proven much more reliable than mice for incurring weight gain in response to antipsychotic treatment. However, this is the case only for female rats as demonstrated in several reports (Kinon et al. 2001; Arjona et al. 2004; Fell et al. 2007; Cooper et al. 2008a;

Fell et al. 2008; Kirk et al. 2009) (see table 1.4). The reason for this discrepancy remains unknown, however the steep growth curve seen in male rats may be one reason why the effects of these drugs are not apparent in male rats.

In humans, a sex-dependent effect of these drugs in causing weight gain is debatable, with some reports suggesting it is more likely to occur in females (Aichhorn et al. 2007) and other suggesting it is more likely in males (Kinon et al. 2001).

However, data on patients is typically hard to interpret due to differences in age, lifestyle and treatment, and a recent meta-analysis reported that there is no conclusive correlation between sex and antipsychotic side effects (Haack et al. 2009).

Animal models of any drug effect present challenges as is inevitable when dealing with a different species. Antipsychotics are no different, and overcoming these challenges is key to developing valid animal paradigms of antipsychotic side effects.

Overall, though not ideal, rodent models of antipsychotic-induced weight gain and metabolic syndrome do reflect the situation observed in human patients (Weston-Green et al. 2011a; Weston-Green et al. 2011b). Therefore, these models are useful in the continued effort to understand the cause of these side effects and to investigate ways to prevent them (Boyda et al. 2010; Weston-Green et al. 2011b). Going forward, efforts should be made to produce models that reflect as close as possible the clinical situation while still producing the relevant side effects.

1.11 Gut microbiota

The gut microbiota comprises the approximate 100 trillion bacteria that reside within the gastrointestinal tract (Lupp and Finlay 2005). Thus the human gut likely represents the most densely populated ecosystem on earth (Molloy 2006). These microorganisms consist mainly of bacteria, though archaea, fungi and viruses are also present (Eckburg et al. 2005). The microbiota has co-evolved with their hosts over the millennia to occupy this environmental niche in a mutually beneficial manner (Ley et al. 2008a).

The role of bacteria in human and health and disease has been noted for many decades (Gibbons and Houte 1975).

However, more recently, there has been a bloom in interest in the microbiota as evidence for a role of the microbiota in numerous physiological and pathophysiological processes mounts. This interest culminated in the establishment of the human microbiome project (HMP), a 5-year project which began its work in 2007 (Peterson et al. 2009).

The HMP is an international initiative of the NIH common fund, set-up to characterise the microbiota of individuals across a range of anatomical sites and to determine if specific changes in the microbiota are associated with disease states (Peterson et al. 2009). To date, the HMP has resulted in several new and enlightening findings linking the microbiota to diseases such as colon cancer, cystic fibrosis and Crohn's disease (Hu et al. 2011; Filkins et al. 2012; Zhang et al. 2012). Furthermore, the HMP has generated a freely available reference database of over 1600 bacterial genomes, a number set to reach 3000 before completion of the HMP.

1.11.1 Establishment of the gut microbiota

The foetus is sterile until birth when colonisation begins. As the progeny moves through the birthing canal, initial colonisation occurs by bacteria originating primarily from the mother's vaginal and then faecal microbiota (Dominguez-Bello et al. 2010). The microbiota continues to establish itself during early post-natal years and by approximately two years of age a relatively stable microbiota resembling that of an adult is established (Palmer et al. 2007). However, differences between infants, adolescents and adults can still observed (Hopkins et al. 2001; Agans et al. 2011).

In addition to age, several other factors apply selective pressures on the development of the gut microbiota (Rawls et al. 2006). These include a number of host factors such as the presence of specific glycolipids on the epithelium or glycans in the mucus which allow specific bacteria to adhere (Backhed 2011).

Similarly, diet has a considerable impact on shaping the gut flora (Tannock and Savage 1974; Hildebrandt et al. 2009). Host immunity also plays a role in determining ones microbiota by killing specific bacterial groups as evidenced by the altered microbial profile observed in mice lacking toll-like receptors (TLRs) or nucleotide–binding oliogomerisation domains (NODs), both of which acts as microbial signatures (Petnicki-Ocwieja et al. 2009; Vijay-Kumar et al. 2010).

1.11.2 Microbiota taxonomy and composition

In taxonomy, bacteria represent one of the three domains of life (along with Eukaryotes and Archea). Bacteria subsequently follow the same ranks as these other domains (though the rank of kingdom is generally disused). Thus, bacteria are ranked; Phylum-

Class-Order-Family-Genus. Additional classification of species and strain follow. Phylum represents the main evolutionary lineage of the bacteria and at present there are 29 phyla, and 23 candidate-phyla characterised. No single classification exists for subsequent assignment, though Bergey's manual of systematic bacteriology is widely considered the authorative guide (Anonymous 2001). Although no definitive classification system exists, the nomenclature of bacteria is regulated under the International Code of Nomenclature of Bacteria (Lapage 1992)

The human microbiota is known to comprise circa 35 000 bacterial species, with an individual typically harbouring 500-1000 of these species. In general, 150-200 species dominate an individual's microbiota, with the remainder consisting of less common species (Eckburg et al. 2005).

Despite this variety at species level, at the phylum level, the adult microbiota is highly conserved across mammals, highlighting the ancient evolutionary pressures and important functional role played by this microbial organ (Backhed et al. 2005a). Indeed, approximately 90% of the gut microbiota is made up of the Gram negative *Bacteriodetes* and the Gram positive *Firmicutes* (Table 1.7).

Phylum	% Representation	Major Genera
Firmicutes	~60-65	Ruminococcus, Clostridium, Lactobacillus, Enterocccus, Peptostreptococcus,
Bacteriodetes	~30-35	Bacteriodes, Prevotellae
Actinobacteria	~5-15	Bifidobacteria

Proteobacteria	~1-5	Escherichia, Desulfovibrio, Heliocobacter
Cyanobacteria/ Verrucomicrobia/ Fusobacteria)	<1	N/A

Table 1.7 Bacterial phyla and genera present in the gut microbiota. *The main phyla found in the mammalian gut microbiota, their proportional representation and the main associated genera. Adapted from (DiBaise et al. 2008; Sekirov et al. 2010; Cho and Blaser 2012).*

The concentration and content of microbiota varies widely throughout the gastrointestinal tract based on the differing environmental conditions with strict anaerobes in the vast majority followed by facultative anaerobes and aerobes (Gordon and Dubos 1970; Sekirov et al. 2010) (Fig.1.7).

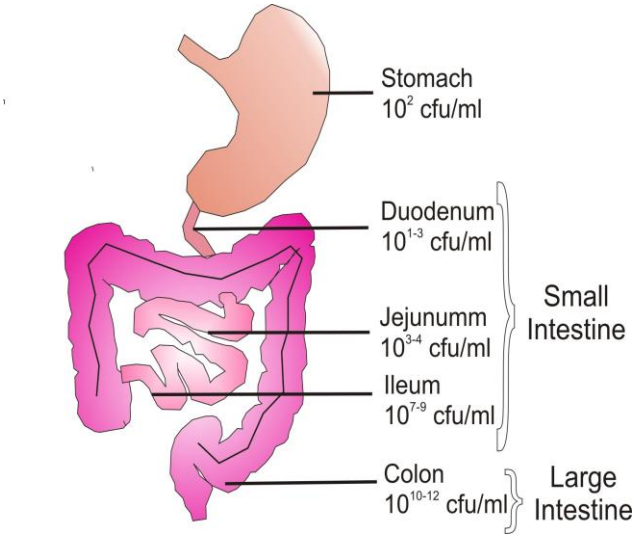


Fig. 1.7 Distribution of bacteria in the gastrointestinal tract. *Concentration of bacteria increases along the gastrointestinal tract (adapted from (DiBaise et al. 2008).*

1.11.3 Gut microbiota and energy regulation

A number of elegant studies have highlighted the extensive role the gut flora can exert on host metabolism and energy regulation. In particular, animal models such as germ-free mice and genetically obese (ob/ob) mice have been utilised to demonstrate the various important effects of the microbiota on body weight.

Germ-free, or axenic, animals lack any microbiota as they are born and raised under sterile conditions. Germ-free mice have up to 40% less body fat than conventionally raised (full compliment of microbiota) mice despite increased food intake (Backhed et al. 2004).

Moreover, colonisation of germ-free mice with the microbiota from ob/ob mice resulted in greater weight gain compared to mice colonised with microbiota from lean mice (Turnbaugh et al. 2006).

In perhaps the most direct and best understood way, the gut flora contributes to energy balance through the fermentation of otherwise indigestible complex polysaccharides (fibres) to simpler, digestible monosaccharides, typically short chained fatty acids (scfa) (Hooper et al. 2002; Macfarlane and Macfarlane 2003).

It is estimated that approximately 10% of our daily caloric intake is produced in this fashion (Flint et al. 2008). The main scfa produced are butyrate, propionate and acetate. These may be used locally or absorbed into the hepatic circulation and subsequently delivered to the liver where they stimulate gluconeogenesis and lipogenesis (Jenkins et al. 1997). This direct symbiosis whereby the gut microbiota are provided with a rich energy

source and subsequently provide the host with energy substrates likely represents the evolutionary underpinning of microbial-host symbiosis.

The microbiota can hence affect a number of hepatic gene pathways intimately involved in lipogenesis and energy storage. These changes appear to result from increased delivery of scfa as well from other indirect mechanisms.

Sterol-regulatory element binding protein 1c (SREBP-1c) and carbohydrate regulatory element binding protein (ChREBP) are two master transcription factors expressed in the liver as well as other tissues including adipose tissue (Uyeda et al. 2002; Ferre and Foufelle 2007).

These genes control the expression of a number of enzymes in response to nutrient signals that subsequently drive metabolism in one direction (usage) or another (storage) by promoting or inhibiting both gluconeogenesis and lipogenesis (see section 1.3). Investigations into the impact of the microbiota on body weight have shown that the microbiota can influence both SREBP-1c and ChREBP as well as their downstream targets, fatty acid synthase (FAS) and acetyl-CoA carboxylase (AAC) (Backhed et al. 2007). As already discussed, these enzymes are crucial for the regulation of lipogenesis and the resultant release of triglycerides into circulation that ultimately are stored in adipocytes contributing to fat deposition and subsequent weight gain.

1.11.3.1 Microbiota and adiposity

As mentioned, germ-free mice have less body fat than their conventional littermates and are resistant to diet-induced obesity (Backhed et al. 2004). The mechanisms underlying

the gut flora's influence on adiposity and thus weight gain involve in part adenosine monophosphate activated kinase (AMPK).

AMPK is highly conserved, highly sensitive cellular fuel gauge, activated when the ratio of ATP to AMP is reduced (such as during exercise) (Hardie and Sakamoto 2006). AMPK activation leads to glucose uptake, lipid metabolism and an increase in the availability of energy substrates to the cell while at the same time shutting off anabolic pathways (Kahn et al. 2005; Steinberg and Kemp 2009).

The microbiota was found to suppress AMPK phosphorylation which is required for AMPK-activation.

Germ-free mice on the other hand had increased levels of phosphorylated AMPK and were subsequently protected from diet-induced obesity (Backhed et al. 2007).

Furthermore, the microbiota can also influence the intestinal expression of fasting-induced adipose factor (Fiaf) (Backhed et al. 2007). Fiaf inhibits lipoprotein lipase (LPL) which is required for the hydrolysis of triglycerides allowing for their storage in adipocytes (Bensadoun and Kompiang 1979; Preiss-Landl et al. 2002). Germ free mice were found to have increased levels of Fiaf and moreover germ-free Fiaf$^{-/-}$ mice were no longer protected from diet-induced obesity (Backhed et al. 2007).

Thus, the microflora can regulate fat storage and as such influence body weight (Fig. 1.8). As already discussed, the adipose tissue is now recognised as an endocrine organ that can release a plethora of adipokines and cytokines to influence both local and systemic inflammation and metabolism. Thus, given the microbiota's established links to adiposity,

it is of little surprise that the gut flora has been found to influence several other aspects of metabolic dysfunction such as inflammation and insulin resistance, as discussed below.

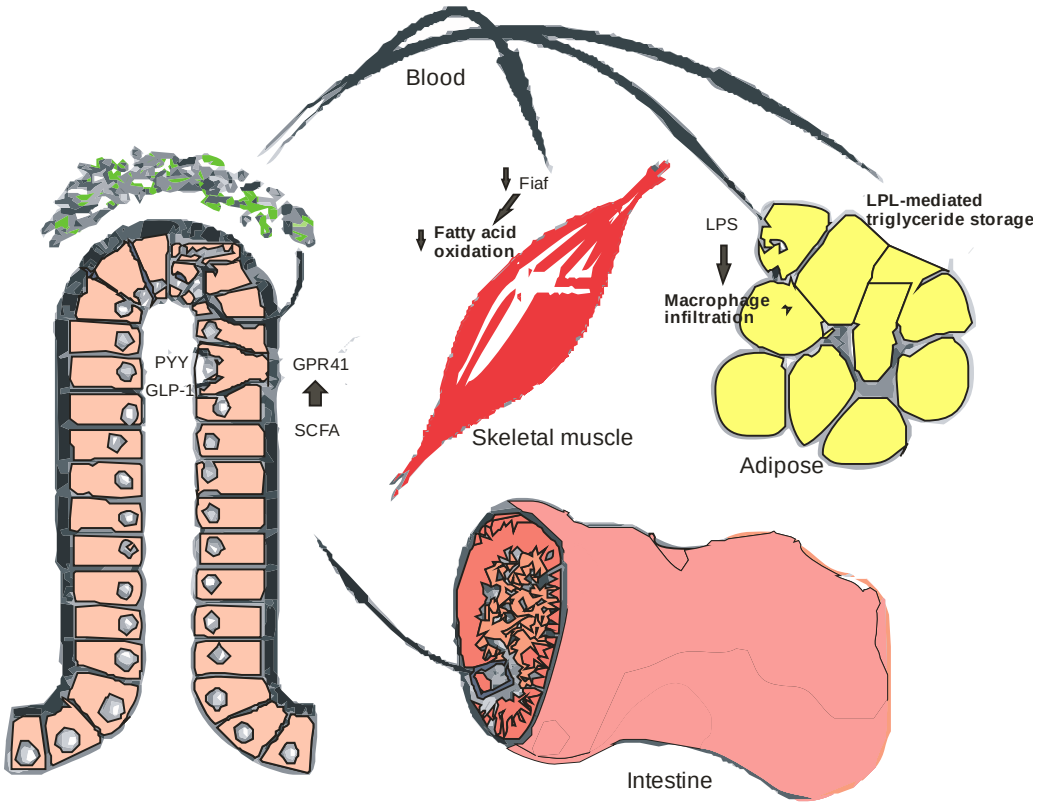


Fig.1.8 Mechanisms linking the microbiota and metabolism *Microbe-produced short chain fatty acids (scfa) are ligands for the G-protein receptor (GPR) 41 which can stimulate the release of hormones peptide-YY (PYY) and glucagon-like peptide (GLP) -1. Microbial suppression of Fiaf (fasting-induced adipose factor) expression in the gut increases lipoprotein lipase (LPL)-mediated triglyceride storage in adipose tissue as well as reduces fatty acid oxidation in skeletal muscle by a yet unidentified mechanism. Furthermore, lipopolysaccharide (LPS) derived from the gut may augment adipose inflammation and reduce insulin sensitivity. Adapted from (Backhed 2011).*

1.11.4 Microbiota and metabolic Disease

Several studies have identified a number of links between the gut flora and aspects of metabolic disease. These include both preclinical and clinical investigations. In addition to effects on adiposity as outlined above, studies have also found that microbiota alterations associated with insulin resistance and inflammation (Bienenstock and Collins 2010; Caesar et al. 2010; Membrez et al. 2010).

Thus, the microbiota's role in the progression and development of metabolic diseases such as Type II diabetes mellitus has received increasing levels of attention. Understanding the mechanisms by which the microbiota influences these metabolic-related processes under both normal and pathological states is an extremely difficult but important challenge in elucidating the full scale of the involvement of the microbiota in human physiology.

1.11.4.1 Microbiota and inflammation

Obesity is now regarded by many as a chronic low-grade inflammatory disease (Das 2001; Bastard et al. 2006a). Inflammation and metabolism are tightly connected and the innate immune system can contribute to obesity and metabolic disease (Pickup 2004; Olefsky and Glass 2010). The microbiota can directly contribute to metabolic inflammation through lipopolysaccharide (LPS) a component of the cell wall of Gram negative bacteria. LPS is a potently inflammatory endotoxin and may enter the circulation via uptake with chylomicrons or via increased intestinal permeability (Caesar et al. 2010). LPS is a ligand for the toll-like receptor 4 (TLR4) receptor which is expressed on macrophages and elsewhere. Activation of TLR4 promotes inflammation and hence contributes to insulin resistance.

Increased plasma levels of LPS, coined metabolic endotoxemia, has been linked to metabolic disorders in both animal models and clinical studies (Cani et al. 2007a; Amar et al. 2008). Moreover, antibiotic treatment attenuated endotoxemia as well as insulin resistance in experimental models (Cani et al. 2008).

It is well established that the microbiota plays a critical role in intestinal inflammation through its effects on several cells including macrophages, CD3+ T cells and CD45+ leukocytes (Abt and Artis 2009; Duerkop et al. 2009). These cells have the capacity to release proinflammatory cytokines into enterohepatic circulation and thus can contribute to systemic low grade inflammation and associated metabolic effects. Indeed, a recent study found changes in intestinal inflammation coincided with the earliest detectable increases in fat mass in mice fed a high-fat diet (Ding et al. 2010).

As mentioned, the microbiota is responsible for the production of the majority of scfa absorbed from the diet. In addition to the already discussed effects on metabolism, scfa can act as direct ligands for a number of G-protein coupled receptors (GPCRs) present on immune cells and thus may directly promote release of proinflammatory factors. These include GPR43, a chemoattractant receptor found on neutrophils (Sina et al. 2009) which has been shown to be regulated by the gut microbiota (Maslowski et al. 2009).

Moreover, GPR43$^{-/-}$ mice were found to be resistant to resistant to diet-induced obesity (Bjursell et al. 2011) further highlighting the complex interaction between the microbiota, inflammation and metabolism. In addition, GPR41, expressed on enteroendocrine cells, is also modulated by short chain fatty acids (s.c.f.a.) and thus may be regulated by the gut microbiota linking both inflammation and adiposity (Samuel et al. 2008).

1.11.4.2 Microbiota and insulin resistance

Germ-free mice have reproducibly been shown to have improved glucose tolerance and insulin sensitivity (Backhed et al. 2004; Rabot et al. 2010).

Moreover, evidence suggests that the gut flora can directly impact on the development of insulin resistance, at least in animal models (Ding et al. 2010; Membrez et al. 2010). Furthermore, antibiotic treatment was shown to attenuate insulin resistance in mice fed a high fat diet (Cani et al. 2008).

1.11.5 Microbiota composition and obesity

Several studies have been focused on identifying links between the composition of the microflora and disease phenotype, in particular, obesity. A surge in the number of such studies has been facilitated by the development of sophisticated, molecular based techniques for analysing the flora (Fraher et al. 2012). Indeed, the number of publications on the gut microbiota rose from approximately 100 per annum in 1998 to over 500 per annum in 2009 (Sekirov et al. 2010).

In their seminal studies, Gordon and colleagues identified an "obese microbiome" in mice, that promotes fat storage and weight gain, and a lean microbiome, that does not (Turnbaugh et al. 2008). The primary difference between these two microbial phenotypes was observed to be a phylum level shift in the ratio between the two dominant phyla, *Firmicutes* and *Bacteriodetes* (Turnbaugh et al. 2008), with an increase in the proportional representation of *Firmicutes* and a concomitant reduction in *Bacteriodetes* found to be associated with the development of obesity in recolonised mice. However, while evidence for the same shift was observed in diet-induced mice, it was found to be temporal and age-related as opposed to directly correlating to obesity (Murphy et al. 2010).

In humans, evidence for such a shift associated with obesity has been somewhat contentious. A study by Turnbaugh et al., found a similar alteration in the major phyla in obese versus lean twins (Turnbaugh et al. 2009). A reduction in *Bacteriodetes* was also observed in obese individuals in a study by Armougom and colleagues (Armougom et al. 2009). Furthermore, Ley et al., observed reduced levels of *Bacteriodetes* in obese subjects and found weight loss correlated with increased levels of same (Ley et al. 2006).

However, other studies have not observed this finding (Duncan et al. 2008) or indeed observed the opposite (Schwiertz et al. 2010). These discrepancies may be due to a number of factors including analysis method and the sequencing power of the different methods employed or dietary factors. It is important note however that these studies did indeed find alterations to the gut flora in association with obesity. These involved changes observed at the genera level, including reduced levels of *Eubacteriumrectale* (Duncan et al. 2008), and decreases in the proportion of *Bifidobacteria* (Schwiertz et al. 2010). This latter finding was recently supported by a study of overweight pregnant women in which a similar reduction in Bifidobacterium was observed (Santacruz et al. 2010).

We are only beginning to routinely see the inclusion of in-depth microbiota sequencing in studies of obesity, and given the massive diversity beyond phylum level of the microbiota coupled with environmental linked variations means we are yet to uncover the true significance and reproducibility of any shift seen in specific phyla, genera or species.

Another major issue yet to be addressed in assessing the microbiota composition in disease versus healthy states is establishing the causal nature of any observed differences.

Diet has long been recognised as a rapid modifier of the gut microbiota (Tannock and Savage 1974). In particular high-fat diets are known to affect the gut flora, thus in human studies of obese subjects, individuals are likely to consume a diet higher in fat than lean counterparts which could account for differences in the flora. This is not to say that these diet-induced alterations to the microbiota are not important, indeed the impact of diet on the gut flora represents a crucial factor in both understanding as well as targeting microbiota-host interactions.

In particular, arguably the most robust finding to date in human obesity is reductions in the levels of *Bacteriodetes* species (Angelakis et al. 2012). Levels of *Bateriodetes* were found to correlate with energy intake rather than obesity (Furet et al. 2010). Intriguingly, the numbers of *Bacteriodetes* were found to dramatically decrease when obese subjects consumed a low-fat or low-carbohydrate diet (Ley et al. 2006). Thus, while diet may be responsible for observed changes in microbial populations of obese individuals, these diet-induced changes may still be involved in the pathogenesis of ensuing disease. This opens up intriguing avenues of potential therapeutic intervention using both dietary and microbial targeted approaches.

In further support of a causative role of the microbiota in the pathogenesis of obesity, Kalliomaki et al. found that alterations in the gut flora of infants predicted their risk of being over weight at seven years of age (Kalliomaki et al. 2008). Moreover, it must be remembered than in animal models which do not have confounders such as diet, microbiota transplantation led to the development of obesity showing a direct link between the composition of the microbiota and weight gain (Turnbaugh et al. 2006).

As we now move in to the metagenomic era, studies have emerged investigating the obesogenic profile of the microbiota. As already mentioned, the microbiota possesses approximately 100 times the number of genes of the human genome. This massive genetic resource accounts for the diverse functions of the gut microbiota emphasises the need to focus on alterations in the genetic potential of the microbiota between obese and lean individuals as well differences in bacterial composition.

Recently, attempts have been made to classify people by their microbiota profile. One such investigation identified three microbial profiles in faecal samples which they coined enterotypes (Arumugam et al. 2011). These enterotypes were identified by their enrichment in *Bacteroides spp.* (enterotype 1), *Prevotella spp.* (enterotype 2) and *Ruminococcus spp.* (enterotype 3). These enterotypes have been shown to be affected by diet, and the *Bacteroides* enterotype is associated with a western diet (Wu et al. 2011). However, direct evidence for a link between this or another enterotype and obesity is currently lacking (Zupancic et al. 2012). Moreover, some have questioned whether the model of enterotypes represents an over-simplistic model, especially given the dynamic nature of the gut microbiota (Jeffery et al. 2012).

Thus, it appears that the microbiota and the host's energy balance affect one another and indeed that the microbiota may act to drive the energy balance equation in either direction (Musso et al. 2010a). Further studies will hopefully elucidate the direct associations between specific bacterial populations and disease pathogenesis allowing for therapeutic targeting of the microbial organ (Jia et al. 2008; Burcelin et al. 2011).

1.11.6 Brain-gut-microbiota axis

The brain gut axis is a bidirectional, integrative link between the CNS and the enteric nervous system (ENS) involving various neural, immune and endocrine mechanisms (Tougas 2000). This communication between the gut and the brain is involved in numerous physiological processes including energy regulation, abdominal pain, bone metabolism and nociception and hence contributes to a persons overall physiological state (Aziz and Thompson 1998; Blackshaw et al. 2007; Romijn et al. 2008; Mayer and Tillisch 2011).

A relationship between emotional state and gut function has long been recognised (Cannon 1909). Stress has been implicated in a range of gastrointestinal disorders including functional dyspepsia, peptic ulcer disease and irritable bowel syndrome (Bhatia and Tandon ’05). The tangible link between gut and brain extends beyond stress, as there is up to 60% co-morbidity between functional gastrointestinal disorders and psychiatric disorders such as depression and mania (Mikocka-Walus et al. 2008). This emphasises the importance of understanding the link between the gastrointestinal tract and the CNS that is the brain-gut axis.

The brain’s limbic system comprises the amygdala, thalamus, hypothalamus and anterior cingulated cortex. This complex neural circuitry receives and integrates external environmental and psychosocial stressors and accordingly activates physiological responses with the ultimate goal of ensuring survival of the individual.

Given the intricate relationship of the intestinal microbiota and the gastrointestinal tract it is almost inevitable that one influences the other and vice-versa.

However, the role the microbiota plays within the brain-gut axis has only recently begun to be investigated and the mechanisms and effects of the CNS on the gut flora remain, as yet, mostly elusive. In turn, the possible influences of the microbiota on central systems i.e. the brain are now becoming the focus of much research and will no doubt become clearer in the near future (Cryan and O'Mahony 2011; Cryan and Dinan 2012).

Hence, the gut microbiota is now integrated into the brain-gut axis (Fig 1.9) The bidirectional relationship between the gut microbiota and the brain is currently of great interest and while the "bottom-up" effects of the gut microbiota remain some-what unclear a number of works have begin to reveal the importance of this connection.

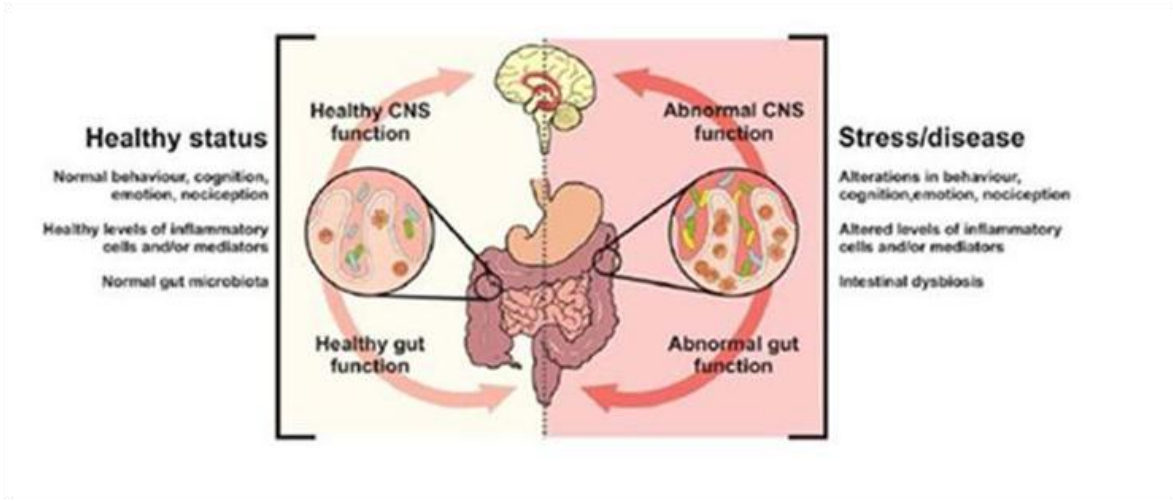


Fig.1.9 Brain-Gut-Microbiota *Axis* *The proposed bidirectional communication between the brain, the gastrointestinal system and the gut microbiota* (Grenham et al. 2011)

1.11.6.1 Microbiota and the CNS

Initial evidence for a link between microbes and the brain comes from the long recognised fact that administration of oral antibiotics improves brain function in patients with hepatic failure who have cognitive impairments or even dementia secondary to the hepatic encephalitis(Cash et al. 2010).

Furthermore, one only has to consider syphilis for a clear example of an infectious bacterium resulting in serious mental impairment (Simon 1985). Thus, work has begun in recent years to investigate the potential impact of the gut flora on central systems.

One area that has yielded interesting findings is studies of the microbiota regarding the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is a neuroendocrine system intimately involved in regulating the body's response to stress as well as being involved in other diverse processes such as immune function and digestion (Tsigos and Chrousos 2002).

A number of animal studies have demonstrated an effect of stress on the composition of the microbiota. O'Mahony et al., showed that maternal separation in rats induced changes to the gut flora of the offspring and this was associated with an altered immune profile (O'Mahony et al. 2009).

Intriguingly, Sudo et al. (2006), showed that germ-free mice had a hyper-responsivity to stress, as seen by increased adreno-corticotropin hormone (ACTH) and corticosterone, demonstrating an effect of microbiota on the stress response (Sudo et al. 2004). This response could be subsequently normalised by the commensal bacteria *Bifidobacterium infantis* but only if administered before six weeks of age, implicating the microbiota in the early development of the HPA axis. This group also went onto show that germ-free mice had lower levels of brain-derived neurotrophic factor (BDNF), noradrenaline and serotonin in certain brain regions which are all key modulators of neurotransmission within the CNS (Sudo 2006).

As already discussed, the gut flora is crucial for the proper development and function of the immune system and hence can impact on the inflammatory state of the host. Inflammation is rapidly becoming recognised as a central feature of numerous pathological states including diabetes (Wellen and Hotamisligil 2005), depression (Raison et al. 2006) and even schizophrenia (Potvin et al. 2008). As such, the role of the gut flora in the production of pro- or indeed anti-inflammatory molecules is of keen interest to the study of several diverse disease states.

1.11.7 Measuring the gut microbiota

In order to identify the members of the intestinal microbiota at any one time a number of techniques may be employed. For many years, the main stay for microbiota analysis was via culture methods. This involves plating of fresh faecal or intestinal material onto chosen media, incubation for a period of time, and then quantitative analysis by microscopy and qualitative analysis by a number of classic biochemical tests. However, estimates put the number of culturable bacteria from the human microbiota at between 10 and 50% (Zoetendal et al. 2004). Thus, while culture techniques were invaluable in times past, procedures with greater capacity for indentifying different bacterial species were sought.

In the last decade the development of molecular techniques have provided this resource, meaning the challenge of characterising this diverse ecosystem is more achievable than ever (O'Sullivan 2000; Fraher et al. 2012). This advancement in technology represents one of the main factors that has led to the recent surge in work carried out in the field and the resultant vast increase in knowledge pertaining to host-microbiota interactions.

Common techniques that are used include fingerprinting techniques such as denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis. These techniques are pcr-based and can be useful for monitoring microbial shifts over time as they relatively quick and inexpensive (Zoetendal et al. 2004). However, such methods lack the depth of sequencing provided by modern platforms.

More recently, the field of metagenomics has come to the fore. Metagenomics may be defined as culture-independent studies of the collective set of genomes of mixed microbial communities present in environmental niches, in plants, or in animal hosts (Petrosino et al. 2009). The Sanger-sequencing method (Sanger et al. 1977) provided a relatively fast method of DNA sequencing and was a key technological development for the progression of biological sciences. Indeed, it is still essentially used in many guises today (e.g. Taq) and was the corner-stone of metagenomic techniques until recently.

The newer pyrosequencing technique was invented in the 1990's based on the detection of a chemoilluminescent pyrophosphate released on nucleotide incorporation (Ronaghi et al. 1998). The technology utilises a "sequencing by synthesis" whereby the template strand is synthesised one base at a time through the use of picotiter plates. This technique is made applicable to microbial metagenomics via knowledge of the 16s rRNA gene and the compilation of large-scale gene banks of identified bacteria.

The 16S rRNA gene is comprised of highly conserved regions interspersed with more variable regions. This allows PCR primers to be designed that are complementary to universally conserved regions.

Amplification, sequencing, and comparison to databases allow for the identification of bacterial species and to asses their respective proportions in a sample. (Wu et al. 2010)

The Roche-454 pyrosequencer is a commercial high-throughput pyrosequencer that uses modern adaptations of pyrosequencing chemistry to provide relatively unambiguous and in-depth metagenomic data of a given sample (Petrosino et al. 2009).

1.12 Linking the gut microbiota and antipsychotic side effects

It is clear from the previous sections that the gut microbiota must be a consideration when attempting to assess and understand global metabolic processes related to obesity and metabolic disease. This is widely seen in animal studies of several immune and metabolic disease models (O'Mahony et al. 2009; Murphy et al. 2010). This integration of the microbiota into systemic physiology may potentially also need to be considered therefore when attempting to understand the aetiology of antipsychotic-induced metabolic effects (Fig. 1.10).

Research into these metabolic effects of antipsychotics, such as olanzapine, have revealed that weight gain alone is not the primary issue when considering patients long term health, and that the peripheral mechanisms involved in developing cardiovascular risk and diabetes must be addressed.

Further understanding of the potential contribution of the gut flora to the multiple metabolic mechanisms involved in the myriad of antipsychotic-associated metabolic effects may allow for preventative or interventive adjunctive therapies targeting the microbiota ultimately improving patient outcomes.

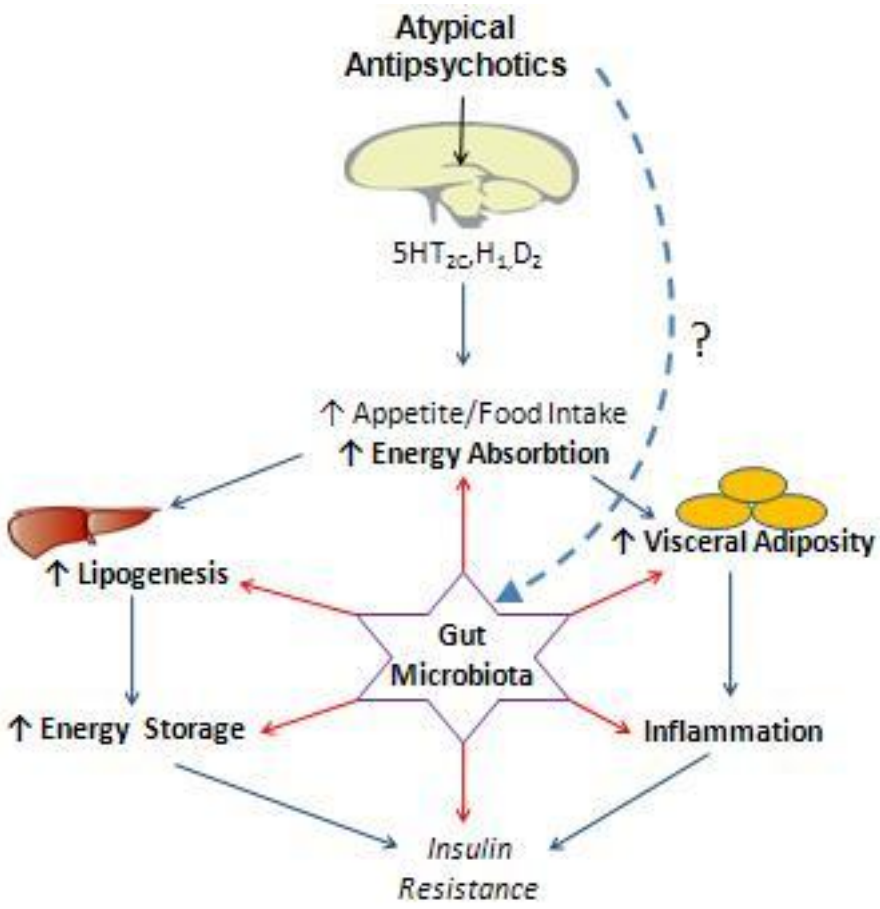


Fig 1.10 Potential links between the functions of the gut microbiota and the pathophysiology of antipsychotic-induced metabolic dysfunction. *The gut microbiota has been independently associated with several metabolic processes involved in antipsychotic-induced dysfunction including energy storage, fat deposition and inflammation.*

1.13 Aims of thesis

This thesis aims to investigate the potential novel contribution of the gut microbiota to the adverse effects of certain antipsychotic compounds and to further elucidate the involvement of the gut microbiota in physiological mechanisms governing body weight, adiposity and diseases thereof.

1.13.1 Can a mouse-based model of antipsychotic weight gain be established?

Germ-free mice offer an unparalleled opportunity to assess the role of the gut microbiota within any particular paradigm. We therefore wanted to establish a murine model of antipsychotic-induced weight gain that could feasibly be replicated in gnotobiotic mice (Chapter 2).

1.13.2 Do rats incur weight gain and metabolic dysfunction in response to olanzapine?

We set out to establish if we could replicate previous findings showing that rats do incur weight gain in response to olanzapine and risperidone, and to establish a suitable protocol within our laboratory (chapter 3/6). We also aimed to investigate previously observed differences between male and female rats in their propensity to incur such effects (chapter 3).

Moreover, we aimed to investigate whether the gut flora was altered in response to atypical antipsychotics in conjunction with other metabolic dysfunction observed. We therefore analysed the microbial composition of faecal or caecal flora of rats chronically treated with olanzapine (chapters 3,4,5) or risperidone (chapter 6).

1.13.3 Does the microbiota potentially represent a therapeutic target for antipsychotic-induced metabolic dysfunction?

To establish if the gut flora pl**a**ys a functional role in antipsychotic induced weight gain and metabolic side effects, we co-administered a broad-spectrum antibiotic cocktail along with olanzapine, chronically, to assess if obliteration of the gut flora in female rats could negate any of the previously observed negative effects of olanzapine (chapter 4).

To further investigate if the gut microbiota potentially represents a therapeutic target for antipsychotic-induced metabolic dysfunction, we assessed if specific, non-absorbable antibiotics could attenuate any of olanzapine's (chapter 5) or risperidone's (chapter 6) metabolic effects.

Chapter 2

Olanzapine increases visceral fat independent of body weight in a sex-specific manner in the mouse

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Abstract

Background: Atypical antipsychotics are associated with increases in body weight which can lead to serious co-morbidities such as type II diabetes and cardiovascular disease. Recent reports suggest peripheral mechanisms may play an important role in such side effects including increases in visceral fat. Moreover, sex differences in the liability of antipsychotic-induced weight gain has been reported though whether this difference extends to other aspects of antipsychotic-induced dysfunction is only beginning to be examined.

Methods: Male and female C57Bl/6 mice were administered one of vehicle, olanzapine (2 mg/kg/d) or olanzapine (4 mg/kg/d) for 21 days, I.P. and body weight and food intake were recorded daily. Body composition was measured by magnetic resonance prior to sacrifice and specific visceral fat deposits were dissected to assess effects of olanzapine on fat deposition in both male and female mice.

Results: Olanzapine treatment in male mice was associated with a reduction in body weight and body fat mass despite temporal increases in food intake. In female mice, olanzapine increased not only total body fat but also visceral and subcutaneous fat despite reductions in overall body weight.

Conclusions: Contrary to the clinical effect, olanzapine caused decreases in body weight in this study. However, olanzapine increased fat deposition in a region and sex-specific manner. Thus, this report, demonstrates the importance of gender in developing models of antipsychotic induced weight gain, and highlights the need to evaluate metabolic side effects beyond overt weight gain, especially increased fat mass.

2.1 Introduction

The discovery of chlorpromazine and the subsequent development of typical antipsychotics in the 1950's and 1960's revolutionized the treatment of psychiatric disorders (Turner 2007). However, these drugs are associated with extra pyramidal side effects (EPS), a debilitating collection of motor disorders including akathisia and tardive dyskinesia (Dayalu and Chou 2008). In the late 1950's, clozapine was developed and was found to carry a greatly reduced risk of EPS and thus was termed atypical. Since then, many more atypical antipsychotics have been developed, all with a lower propensity to cause EPS.

While atypical antipsychotics were a breakthrough in terms of EPS liability, they brought with them their own constellation of serious metabolic side effects. Significant body weight gain (>7% from baseline) occurs in as many as 50% of patients receiving an atypical antipsychotic (Citrome et al. 2011b). Furthermore, complications such as glucose dysregulation and dyslipidemia commonly occur and can be seen with or without overt weight gain (Eder et al. 2001; Oriot et al. 2008; Patel et al. 2009; Perez-Iglesias et al. 2009)(for review see (Newcomer 2005).

The mechanisms underlying these metabolic side effects are not well understood, though in recent years it has become apparent that both central and peripheral mechanisms are involved. The various pathways affected by antipsychotics appear to play an integrated role in causing the myriad of metabolic complications which ultimately lead to co-morbidities such as type II diabetes mellitus (Newcomer 2004; Chintoh et al. 2008a; Sacher et al. 2008; Chintoh et al. 2009a).

In particular, much focus has been placed on expansion of visceral fat mass as a major contributor to the development and progression of metabolic disease in general (Demerath et al. 2008). This is due to the realisation that the adipose tissue is an active endocrine organ rather than simply an energy repository (Trayhurn and Wood 2004; Wisse 2004).

Moreover, evidence for effects of antipsychotics on visceral fat suggests that the adipose tissue may be an important source of antipsychotic-induced metabolic defects (Victoriano et al. 2010).

One factor recently found to be involved in fat deposition and the aetiology of obesity, in mice at least, is the gut microbiota (Backhed et al. 2004). The gut microbiota represents the approximate 100 trillion bacteria that live symbiotically within the gastrointestinal tract. This microbial organ contains 100 times the number of genes in the human genome and hence has a huge capacity to influence human physiology via a number of mechanisms (Turnbaugh et al. 2007; Ley et al. 2008b; Bienenstock and Collins 2010; Backhed 2011; Burcelin et al. 2011). Whether the gut microbiota contributes to the metabolic effects of any or all antipsychotic drugs is thus far unexplored.

Mice offer a unique opportunity to investigate the role of the gut microbiota in a given model due to the availability of germ-free mice (mice lacking any microbiota), first developed in the early 1960's (Trexler 1961).

However, in order to investigate if the gut microbiota plays a significant role in antipsychotic induced metabolic dysfunction using a tool such as germ-free mice, we first needed to establish a reproducible and clinically relevant mouse-model of antipsychotic induced metabolic effects.

An additional unresolved question in relation to antipsychotic-induced metabolic effects is that of sex. Several reports have suggested that females have an increased liability for such adverse effects compared to males (Hakko et al. 2006; Verma et al. 2009). Clear differences in body weight between males and females in animal models have been demonstrated (Albaugh et al. 2006), however, direct comparisons between male and females in other aspects of metabolic dysfunction induced by atypical antipsychotics have been lacking.

Thus, we investigated the effects of olanzapine on body weight and body composition in male and female C57Bl/6 mice.

2.2 Methods

Animals

Male and female C57Bl/6 mice were used (Harlan, UK). Mice receiving the same drug treatment were housed in pairs. Animals were maintained on a 12 hour light/dark cycle with lights on at 7:30 am. Animals had access to standard chow and water *ad libitum*. All experiments were approved by the Animal Experimentation Ethics Committee (AEEC) of University College Cork and carried out in accordance with the Cruelty to Animals Act 1876 and European Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

Drug administration

Olanzapine (Discovery Fine Chemicals, Ireland) was prepared fresh daily and was dissolved in a minimal amount of glacial acetic acid (approx 0.1 ml) and then made to volume and pH adjusted to approximately 6 with 1M NaOH. Vehicle consisted of distilled water acidified with glacial acetic acid and pH adjusted to 6 with 1M NaOH. Drug and vehicle were administered at an injection volume of 10 ml/kg.

Treatment groups

Mice (n=8) received vehicle, olanzapine (2 mg/kg/d) or olanzapine (4 mg/kg/d) via twice-daily (B.I.D) intra-peritoneal injection for 21 days. Injections took place between 9:00 am and 10:00 am and 4:00pm and 5:00pm.

Daily measurement

Body weight and food intake were measured daily to the nearest 0.01g using an electronic balance.

Body composition

Body composition was analysed in conscious animals using a minispec nuclear magnetic resonance body composition analyser (Bruker Optics, MA, U.S.). Briefly, mice were placed carefully into a clear plastic tube which was then inserted horizontally into the minispec machine.

The analysis takes 20-30 seconds before the mouse was removed from the tube, the tube cleaned and the next animal placed inside.

Body composition was further assessed by careful dissection of subcutaneous, mesenteric, peri-uterine and peri-renal fat deposits which were excised and weighed to the nearest 0.0001g on an electronic balance.

Statistical analysis

Body weight and food intake were analysed using repeated measures ANOVA. Where sphericity was violated, Greenhouse-geisser correction was applied. Body composition was analysed using one way ANOVA, with LSD post hoc test used for further analysis where appropriate. LSD was selected as an appropriate post-hoc test due the relatively small N numbers and expected effect sizes.

2.3 Results

Body weight

Time had a significant effect on body weight, ($F_{(3.63,149.06)}$ = 81.641, $p < 0.001$). Drug treatment also had a significant overall effect ($F_{(2,41)}$ = 9.66, $p < 0.001$) and there was a significant time x drug interaction ($F_{(7.27,149.06)}$ = 2.803, $p < 0.01$). Gender did not have a significant overall effect ($F_{(1,41)}$ = 0.846, $p = 0.363$).

In male animals, post-hoc analysis revealed that mice treated with olanzapine (2 mg/kg) or olanzapine (4mg/kg) had significantly reduced weight gain on days 2-10 (inclusive) ($p < 0.001$ to $p < 0.05$) (Fig. 2.1A). Female mice receiving olanzapine (2 mg/kg) displayed reduced body weight gain compared to vehicle treated mice on days 2-5 and days 9-22 (inclusive) ($p < 0.01$ to $p < 0.05$) while those receiving olanzapine (4 mg/kg) displayed reduced body weight gain compared to vehicle treated mice on days 2-22 inclusive ($p < 0.001$ to $p < 0.05$) (Fig. 2.1B).

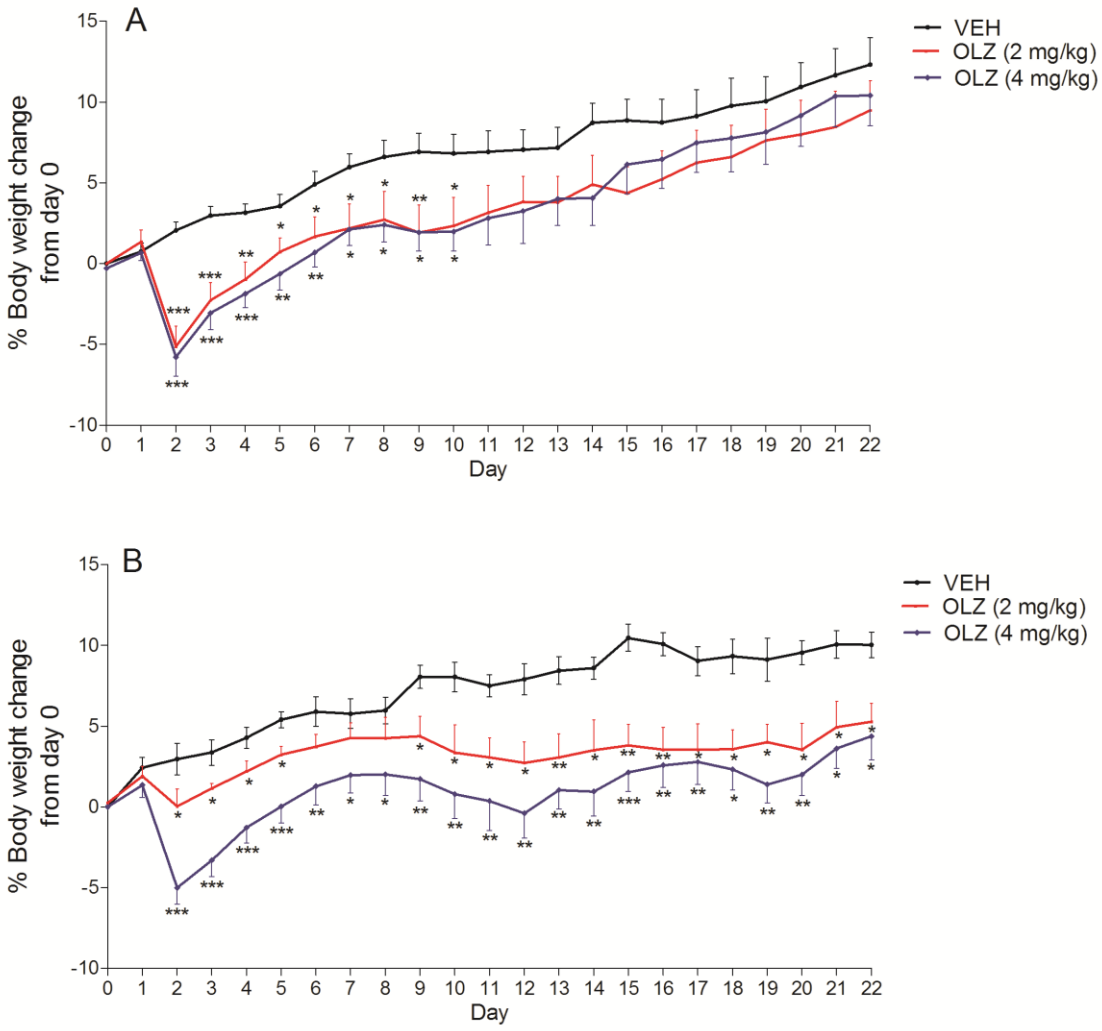


Fig. 2.1 Effect of olanzapine on body weight in C57Bl/6 mice. *Percent body weight change following 21 days vehicle (VEH), olanzapine (OLZ) (2 mg/kg) or olanzapine (4 mg/kg) administration in (A) Male C57Bl/6 mice and (B) Female C57Bl/6 mice. Data expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p <0.001 compared to vehicle treated group.*

Food Intake

Food intake was not significantly affected by time ($F_{(2,372)}$ = 2.618, p = 0.08). Gender did significantly affect food intake ($F_{(2,36)}$ = 23.547, p < 0.001) and there was a significant time x gender interaction, ($F_{(2,72)}$ = 4.74, p < 0.05). Drug treatment did not have an overall effect ($F_{(2,36)}$ = 2.443, p = 0.101).

Further analysis revealed that in male mice receiving OLZ (2 mg/kg) there was evidence of increased food intake which was significantly increased compared to mice receiving OLZ (4 mg/kg) in week 3 ($p < 0.5$) (Fig. 2.2A). In the females, mice receiving olanzapine (2 mg/kg) had increased food intake in week 2 compared to both the vehicle treated animals and those receiving olanzapine (4 mg/kg) ($p < 0.05$) (Fig. 2.2B).

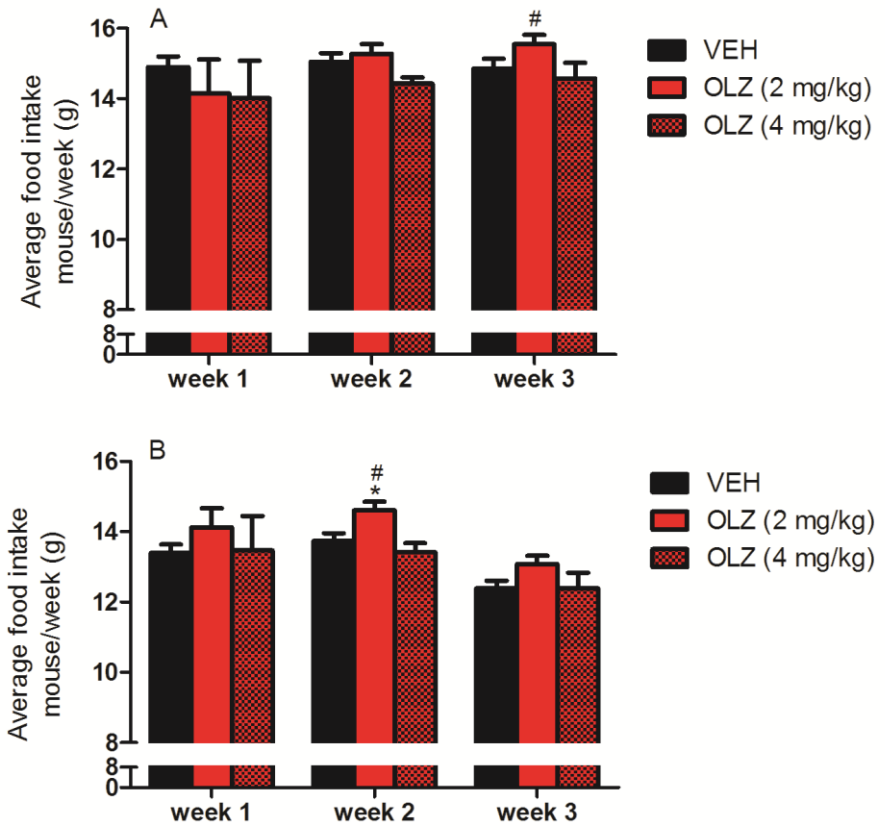


Fig. 2.2 Effect of olanzapine on food intake in C57Bl/6 mice. *Average weekly food intake following administration of vehicle (VEH), olanzapine (OLZ) (2 mg/kg) or olanzapine (4 mg/kg) in (A) Male C57Bl/6 mice and (B) Female C57Bl/6 mice. Data expressed as mean ± S.E.M. *p < 0.05 versus vehicle treated group, #p < 0.05 versus olanzapine (4 mg/kg) treated group.*

Body Composition

Body fat percentage was significantly affected by gender ($F_{(1,42)} = 88.64$, $p < 0.001$) but not drug treatment, ($F_{(2,42)} = 1.893$, $p = 0.163$). There was however a significant gender x drug interaction ($F_{(2,42)} = 5.561$, $p < 05$).

In the male mice, both olanzapine (2 mg/kg) and olanzapine (4 mg/kg) resulted in a significant reduction in body fat percentage compared to vehicle treated mice ($p < 0.05$) (Fig. 2.3A). In the female mice, the opposite was seen with mice treated with olanzapine (2 mg/kg) displaying significantly increased body fat compared to both vehicle treated mice ($p < 0.01$) and mice receiving olanzapine (4 mg/kg) ($p < 0.05$) (Fig 2.3B).

Total body lean mass was not affected by drug treatment ($F_{(2,42)} = 1.401$, $p = 0.258$) while gender did have a significant effect ($F_{(1,42)} = 62.929$, $p < 0.001$). There were no significant differences in lean mass observed between treatment groups in the male (Fig. 2.4C) or female mice (Fig. 2.4D).

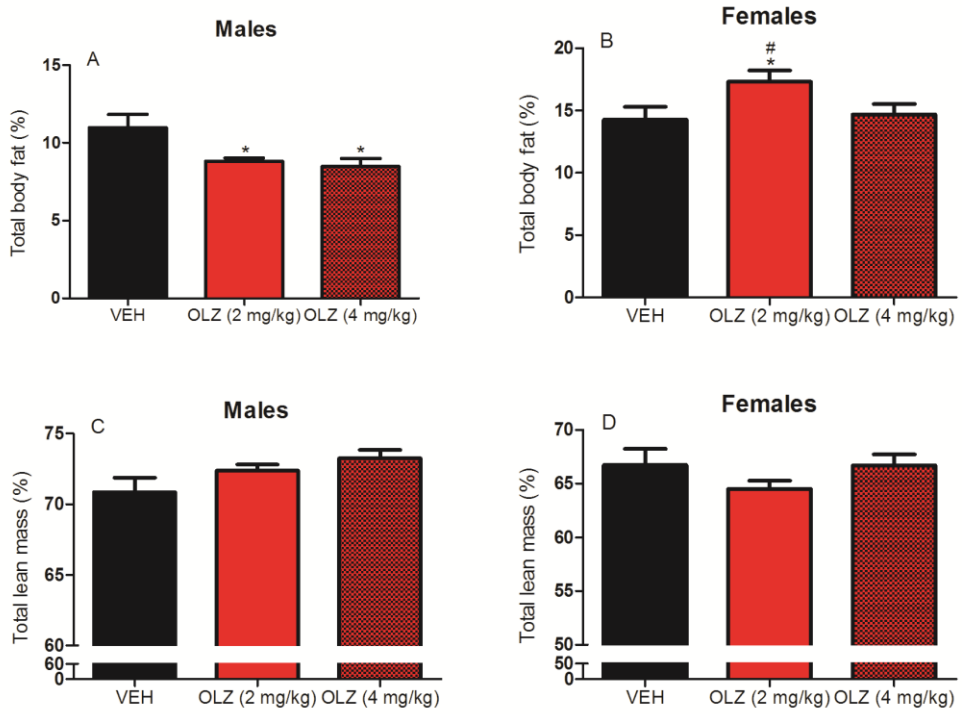


Fig. 2.3 Effect of olanzapine on body composition in C57Bl/6 mice. *Effect of vehicle, olanzapine (2 mg/kg) or olanzapine (4 mg/kg) administration for 21 days on percent body fat percentage in (A) Male and (B) Female C57Bl/6 mice and on lean mass percentage in(C) Male and (D) Female C57Bl/6 mice. Data expressed as mean ± SEM. *p < 0.05 versus vehicle treated mice, $^{\#}p < 0.05$ versus olanzapine (4 mg/kg) treated mice.*

Fat distribution

Olanzapine treatment was found to have a significant effect on visceral fat mass ($F_{(2,42)}$ = 4.827, $p < 0.05$) as did gender ($F_{(1,42)}$ = 73.865, $p < 0.001$). No differences in visceral fat were seen for male mice in visceral (Fig 2.4A) however female mice treated with olanzapine (2 mg/kg) or olanzapine (4 mg/kg) had increased visceral fat compared to vehicle treated mice ($p < 0.05$) (Fig.2.4B).

Subcutaneous fat was also affected by drug treatment ($F_{(2,42)}$ = 3.109, $p < 0.05$) and gender, ($F_{(1,42)}$ = 43.555, $p < 0.001$).

Male mice did not show any difference in subcutaneous fat (Fig 2.4C) but the female mice receiving olanzapine (2mg/kg) had increased subcutaneous fat compared to the vehicle and olanzapine (4 mg/kg) treated mice ($p < 0.05$) (Fig. 2.4D).

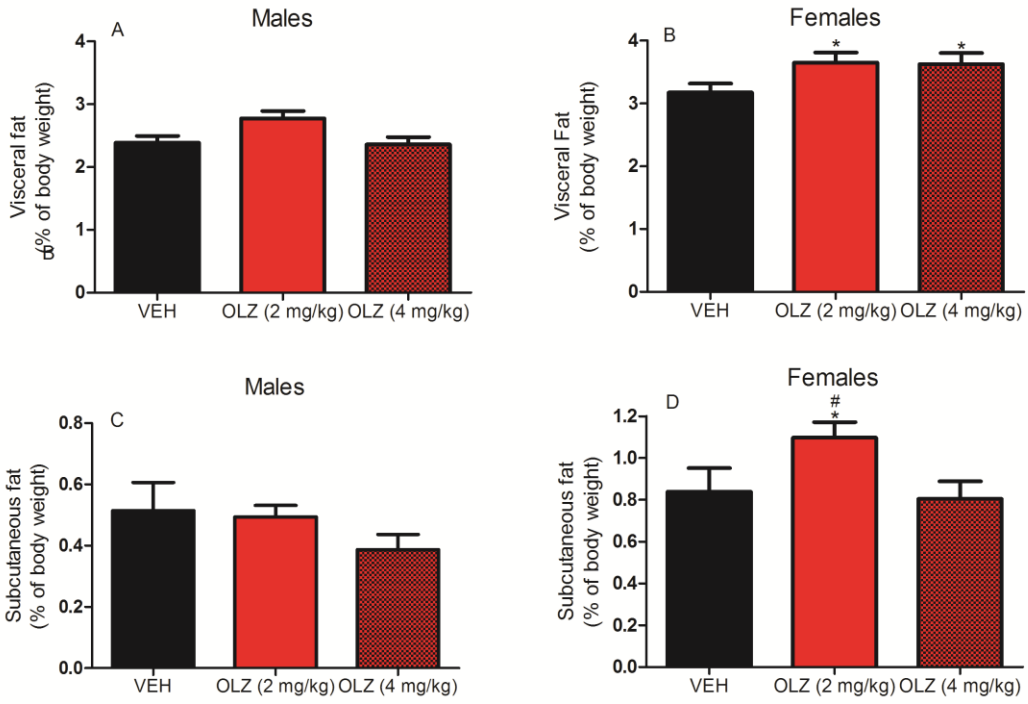


Fig. 2.4 Effect of olanzapine on fat deposition in C57Bl/6 mice. *Effect of vehicle (veh), olanzapine (OLZ) (2 mg/kg) or olanzapine (4 mg/kg) administration for 21 days on visceral fat in (A) Male and (B) Female C57Bl/6 mice and on subcutaneous fat mass in (C) Male and (D) Female C57Bl/6 mice. Data expressed as mean ± SEM. *p < 0.05 versus vehicle treated group, #p < 0.05 versus olanzapine (4 mg/kg) treated mice.*

To further assess fat distribution, the individual deposits were dissected and weighed. Gender had a significant effect on fat mass in all of the areas measured: gonadal ($F_{(1,42)}$ = 68.947, $p < 0.001$); mesenteric ($F_{(1,42)}$ = 16.381, $p < 0.001$) and renal ($F_{(1,42)}$ = 41.914, $p < 0.001$). In the case of gonadal fat there was also a significant gender x drug interaction ($F_{(2,42)}$ = 5.587, $p < 0.01$).

Further analysis revealed that in the male mice, treatment with olanzapine (2 mg/kg) led to increased levels of peri-renal fat compared to vehicle treated mice ($p < 0.05$) (Fig. 2.6A) while the other fat deposits were unaltered (Fig. 2.6 B-C).

In the female mice, peri-renal fat was not altered following olanzapine treatment (Fig. 2.5B). However, female mice receiving olanzapine (2 mg/kg) or olanzapine (4 mg/kg) had increased gonadal fat mass compared to vehicle treated mice (Fig. 2.6D). No change in mesenteric fat was observed in female mice (Fig. 2.6F).

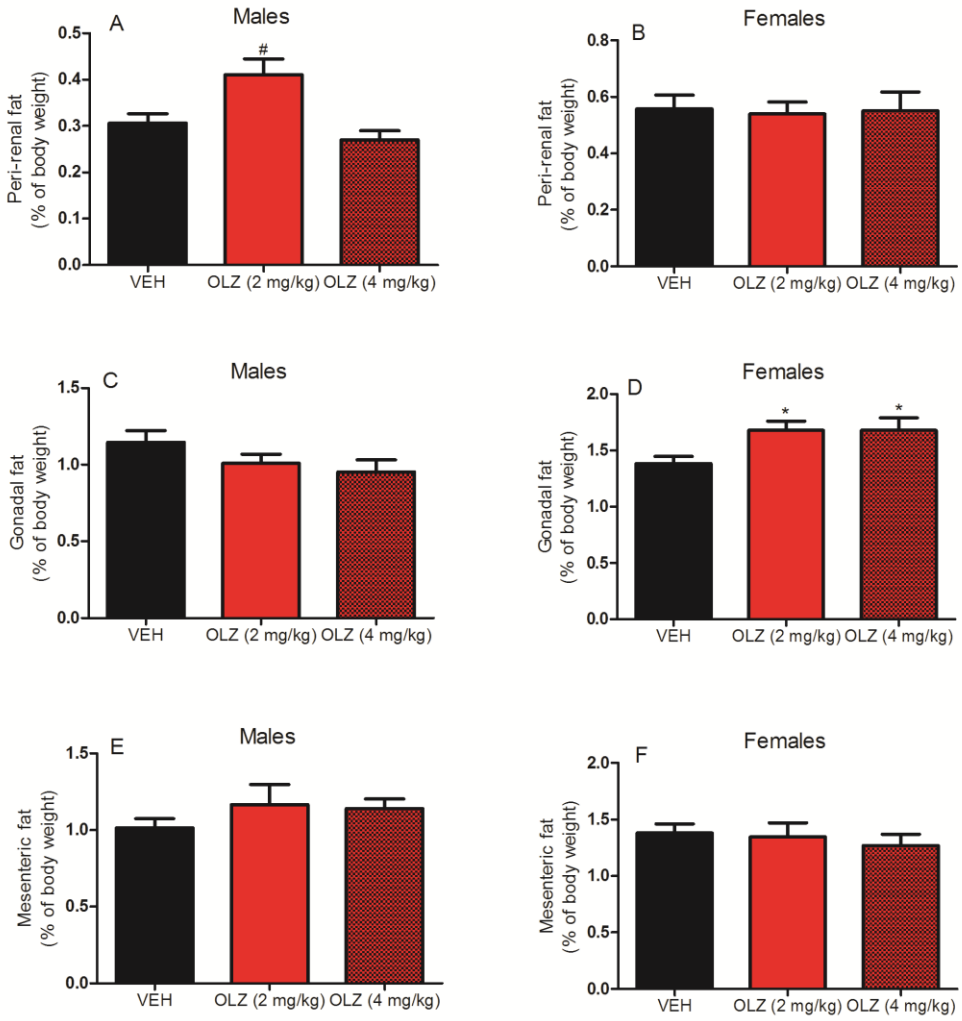


Figure 2.5 Effect of olanzapine on regional fat deposition in C57Bl/6 mice. *Effect of vehicle (VEH), olanzapine (OLZ) (2 mg/kg) or olanzapine (4 mg/kg) on peri-renal (A-B), gonadal (C-D) and mesenteric (E-F) fat deposits in male and female C57Bl/6 mice. Data expressed as mean ± S.E.M. *p < 0.05 versus vehicle treated mice, $^{\#}p < 0.05$ versus olanzapine (4 mg/kg) treated mice.*

2.4 Discussion

In the present study, we set out to establish a murine model of antipsychotic-induced weight gain and metabolic dysfunction. This has proven to be difficult in the past, as a number of studies have reported a failure of mice to gain excessive weight when administered antipsychotics (Albaugh et al. 2006; Shertzer et al. 2010). Studies which have produced weight gain in murine models have relied on high doses and long treatment protocols which arguably do not represent the clinical setting in a relevant fashion (Cope et al. 2005; Coccurello et al. 2008).

Herein, the atypical antipsychotic olanzapine did not induce weight gain in mice. Indeed, somewhat surprisingly, olanzapine was associated with decreased body weight. This is however not without precedent, as clozapine, an atypical antipsychotic associated with severe weight gain in patients (Allison et al. 1999) has repeatedly been found to induce reduced weight gain in rodents (Cheng et al. 2005; Yuan et al. 2008).

Furthermore, the initial injection of olanzapine was associated with acute hypophagia leading to reductions in body weight. This peculiar finding was seen in a previous study (Yoon et al. 2010), suggesting that a species-specific hypophagic effect of acute olanzapine may exist and this may have precluded weight gain.

The possible role of the gut microbiota in antipsychotic-induced weight gain thus remains to be explored. This study suggests that future studies investigating if the gut microbiota contributes to the metabolic liabilities of antipsychotics will likely have to employ different species other than mice as they do not reflect the clinical setting in a reproducible fashion.

However, given the complex and multifactorial nature of the mechanisms underlying the side effects of antipsychotics, the study described herein is not without potential implications.

Increased food intake is the primary factor behind initial weight gain observed in patients and animals receiving atypical antipsychotics such as olanzapine (Kluge et al. 2007; Davoodi et al. 2009; Fountaine et al. 2010). This hyperphagia is caused by antagonism of numerous receptors which are intimately involved in the regulation of food intake, including dopamine D_2, serotonin 5-HT_{2C} and histamine H_1 receptors (Nasrallah 2008). Both male and female olanzapine treated mice displayed evidence of increased food intake at the lower, 2 mg/kg, dose. These increases, while not sufficient to produce overall body weight gains, suggest olanzapine was exerting a central effect reminiscent of that seen in patients.

Crucially, research into the mechanisms underlying antipsychotic association with co-morbidities such as type II diabetes mellitus has revealed that several overlapping factors are involved, both central and peripheral (Basson et al. 2001a; Houseknecht et al. 2007; Jassim et al. 2011). Furthermore, it is important to note that weight gain *per se* is not necessary for metabolic abnormalities to develop in patients or animals receiving antipsychotics (Zhang et al. 2004; Minet-Ringuet et al. 2006) or indeed in the general population (Alberti et al. 2009).

Recently, emphasis has been placed on the expansion of visceral fat as a critical factor in metabolic disease (Demerath et al. 2008). Visceral, or abdominal, fat is no longer thought of as a simple storage depot, but rather as an endocrine organ (Galic et al. 2010).

There are several converging mechanisms that make increased visceral adiposity particularly dangerous, including the release of pro-inflammatory mediators such as cytokines: TNF, IL-6, IL-1β, and hormones such as leptin. Together with the release of free fatty acids into circulation, these factors all contribute to a vicious cycle of metabolic dysfunction instigating insulin resistance which leads to further fat expansion and exacerbating metabolic dysregulation.

Intriguingly, the female mice receiving olanzapine (2 mg/kg) displayed significantly greater body fat percentage than vehicle treated mice. Furthermore, female mice receiving either dose of olanzapine displayed increased levels of visceral fat. In addition, female mice receiving olanzapine (2 mg/kg) displayed increased levels of subcutaneous fat. Thus, this increase in subcutaneous fat at only the lower dose of olanzapine likely explains why an overall increase in body fat percentage was observed only at this dose.

We further investigated potential effects of olanzapine on visceral fat by dissecting three discrete visceral regions, namely; gonadal, renal and mesenteric fat deposits. We found both doses of olanzapine increased gonadal (peri-uterine) fat in female mice. Interestingly, in the male mice, increases in peri-renal fat were observed in response to olanzapine suggesting that despite limited effects on body weight and fat mass, the male mice were susceptible to certain effects of olanzapine.

These increases in visceral fat without increased body weight are not too surprising as normal weight individuals can have increases in visceral fat mass and this has also been seen in rodents receiving antipsychotics (Minet-Ringuet et al. 2006).

Moreover, a number of studies have observed increases in adipose mass and adipose dysfunction in male rodents in response to antipsychotics in the absence of effects on body weight (Albaugh et al. 2010; Victoriano et al. 2010).

As already mentioned, increased visceral fat is a key step in the development of metabolic disease, and is not necessarily associated with body weight gain. Hence, these findings suggest that while not sufficient to replicate the weight gain observed in humans, olanzapine's effects on fat mass in this study are potentially highly clinically relevant.

In particular, the sex-specific nature of the alterations may be of importance when assessing the metabolic effects of olanzapine in both animal and clinical studies.

The observed sex differences in the effects of olanzapine on fat deposition are extremely interesting as many, if not all, studies in patients have found a gender bias, with females exhibiting a greater propensity towards the weight gain liabilities of antipsychotics (Hakko et al. 2006; Aichhorn et al. 2007; Haack et al. 2009).

This difference may be due to gender differences in drug pharmacokinetics (Harris et al. 1995; Beierle et al. 1999) as females exhibit higher plasma levels of olanzapine for a given dose compared to males (Callaghan et al. 1999; Kelly et al. 1999; Seeman 2004). Sex differences in metabolic enzyme systems such as cytochrome enzymes CYP3A4 and CYP1A2 (Parkinson et al. 2004) also exist. CYP1A2 is the main metabolising enzyme for olanzapine, and is less active in women than men (Kelly et al. 1999; Gex-Fabry et al. 2003) and polymorphisms in CYP1A2 are associated with reduced liability for metabolic side effects of antipsychotics (Laika et al. 2010).

Moreover, sex-differences in hepatic drug metabolism are more robust in rodents than humans (Waxman and Celenza 2003). Thus, the sex-specific differences in drug pharmacokinetics and pharmacodynamics may impact on side effect susceptibility in humans, and this may be exaggerated in experimental models.

Thus, we have shown that while this model was not sufficient to investigate the effects olanzapine on body weight, a number of potentially relevant observations were made. A potentially important sex-difference in the nature of olanzapine-induced alterations in visceral fat deposition was revealed. Critically, this occurred in the absence of weight gain, drawing attention to the importance of considering potential metabolic complications without evidence of overt weight gain in patients.

An unanswered question from this study is the impact of the gut microbiota in antipsychotic-induced alterations in body weight and fat mass. This study suggests that other species, such as the rat, may represent a better choice for attempting to unravel such a question.

Chapter 3

Gender-Dependent Consequences of Chronic Olanzapine in the Rat: Effects on Body weight, Inflammatory, Metabolic and Microbiota Parameters

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Abstract

Background: Atypical antipsychotic drugs (AAPDs) such as olanzapine have a serious side effect profile including weight gain and metabolic dysfunction and a number of studies have suggested a role for gender in the susceptibility to these effects. In recent times, the gut microbiota has been recognised as a major contributor to the regulation of body weight and metabolism. Thus, we investigated the effects of olanzapine on body weight, behaviour, gut microbiota and inflammatory and metabolic markers in both male and female rats.

Methods: Male and female rats received olanzapine (2 mg/kg/d or 4 mg/kg/d) or vehicle for 3 weeks. Body weight, food and water intake were monitored daily. The faecal microbial content was assessed by 454 pyrosequencing. Plasma cytokines, as well as expression of genes including sterol-regulatory element binding protein-1c (SREBP-1C) and CD68 were analysed.

Results: Olanzapine induced significant body weight gain in the female rats only. Only female rats treated with olanzapine (2 mg/kg) had elevated plasma levels of IL-8 and IL-1β while both males and females had olanzapine-induced increases in adiposity and evidence of macrophage infiltration into adipose tissue. Furthermore, an altered microbiota profile was observed following olanzapine treatment in both genders.

Conclusions: This study furthers the theory that gender may impact on the nature of, and susceptibility to, certain side effects of antipsychotics. In addition, we demonstrate, what is to our knowledge the first time, an altered microbiota associated with chronic olanzapine treatment.

3.1 Introduction

Atypical antipsychotics (AAPDs) such as olanzapine represent the mainstay of treatment for schizophrenia and bipolar disorder. AAPDs are a diverse drug class grouped together based on their lack of extra-pyramidal symptoms (EPS) associated with typical antipsychotics (De Oliveira and Juruena 2006). AAPDs however are associated with their own side effects, most notably weight gain and metabolic dysfunction (Birkenaes et al. 2008; Chintoh et al. 2008b; Oriot et al. 2008; Perez-Iglesias et al. 2009; Albaugh et al. 2010). Clinically significant weight gain (> 7%) often occurs in greater than 50% of patients receiving an atypical antipsychotic (Ahmer et al. 2008; Patel et al. 2009; Citrome et al. 2011a; Citrome et al. 2011b). This has ramifications for the patient in terms of metabolic and cardiovascular disease co-morbidity as well treatment compliance (Nasrallah 2003; Farwell et al. 2004; Cohen and Correll 2009; Correll et al. 2009; Starrenburg and Bogers 2009).

A number of factors such as base-line weight (Basson et al. 2001a; Gebhardt et al. 2009), therapeutic outcome (Basson et al. 2001a; Meltzer et al. 2003b) and gender (Aichhorn et al. 2007; Haack et al. 2009), have been proposed to confer susceptibility to the metabolic effects of antipsychotics. In the majority of cases, females have been found to have a higher prevalence of AAPD induced weight gain (Hakko et al. 2006; Aichhorn et al. 2007; Haack et al. 2009; Verma et al. 2009). However, exceptions exist, where men have increased weight gain (Basson et al. 2001a) or no gender bias has been apparent (Lee et al. 2004).

Intriguingly, in rat (and to a lesser extent mouse) models of antipsychotic-induced weight gain, there appears to exist gender-dependent effects with female rats showing more robust weight gain following treatment compared to males (Albaugh et al. 2006; Choi et al. 2007). This has led some to challenge the relevance of these models (Pouzet et al. 2003). However, recent studies have demonstrated that male rats do incur a number of the detrimental metabolic effects in the absence of weight gain (Minet-Ringuet et al. 2006; Victoriano et al. 2009; Albaugh et al. 2010) and can even incur weight gain with extended protocols (Shobo et al. 2011).

Overall therefore, gender differences in animal models may be more relevant to the clinical setting than previously thought (Weston-Green et al. 2011b) and while current models are not perfect, they are extremely important in tackling the problems associated with AAPDs (Boyda et al. 2010).

It is important to note that AAPDs such as olanzapine can lead to the development of Type II diabetes mellitus with or without the presence of overt weight gain (Newcomer 2004; Kim et al. 2010). Therefore, direct and indirect metabolic actions of these drugs are important considerations when assessing their overall metabolic impact. Inflammation, for instance, is an important factor in the development of obesity and the metabolic syndrome (Das 2001; Bastard et al. 2006a) and a correlation between increased cytokine production and AAPD-induced weight gain has been observed (Kluge et al. 2009).

The gut microbiota comprises the approximate 10^{13}-10^{14}bacteria which reside within the gastrointestinal tract and exist in a symbiotic relationship with the host.

Recently, a role for the gut microbiota in body weight, metabolism and systemic inflammation has begun to be elucidated (Backhed et al. 2004; Ley et al. 2005; Turnbaugh et al. 2006; Backhed et al. 2007; Clarke et al. 2010; Murphy et al. 2010; Bailey et al. 2011). Furthermore, as with many other systems, a definitive gender divide exists in the composition of the gut microbiota in both humans and animals (Mueller et al. 2006; Fushuku and Fukuda 2008). Moreover, the microbiota can have marked effects on the brain-gut axis (Cryan and O'Mahony, 2011; Bravo et al., 2011). It is currently unclear whether chronic AAPD treatment can affect microbiota composition in addition to affecting body weight, metabolism and systemic inflammation.

Thus, we investigated the impact of chronic olanzapine treatment on metabolic, inflammatory and microbiome parameters and assessed whether there was a sexually dimorphic response.

3.2 Methods

Animals

Male and female Sprague-Dawley rats, initially weighing approximately 200g, were used (Harlan, UK). Animals were habituated to the animal facility for one week. They were housed four per cage (56x38x17 cm), allowed access to standard chow and water *ad libitum* and kept on a 12 hour light-dark cycle with lights on at 7.30am. All experiments were approved by the Ethical Committee of University College Cork (#2010/013) and carried out in accordance with the Cruelty to Animals Act 1876.

Drug administration

Olanzapine (Discovery Fine Chemicals, UK) was dissolved in a minimal amount of glacial acetic acid (approx 0.1ml) and then made to volume with deionised water and pH adjusted to approximately 6.0 with 0.1M NaOH. Vehicle consisted of distilled water acidified with glacial acetic acid and pH adjusted with 0.1M NaOH. All solutions were prepared fresh daily. The treatments were administered via intra-peritoneal injection B.I.D. with the first injection between 9.00am and 10.00am and the second injection between 4.00pm and 5.00pm.

Treatment groups

Rats (n=8) received vehicle, olanzapine 2 mg/kg/day or olanzapine 4 mg/kg/day for 21 days. All groups were weight matched prior to treatment commencing. Doses were selected on the basis they reflect therapeutic concentrations and have been shown to induce weight-gain and metabolic side-effects previously (Kapur et al. 2003; Cooper et al. 2005; Fell et al. 2005).

Daily measurements

Body weight, food intake and water intake were measured each morning to the nearest 0.01g using an electronic balance. This was carried out prior to the first injection.

Locomotor activity

On day 22, animals were allowed one hour to habituate to the testing room (13.00-14.00) before being placed into the centre of a rectangular plastic box (60x50x40 cm). The behaviour was recorded via an overhead camera for 30 minutes (14.00-14.30) and locomotor activity was analysed using a tracking software system (Ethovision, Noldus, The Netherlands).

Sample collection

Animals were sacrificed by decapitation and trunk blood was collected in EDTA coated tubes and centrifuged for fifteen minutes at 6000 rpm. The plasma supernatant was aliquoted and stored on dry ice. The brain was quickly excised and dissected and each brain region was initially stored in RNALater for 24 hours. The gonadal, mesenteric and subcutaneous fat deposits were carefully excised and weighed to the nearest 0.0001g. The gonadal and mesenteric deposits were added together as a measure of visceral fat. The frontal lobe of the liver was snap-frozen in isopentane and stored on dry ice. All samples were frozen at -80^{0}C for later analysis. Faecal pellets were collected directly from the animals on day 22 on dry ice and quickly stored at -80^{0}C.

Plasma analysis

Concentrations of the cytokines tumour necrosis factor alpha (TNF-α), interleukin-8, (IL-8), interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) were analysed using a commercially available electrochemiluminescence multiplex system (MSD, Gaithersburg, MD, USA). The highly sensitive assay has a range of 9.8-40,000 pg/ml. The plates were analysed on a SECTOR Imager 2400 from Mesoscale Discovery. 25µl of plasma was used for each well and all samples were analysed in duplicate. Plasma leptin was analysed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA, USA). The assay range of this assay is 0.2 ng/ml to 30 ng/ml. 10 µl of plasma was used for each well and all samples were analysed in duplicate.

Plasma ghrelin was measured using a mouse/rat total ghrelin multi-array assay from Mesoscale discovery (MSD, Gaithersburg, MD, USA). The assay has a sensitivity of 11-5000 pg/ml. All samples were measured in duplicate and analysed on a SECTOR Imager 2400 from Mesoscale Discovery.

Gene expression analysis

RNA from brain and liver samples was extracted for gene analysis using a commercially available kit (Agilent Technologies, CA, USA). Qiagen RNeasy Lipid Mini Kit was used for adipose tissue (QIAGEN, Valencia, CA, USA). mRNA was reverse transcribed using High capacity cDNA reverse transcription Kit (Applied Biosystems) in a G-storm thermocycler (G-storm, Surrey , UK). Gene expression was analysed using TaqMan Gene Expression Assays and the AB7300 system (Applied Biosystems). The expression value of each gene was normalised to that of β-actin. All samples were analysed in triplicate.

Microbial community composition: pyrosequencing

For analysis of the microbial community composition, total DNA was extracted from the faecal pellets of two rats per cage using the QIAamp DNA stool mini kit according to the manufacturer's instructions (Qiagen, West Sussex, UK) coupled with an initial bead-beating step. Universal 16s rRNA primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region, i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were used for Taq-based PCR amplification. Sequencing was performed on a Roche 454 GS-FLX using Titanium chemistry by the Teagasc454SequencingPlatform. Resulting raw sequences reads were quality trimmed as previously described (Claesson et al., 2009) Trimmed FASTA sequences were then BLASTed (Altschul et al. 1997) against a previously published 16s rRNA-specific database (Urich et al. 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson et al. 2007a; Huson et al. 2007b). MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarization. A bit-score of 86 was selected as previously used for 16s ribosomal sequence data (Urich et al. 2008). Phylum and family counts for each subject were extracted from MEGAN. Clustering and alpha diversities were generated with the MOTHUR software package (Schloss et al., 2009).

Statistical analysis

Two-way repeated measures analysis of variance (ANOVA) was used to analyse body weight change, food intake and water intake and locomotion, with gender, treatment as factors. Two-way ANOVA was used for gene and cytokine analysis with gender and treatment as factors. As a possible effect gender was the central hypothesis of this study, males and females were analysed separately, regardless of whether there was an overall effect of gender. Due to the increased number of groups, further analysis was carried out using Tukey's post-hoc test in order to protect against Type I errors caused by increased comparisons. $p < 0.05$ was considered statistically significant.

3.3 Results

Body weight gain

The effect of olanzapine on body weight gain was significantly affected by gender ($F_{(1,42)}$ = 26.906, $p < 0.001$) and time ($F_{(2.85,119.56)}$= 271.878, $p < 0.001$). There was a significant interaction between gender and treatment ($F_{(2,42)}$ = 4.883 $p = 0.01$) as well as between gender and time ($F_{(2.85,119.56)}$ = 34.425, $p < 0.001$) and a gender X treatment X time interaction ($F_{(5.69,119.56)}$ = 15.101 $p < 0.001$).

The increase in the weight in the female rats was evident within the first days of treatment, with significant increases observed in animals given olanzapine (2 mg/kg) on days 5 to 15 and days 19-23 inclusive ($p < 0.05$ to $p < 0.001$). Females treated with olanzapine (4 mg/kg) displayed significant weight gain on days 3 to 15 inclusive ($p < 0.05$ to $p < 0.001$) but subsequently showed a reduction in body weight returning to normal levels such that the animals receiving olanzapine (2 mg/kg) were significantly increased compared to those receiving olanzapine (4 mg/kg) on days 21-23 inclusive ($p < 0.05$) (Fig. 3.1a). In the male rats, no difference between treatment groups was observed (Fig. 3.1b).

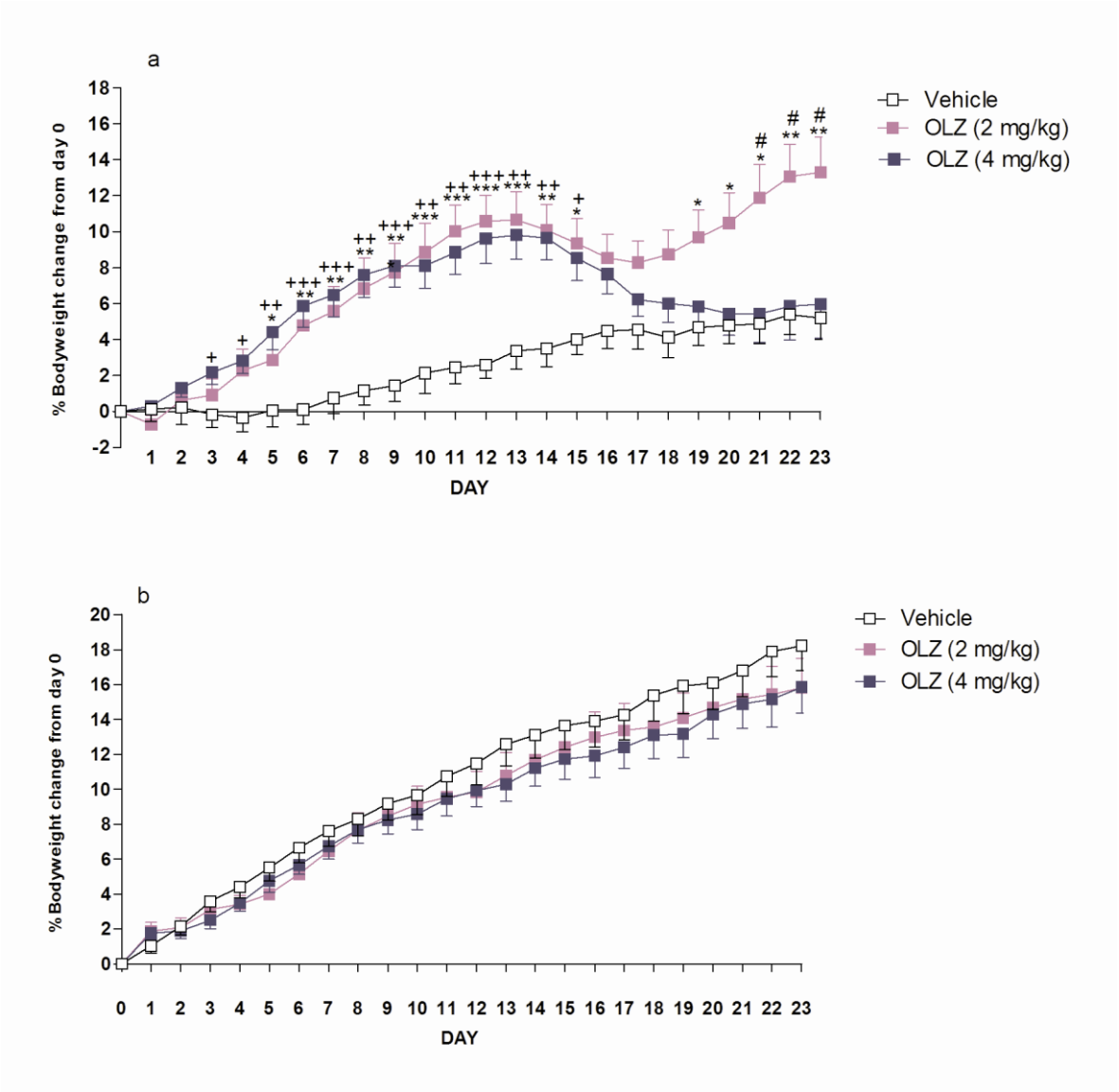


Fig. 3.1 Effect of olanzapine on body weight in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2 mg/kg and 4 mg/kg) on percentage body weight gain in (a) female rats and (b) male rats treated for 21 days B.I.D. First injection on day 1. Data shown represents mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 OLZ (2 mg/kg) significantly different versus vehicle group. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ 4 mg/kg significant versus vehicle group. $^{\#}p < 0.05$, OLZ (2 mg/kg) significant versus OLZ (4 mg/kg) group.*

Food and Water intake

Olanzapine treatment significantly affected food intake ($F_{(2,90)} = 21.212$, $p < 0.001$). There was also a significant effect of gender ($F_{(1,90)} = 530.485$, $p < 0.001$) and a significant time X gender X treatment interaction ($F_{(3.34,150.66)} = 4.28$, $p < 0.01$). Hyperphagia was observed in the female rats in both treatment groups compared to vehicle in week one (2 mg/kg $p < 0.05$, 4 mg/kg $p < 0.001$) and week 2 (2 mg/kg $p < 0.05$ 4 mg/kg $p < 0.01$). The observed increases in the rats treated with olanzapine (4mg/kg) was significantly greater than those receiving olanzapine (2 mg/kg) group in week one ($p < 0.05$). In week 3, hyperphagia persisted in the animals treated with olanzapine (2 mg/kg) ($p < 0.05$) but was significantly reduced in those receiving olanzapine (4 mg/kg) compared to vehicle treated rats ($p < 0.01$) and the olanzapine (2 mg/kg) group ($p < 0.01$). In the male rats, the olanzapine (4 mg/kg) group displayed a significant increase in food intake compared to the vehicle group in week 1 ($p < 0.05$) but not in the subsequent weeks (Fig. 3.2a).

Olanzapine treatment also induced significant increases in water intake ($F_{(2,90)} = 6.573$, $p < 0.01$) with a further significant effect of gender ($F_{(1,90)} = 51.397$, $p < 0.001$) and time ($F_{(2,180)} = 14.637$, $p < 0.001$). There was also a significant gender X treatment interaction ($F_{(2,90)} = 4.895$, $p < 0.01$). The water intake followed a similar pattern to food intake with increases seen in the female rats treated with olanzapine (2 mg/kg and 4 mg/kg) compared to vehicle treated rats in week one ($p < 0.05$, $p < 0.01$) respectively and week two ($p < 0.001$, $p < 0.05$). In week three, only the female rats receiving olanzapine (2 mg/kg) displayed significant increases versus vehicle treated animals ($p < 0.05$). The male rats did not show differences in any of the weeks (Fig. 3.2b).

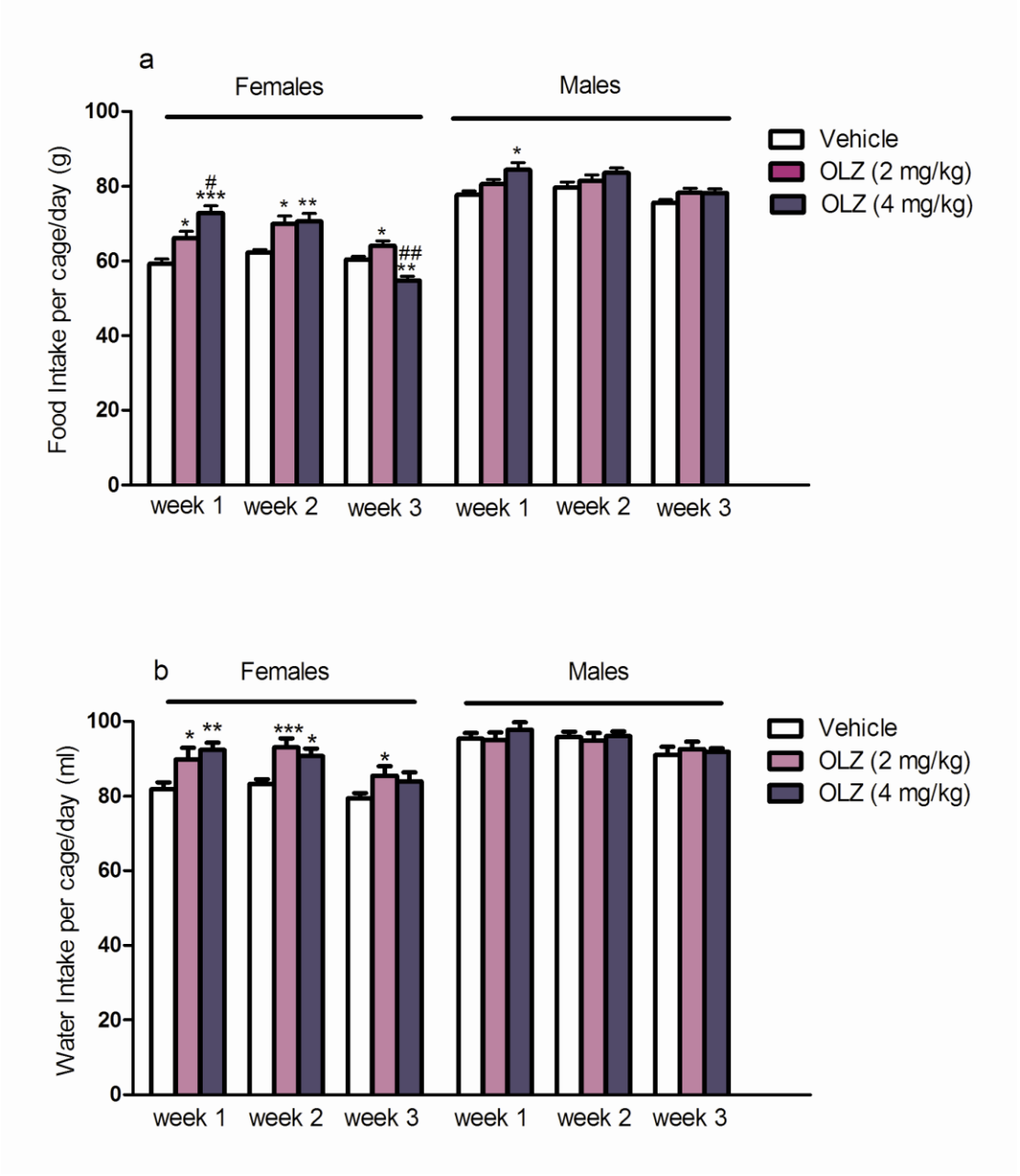


Fig. 3.2 Effect of olanzapine on food and water intake in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2mg/kg and 4 mg/kg) on (a) food intake and (b) water intake in female and male rats treated for 21 days B.I.D. Data shown represents mean ± SEM. Food and water intake shown as amount consumed per cage per day. *p < 0.05, **p < 0.01, ***p < 0.001 significantly different versus vehicle group of same gender. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus OLZ (2 mg/kg) group of same gender.*

Locomotor activity

In the locomotor activity test there was a significant effect of time ($F_{(3.45,144.68)} = 221.217$, $p < 0.001$) and of treatment ($F_{(2,42)} = 7.264$, $p < 0.01$). Post-hoc analysis showed there was a reduction in locomotion in female rats treated with olanzapine (2 mg/kg) between both 15 and 20 minutes ($p < 0.001$) and between 20 and 25 minutes ($p < 0.05$). The female rats treated with olanzapine (4 mg/kg) only displayed decreased activity between 15 and 20 minutes ($p < 0.05$).

The male rats treated with olanzapine (2 mg/kg and 4 mg/kg) exhibited reduced locomotor activity between the 5 minute and ten minute time points ($p < 0.05$), ($p < 0.01$) respectively. The male rats treated with olanzapine (2 mg/kg) further showed reduced movement between 15 and 20 minutes and 20 and 25 minutes ($p < 0.05$) (Fig. 3.3).

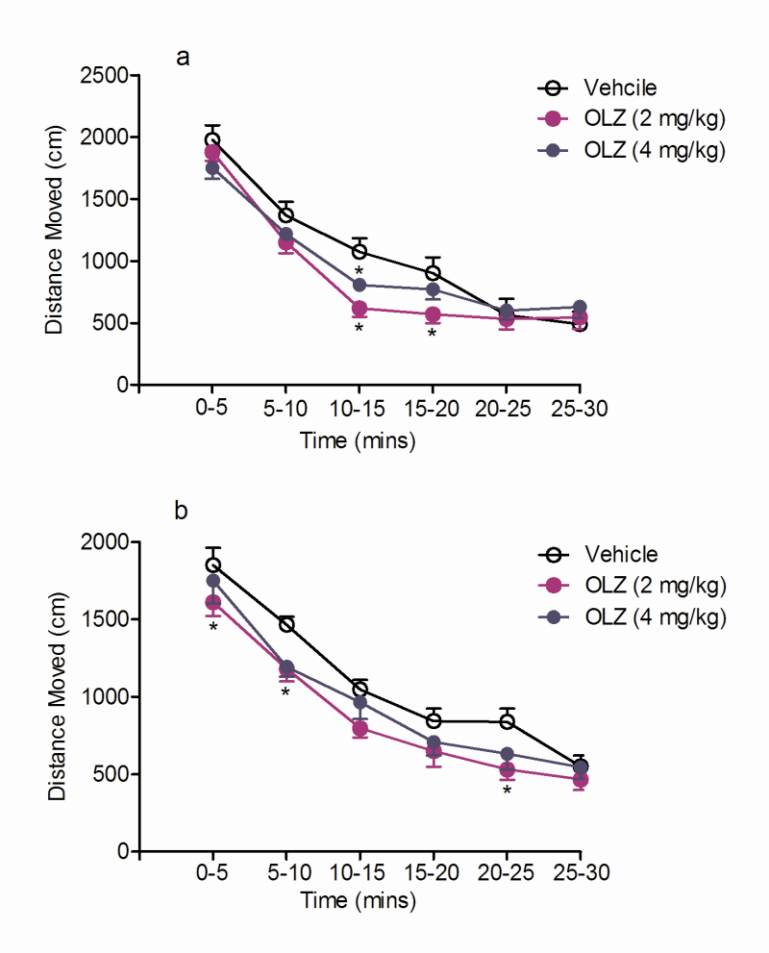


Fig. 3.3 Effect of olanzapine on locomotion in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2 mg/kg and 4 mg/kg) on locomotor activity in (a) female and (b) male rats treated for 21 days B.I.D. Locomotion measured as distance moved. Data shown represent mean ± SEM. *p < 0.05 versus vehicle treated rats.*

Adipose Tissue

Olanzapine treatment significantly increased visceral fat mass ($F_{(2,42)}$ = 16.042, p < 0.001) and there was a significant gender X treatment interaction ($F_{(2,42)}$ = 6.147 p < 0.01). Female rats treated with olanzapine (2 mg/kg and 4 mg/kg) had significantly increased visceral fat mass compared to vehicle treated animals (p < 0.01, p < 0.05) respectively.

The male rats receiving olanzapine (4 mg/kg) group showed significant increases in visceral fat compared to both the male vehicle treated group ($p < 0.001$) and the male 2 mg/kg treated group ($p < 0.05$) (Fig. 3.4).

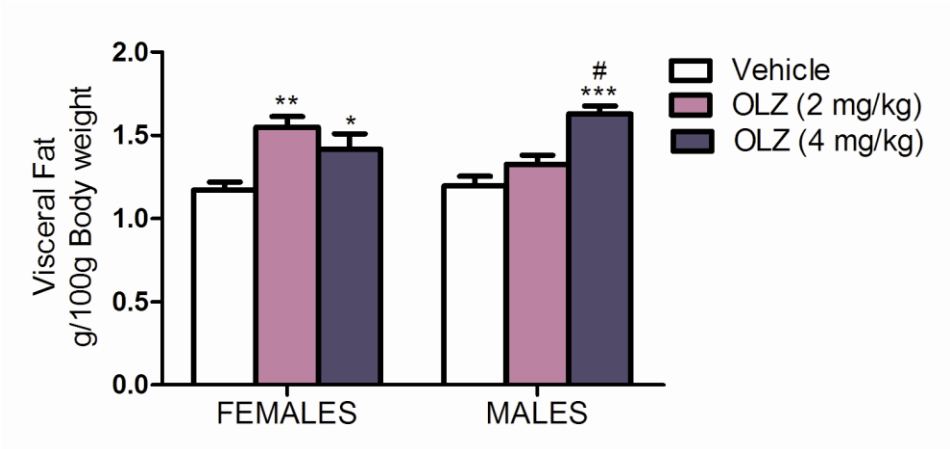


Fig.3.4 Effect of olanzapine on visceral fat deposition in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2mg/kg and 4 mg/kg) on proportion of visceral fat (gonadal + mesenteric fat deposits) in female and male rats treated for 21 days B.I.D. Data shown represents mean ± SEM. **p < 0.01, ***p < 0.001 significantly different versus vehicle group of same gender. #p < 0.05 significantly different versus OLZ (2 mg/kg) group of same gender.*

Olanzapine treatment also had a significant effect on CD68 expression ($F_{(2,38)} = 8.825$, $p < 0.001$) with an effect of gender ($F_{(1,38)} = 16.046$ $p < 0.001$). Female rats treated with olanzapine (4 mg/kg) displayed significantly increased levels of CD68 mRNA compared to vehicle treated rats ($p < 0.05$). In the male rats, olanzapine treatment (2 mg/kg and 4 mg/kg) resulted in increased levels of CD68 mRNA expression compared to vehicle treated animals ($p < 0.05$) (Fig. 3.5a).

Interleukin (IL)-6 mRNA expression was significantly affected by gender ($F_{(1,37)} = 26.511$ $p < 0.001$) and there was a significant gender X treatment interaction ($F_{(2,37)} = 3.886$, $p < 0.05$).

The female animals receiving olanzapine (4 mg/kg) had increased levels compared to the animals receiving olanzapine (2 mg/kg) ($p < 0.05$). The male rats treated with olanzapine did not show significant increases, though those treated with olanzapine (2 mg/kg) had a 4 fold increase compared to vehicle treated rats.

The male vehicle treated rats had significantly lower expression compared to female vehicle treated animals ($p < 0.05$) (Fig. 3.5b).

Sterol-regulatory element binding protein 1c (SREBP-1c) expression was significantly affected by olanzapine treatment ($F_{(2,34)} = 4.90$, $p < 0.05$). There was also a significant effect of gender ($F_{(1,34)} = 39.189$, $p < 0.001$), and a significant gender, treatment interaction ($F_{(2,34)} = 3.481$, $p < 0.05$). In female rats, those treated with olanzapine (4 mg/kg) had a significant reduction in the mRNA expression of SREBP-1c compared to both the vehicle ($p < 0.05$) and olanzapine (4 mg/kg) groups ($p < 0.01$). This reduction was not seen in the male rats, though the male vehicle group had significantly lower levels than female vehicle treated animals ($p < 0.05$) (Fig. 3.5c).

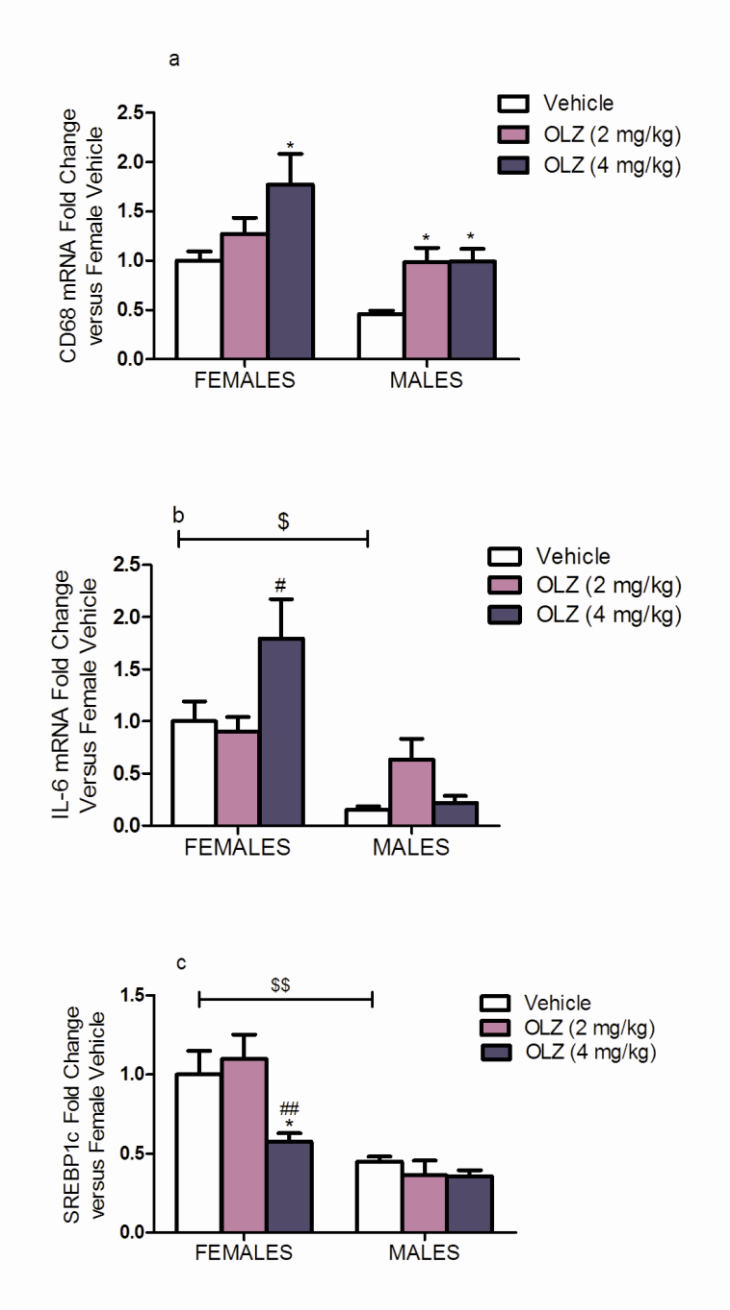


Fig. 3.5 Effect of olanzapine on gene expression in adipose tissue in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2 mg/kg and 4 mg/kg) on (a) CD68 mRNA expression (b) IL-6 mRNA expression and (c) SREBP-1c mRNA expression in female and male rats. Data shown represents mean ± SEM. *p < 0.05 significantly different versus vehicle group of same gender. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significantly different versus OLZ (2 mg/kg) group of same gender. $^{\$}p < 0.05$, $^{\$}p < 0.01$ female vehicle group versus male vehicle group.*

Liver

Liver weight as a percentage of body weight was significantly affected by olanzapine treatment ($F_{(2,42)}$ = 4.512, p< 0.05) and gender ($F_{(1,42)}$ = 26.466, p < 0.001). The female rats treated with olanzapine (2 mg/kg) were found to have significantly increased liver weight compared to the vehicle treated rats (p < 0.05). No differences were observed in the male rats (Fig. 3.6).

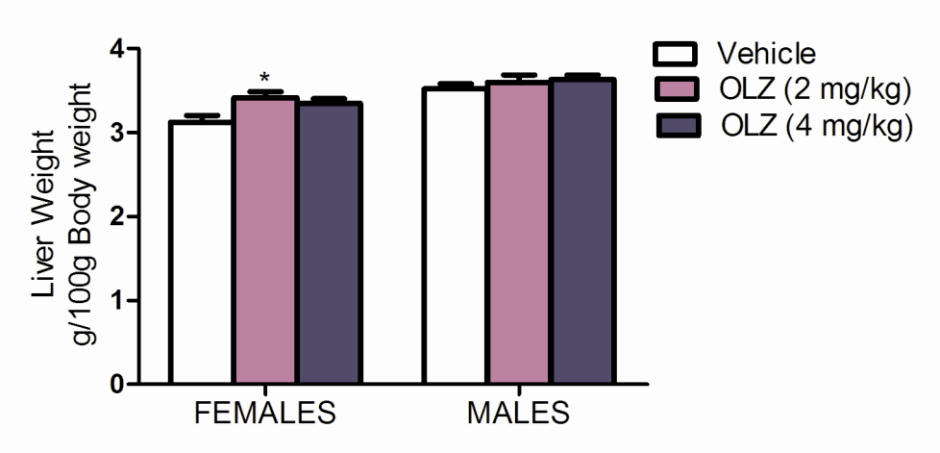


Fig. 3.6 Effect of olanzapine on liver weight in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2 mg/kg and 4 mg/kg) on relative liver weight in female and male rats treated for 21 days B.I.D. Data shown represents mean ± SEM. *p < 0.05 significantly different versus vehicle group of same gender.*

SREBP-1c mRNA expression in the liver was not affected by treatment but was significantly affected by gender ($F_{(1,42)}$ = 143.439 p < 0.001). The male and female vehicle treated groups differed significantly from one another (p < 0.001) (Table 3.1).

Carbohydrate regulatory element binding protein (ChREBP) mRNA expression was significantly affected by gender ($F_{(1,46)}$ = 50.085 p< 0.001) and male vehicle treated rats were significantly different from female vehicle treated animals (Table3.1).

TNF mRNA in the liver showed no significant differences following olanzapine treatment (Table 3.1).

Gender	**Female**			**Male**		
Olanzapine	*Vehicle*	*2 mg/kg*	*4 mg/kg*	*Vehicle*	*2 mg/kg*	*4 mg/kg*
SREBP-1c	1.0±0.16	0.73±0.07	0.70±0.08	3.26±0.16*	3.57±0.33	3.42±0.44
Chrebp	1.0±0.16	1.09±0.17	0.99±0.17	2.40±0.21*	2.67±0.26	2.70±0.47
TNF	1.0±0.09	0.89±0.14	0.90±0.08	1.82±0.42	0.89±0.20	0.86±0.11

Table 3.1 Effect of chronic olanzapine on hepatic gene expression in Sprague-Dawley rast. *Relative gene expression of SREBP-1c, ChREBP and TNF in liver of olanzapine and vehicle treated rats. Values normalised to female vehicle group. *p < 0.01 versus female vehicle group SREBP= sterol regulating element binding protein,ChREBP= carbohydrate regulating element binding protein, TNF= tumor necrosis factor*

Plasma cytokines and leptin

Olanzapine treatment had a significant effect on the circulating plasma levels of IL-8 ($F_{(2,41)}$ = 3.613 p < 0.05). The female rats treated with olanzapine (2 mg/kg) had increased levels compared to vehicle treated rats (p < 0.01) (Fig. 3.7a).

Plasma levels of TNF-α were significantly affected by gender ($F_{(1,41)}$ = 102.024 p < 0.001) and there was a significant gender X treatment interaction following olanzapine treatment ($F_{(2,41)}$ = 7.049, p < 0.01). Male rats treated with olanzapine (4 mg/kg) showed a reduction in circulating levels of TNF-α (p < 0.05) (Fig. 3.7b).

Plasma IL-6 levels were significantly affected by treatment ($F_{(1,41)}$ = 4.005, p < 0.05), and gender ($F_{(1,41)}$ = 7.473, p < 0.01). There was a significant gender X treatment interaction ($F_{(2,41)}$ = 5.86, p < 0.01).

Male animals treated with olanzapine (2 mg/kg and 4 mg/kg) had lower levels of IL-6 compared to the male vehicle treated rats ($p < 0.05$, $p < 0.01$) respectively. The vehicle treated male animals had significantly higher levels than female vehicle treated rats ($p < 0.01$) (Fig. 3.7c).

IL-1β plasma levels were significantly affected by gender ($F_{(1,41)} = 5.575$, $p < 0.05$) and there was a significant gender X treatment interaction ($F_{(2,41)} = 4.93$, $p < 0.05$). In the female rats, those receiving olanzapine (2 mg/kg) had significantly elevated levels compared to vehicle treated rats ($p < 0.05$). The male vehicle treated rats had significantly higher levels than the female vehicle treated rats ($p < 0.01$) (Fig. 3.7d).

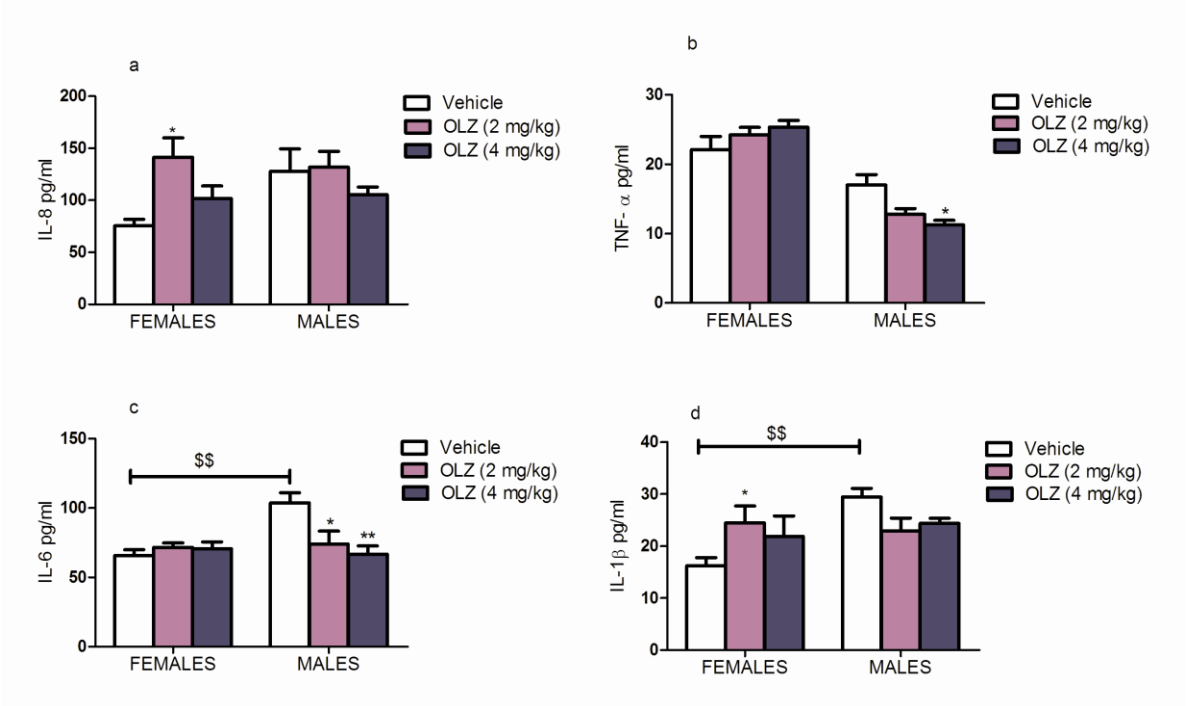


Fig. 3.7 Effect of chronic olanzapine on plasma cytokines in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2 mg/kg and 4 mg/kg) on plasma levels of (a) IL-8 (b) TNF-α (c) IL-6 and (d) IL-1β in female and male rats treated for 21 days B.I.D. Data shown represents mean ± SEM. *p < 0.05, **p < 0.01 significantly different versus vehicle group of same gender. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, significant difference between female vehicle group and male vehicle group.*

Plasma leptin levels showed a significant effect of gender ($F_{(1,42)} = 12.636$, $p < 0.001$). The male vehicle treated animals had higher levels than female vehicle treated rats ($p < 0.05$) (Fig. 3.8).

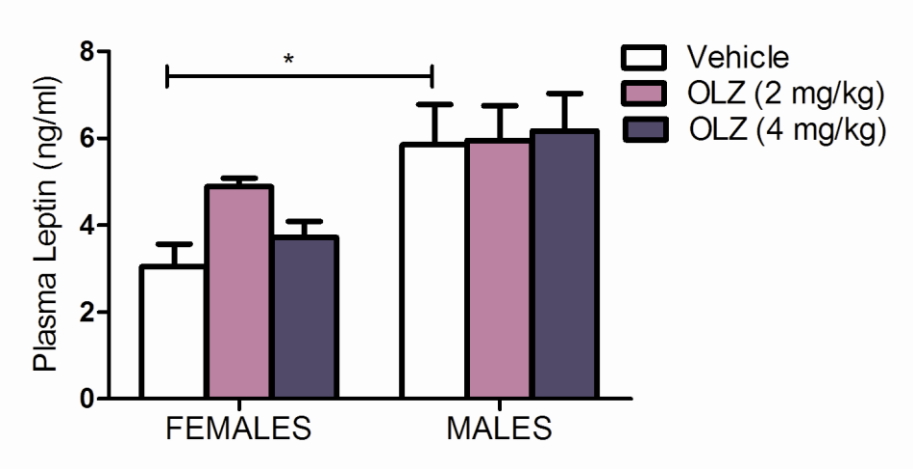


Fig.3.8 Effect of chronic olanzapine on plasma leptin in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2mg/kg and 4 mg/kg) on plasma levels of leptin in female and male rats treated for 21 days B.I.D. Data shown represents mean ± SEM. *p < 0.05 female vehicle group versus male vehicle group.*

Peripheral and central ghrelin

Olanzapine significantly affected plasma levels of total ghrelin ($F_{(2,42)} = 3.143$, $p = 0.05$). There was also a significant effect of gender ($F_{(1,42)} = 9.109$, $p < 0.01$). The female rats treated with olanzapine (2 mg/kg) had reduced circulating levels of ghrelin ($p < 0.05$) and those receiving olanzapine (4 mg/kg) also displayed a trend for reduced levels ($p = 0.068$). No significant effects were observed between male groups (Fig. 3.9).

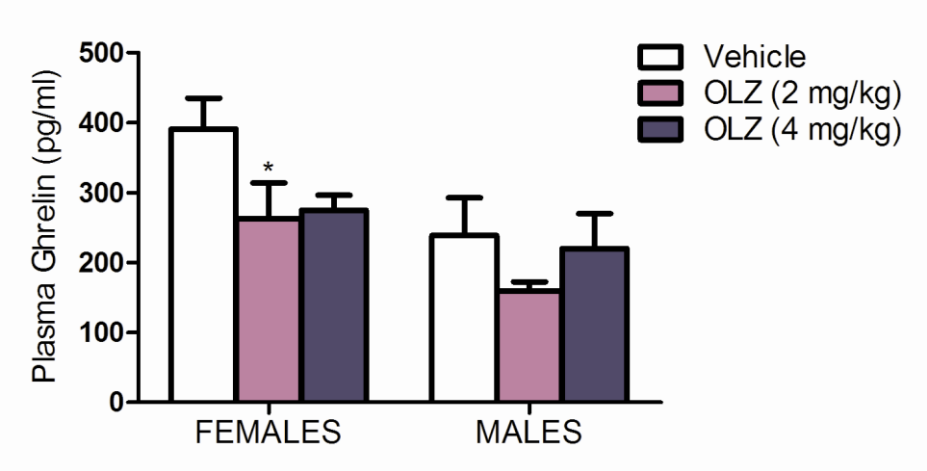


Fig. 3.9 Effect of chronic olanzapine on plasma ghrelin in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2mg/kg and 4 mg/kg) on plasma levels of total ghrelin in female and male rats treated for 21 days B.I.D. Data shown represents mean ± SEM. *p < 0.05 significantly different versus vehicle group of same gender.*

Hypothalamic expression of the Ghrelin 1a receptor mRNA was significantly affected by gender ($F_{(1,35)}$ = 13.68, p < 0.01) and there was a significant gender X treatment interaction ($F_{(2,35)}$ = 6.973, p<0.01). The male rats treated with olanzapine (4 mg/kg) had significantly higher levels than the vehicle treated rats (p < 0.05) (Fig. 3.10).

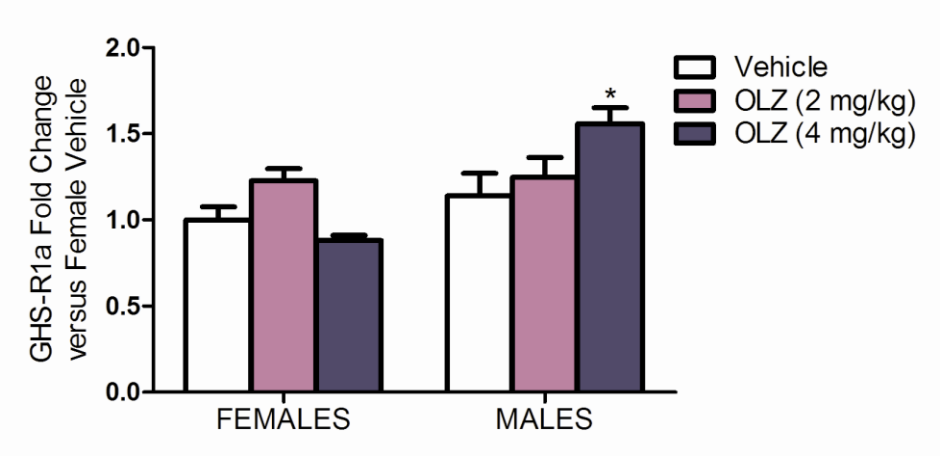


Fig. 3.10 Effect of chronic olanzapine on hypothalamic ghrelin receptor expression in Sprague-Dawley rats. *Effect of olanzapine (OLZ) (2mg/kg and 4 mg/kg) on growth hormone secretagogue 1a receptor mRNA expression in the hypothalamus in female and male rats treated for 21 days. Data shown represents mean ± SEM. *p < 0.05 significantly different versus vehicle group of same gender.*

Gut Microbiota

The effects of chronic olanzapine on the microbial composition of the gut microbiota of the rats was elucidated through high throughput pyrosequencing (Roche-454 Titanium) of 16S rRNA (V4) amplicons generated from faecal DNA obtained at study termination. Species richness, coverage, and diversity estimations were calculated for each data set. At the 97% similarity level, the Shannon index, a metric for community diversity, revealed a high level of overall biodiversity within all samples with values exceeding 4.2. The Good's coverage at the 97% similarity level ranged between 84-98% for all datasets. The Chao1 richness values indicate good sample richness throughout.

Assessment of the faecal microbiota, in terms of microbial phyla, revealed that olanzapine treatment in the female rats seemed to be associated with increased levels of *Firmicutes* following olanzapine 2 mg/kg (72.11 % versus 84.06 %) and olanzapine 4 mg/kg (72.11% versus 88.12%), increases of 11.95 % and15.99 % respectively. Olanzapine treatment of 2 mg/kg and 4 mg/kg also appeared to reduce diversity compared to vehicle treated rats evidenced by reductions in the less represented phyla *Actinobacteria* (3.72 % versus 0.34 and 0.15 %) respectively and *Proteobacteria* (1.60 % versus 0.15 and 0.77 %) respectively. Animals treated with olanzapine 4 mg/kg also displayed evidence of reduced *Bacteriodetes* (17.57 % versus 10.88 %) (Fig. 3.11a).

In the male rats, olanzapine treatment (2 mg/kg) appeared to impact the microbiota minimally with an apparent reduction in *Proteobacteria* (3.15 % versus 0.94 %). Olanzapine treatment of 4 mg/kg however seemed to cause an increase in *Firmicutes* (82.66 % versus 91.63 %) and a reduction on *Bacteriodetes* of a similar magnitude (14.08 % versus 7.97 %) (Fig. 3.11b).

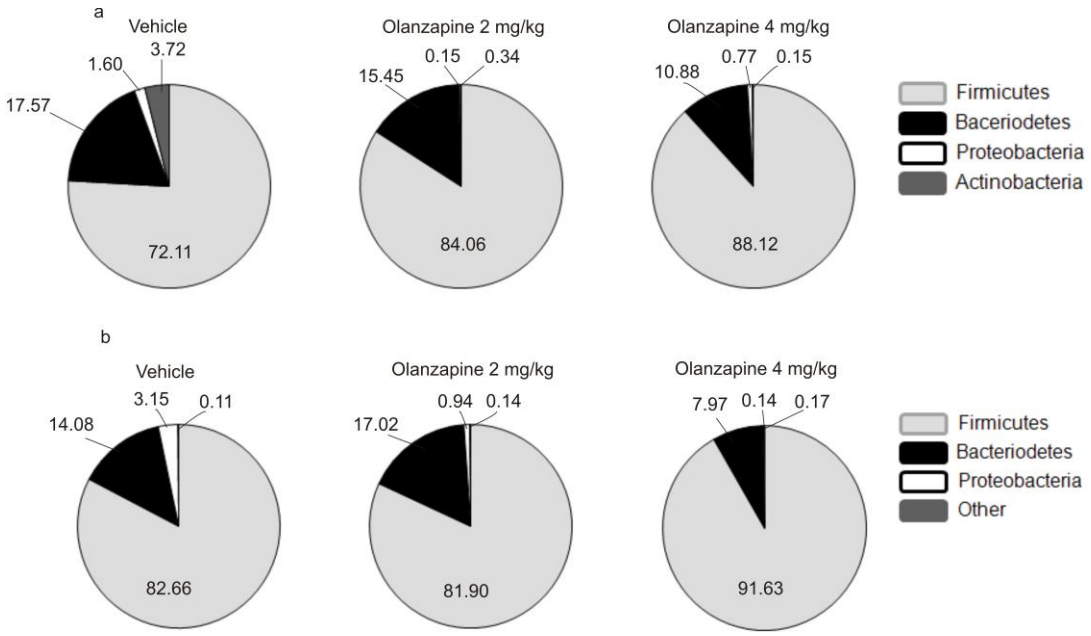


Fig. 3.11 Effect of chronic olanzapine on faecal microbiota composition of Sprague-Dawley rats. *Proportional composition of the faecal microbiota following 21 days of olanzapine treatment (2 mg/kg or 4 mg/kg) in (a) Females and (b) Males. Data represents the cumulative DNA of one pellet per cage for each group.*

Correlation Analysis

In order to assess the possible relationship between the main physical alterations induced by olanzapine treatment in our model, and possible biochemical correlates, we carried out correlation analysis on body weight gain and a number of biochemical plasma markers. For the female rats, a significant correlation was found between body weight gain and plasma leptin, (Pearson correlation co-efficient = 0.457, r^2 =0 .205, $p < 0.05$) (Fig. 3.12a). A significant correlation was also found for body weight gain and plasma ghrelin (Pearson correlation co-efficient = -0.429, r^2 = 0.185, $p < 0.05$). (Fig. 3.12b). A significant correlation was also observed for body weight gain and plasma IL-8 levels (Pearson correlation co-efficient = 0.702, r^2 = 0.493, $p < 0.001$) (Fig. 3.12c).

Furthermore, a significant correlation was found between visceral fat mass and plasma IL-8 (Pearson correlation co-efficient = 0.550, $r^2 = 0.303$, $p < 0.01$) (Fig. 3. 12d).

In the male rats, no significant correlation was observed between any of the measured physical and biochemical parameters.

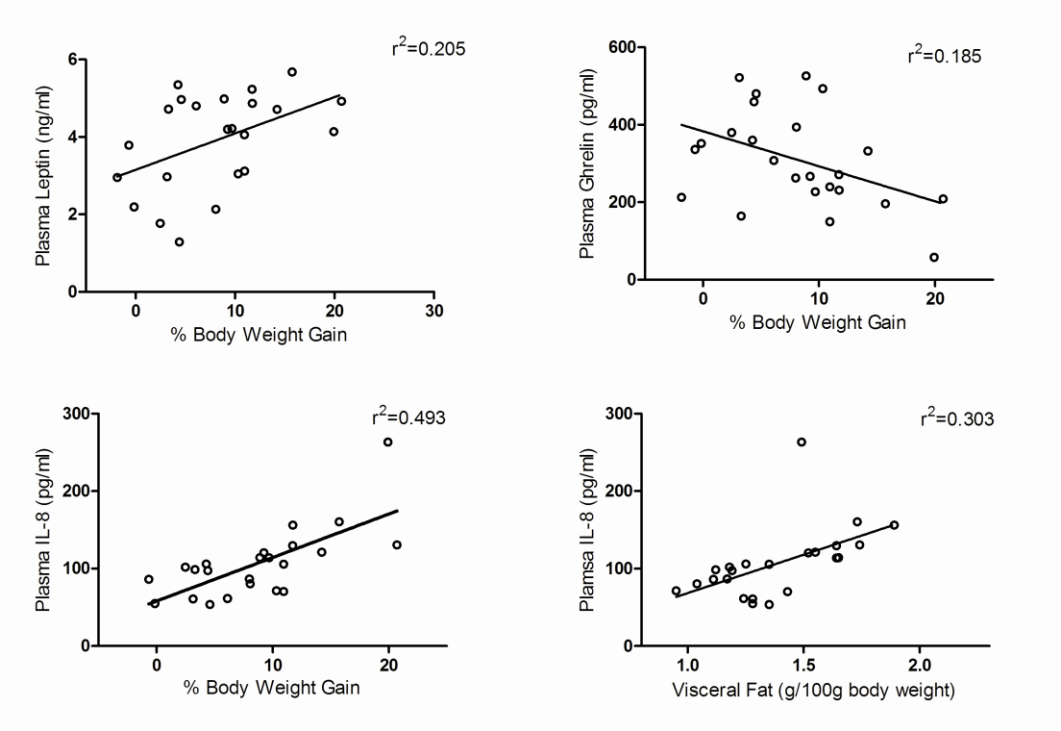


Fig.12 Correlation analysis. *Correlation between percent body weight gain at day 23 and (a) plasma leptin, (b) plasma ghrelin (c) plasma IL-8. (d) Represents correlation analysis between visceral fat and plasma IL-8.*

3.4 Discussion

Here we show that olanzapine had significant effects on a number of physiological, inflammatory and microbial parameters in the rat and that many, but not all of these were more pronounced in females compared to males. Olanzapine induced rapid weight gain in female rats and not in male rats which is consistent with previous reports (Albaugh et al. 2006; Choi et al. 2007). Both male and female rats treated with olanzapine did however exhibit increased visceral fat, though in the males this was the case only at the higher dose. We also show, to our knowledge for the first time, specific alterations to the gut microbiota as a result of antipsychotic treatment, suggesting that microbiota may contribute to AAPD-induced metabolic dysfunction.

The reason for the gender difference in body weight-gain at a preclinical level is currently unknown and its significance to the clinical presentation of AAPD-induced metabolic alterations is contentious. One reason for this being that clozapine , an antipsychotic that also causes considerable weight gain in humans, does not appear to do so in rats (Cooper et al. 2008b). There is considerable evidence however to suggest that females are more liable to incur antipsychotic induced weight gain (Hakko et al. 2006; Aichhorn et al. 2007; Haack et al. 2009) although this may reflect gender differences in drug pharmacokinetics (Harris et al. 1995; Beierle et al. 1999). In the present study we observed a number of gender differences in baseline levels of the plasma cytokines IL-1β and IL-6 as well as local levels of IL-6 in the adipose tissue which may impact on susceptibility to the effects of AAPDs. Gender dimorphism in immune function including cytokine release is well documented and our findings suggest these may have implications for antipsychotic side-effects (Cannon and Pierre 1997; Bao et al. 2002; Yokoyama et al. 2005).

The complex nature of body weight regulation may explain why we did not observe a dose-response relationship in weight-gain with olanzapine treatment. This is supported by clinical findings in which lower doses are not necessarily associated with lower weight gain (Citrome et al. 2009a). The mechanisms by which olanzapine causes weight gain as in the female rats in this study are unclear, but are largely attributed to its diverse pharmacological receptor profile (Roth et al. 2003; Matsui-Sakata et al. 2005; Newcomer 2005; Silvestre and Prous 2005; Reynolds et al. 2006). Antagonism of central receptors including serotonin 5-HT_{2C} and histamine H_1 receptors, which play pivotal roles in appetite regulation as well as long-term energy balance (Tsuda et al. 2002; Masaki et al. 2004; Lam et al. 2008) have been particularly implicated in weight gain associated with antipsychotic treatment (Reynolds et al. 2002; Kroeze et al. 2003; Reynolds et al. 2006; Deng et al. 2010). Hyperphagia was observed in the female rats and is believed to drive initial weight gain (Thornton-Jones et al. 2002). This is also seen in clinical studies in which increased appetite is commonly reported by patients initiating olanzapine therapy (Basson et al. 2001b; Kluge et al. 2007; Treuer et al. 2009) and in non psychotic controls (Fountaine et al. 2010).

Male and female animals treated with olanzapine displayed a significant accretion of visceral fat. Interestingly, the female rats treated with olanzapine (4 mg/kg) returned to control body weight but still had increased visceral fat. Furthermore the male rats treated with olanzapine (4 mg/kg) did not show increases in body weight gain but did however show increased adiposity.

This finding supports clinical and pre-clinical studies which found increased adiposity following olanzapine treatment with (Raskind et al. 2007; Ader et al. 2008; Victoriano et al. 2009) and without weight gain (Victoriano et al. 2009). Increased visceral mass is considered a key factor in the development of the metabolic syndrome and in particular the development of insulin resistance (Bjorntorp 1991; Demerath et al. 2008). Thus, these data emphasise that the metabolic threat posed by olanzapine goes beyond merely increases in body weight gain.

Gonadal adipose tissue of female rats treated with olanzapine (4 mg/kg) and male rats treated with olanzapine (2 mg/kg and 4 mg/kg) displayed increased CD68 expression. CD68 is a glycoprotein which represents a marker of macrophage presence. Macrophage infiltration of adipose tissue is considered a key step in the development of obesity-related inflammation and subsequent insulin resistance (Xu et al. 2003). Interestingly, CD68 expression did not mirror weight gain, even in the females. Thus, this suggests that olanzapine can predispose toward a pro-inflammatory state independent of effects on bodyweight *per se*. This may have important connotations for patient monitoring following the prescription of AAPDs.

The adipose tissue of female rats treated with olanzapine (4 mg/kg) also displayed inflammation with increased IL-6 gene expression. Though not significant, the male rats receiving olanzapine (2 mg/kg) also displayed the same trend. Like CD68, IL-6 did not follow the pattern of weight gain.

However, sorted cell gene analysis of adipose tissue has previously suggested that macrophages and adipocytes secrete roughly equal amounts of IL-6, thus macrophage infiltration indicated by elevated CD68 expression likely led to elevated IL-6 expression (Wisse 2004). *In vitro* data suggests that IL-6 can directly confer insulin resistance (Rotter et al. 2003) and levels are associated with increased risk of Type II diabetes (Pradhan et al. 2001).

This further suggests therefore that olanzapine can confer risk of such metabolic abnormalities without overt weight gain and that IL-6 may be one mediator of this disguised threat.

The female rats further displayed a pro-inflammatory phenotype with IL-8 and IL-1β being significantly elevated in plasma in the olanzapine (2 mg/kg) group. Increased circulating levels of each of these cytokines has been associated with obesity and implicated in insulin resistance (Straczkowski et al. 2002; Kim et al. 2006). Conversely, male rats treated with olanzapine displayed an anti-inflammatory phenotype with reductions in IL-6 and TNF-α observed. While this is initially surprising, anti-inflammatory effects of antipsychotics have been recognised for some time (Chedid 1954). Olanzapine has been shown to suppress TNF-α and Il-6 production in mice treated with lipopolysaccharide (Sugino et al. 2009). This discrepancy in circulating cytokines may reflect differences in their primary source of the cytokines (Trayhurn and Wood 2004) which could potentially account for the lack of an observed increase in the males and higher dose females.

Together, these findings imply systemic inflammation associated with olanzapine occurs primarily as a result of body weight gain.

Intriguingly, the plasma levels of IL-8 in the female rats showed a significant correlation with body weight gain and visceral fat mass implicating this cytokine in particular as a possible link between inflammation and body weight gain and vice-versa and may potentially be a biomarker for recognising the induction of AAPD metabolic side effects.

This systemic inflammation may also act to impair metabolism leading to insulin resistance and increased risk of metabolic syndrome and diabetes as a secondary effect. This emphasises the double–edged risk olanzapine confers on metabolic function with weight gain inducing systemic inflammation and the direct actions of the drug impacting on local inflammatory responses both of which can converge to induce insulin resistance.

Further disruption to normal metabolic functioning was evidenced by reductions in sterol-regulatory binding protein-1c (SREPB-1c) gene expression in the adipose tissue of female rats treated with 4 mg/kg of olanzapine. SREBP-1c is a key regulatory transcription factor which controls a number of genes involved lipid metabolism (Ferre and Foufelle 2007). Reduced expression of SREBP-1c in adipose tissue has been observed in obese patients, and subsequent weight loss was associated with increased expression (Kolehmainen et al. 2001). These reductions are likely to be secondary to insulin resistance, as insulin is the major regulator of SREBP expression. However, antipsychotics have been shown to activate SREBP in vitro (Ferno et al. 2006; Raeder et al. 2006; Yang et al. 2007) and in a recent in vivo study of risperidone(Lauressergues et al. 2010).

A recent study also demonstrated down regulation of SREBP-1c following an initial up-regulation after acute olanzapine treatment (Jassim et al. 2011).

A study of clozapine administration was also associated with acute increases in SREBP and associated genes followed by a sustained down regulation (Ferno et al. 2009). Thus the long term affects of antipsychotics on SREBP system are not yet clear but seem to involve feed back mechanisms and this finding further supports the theory that olanzapine can directly affect lipid handling in the adipose tissue and thus directly contribute to fat deposition and dyslipidemia independent of weight gain (Ferno et al. 2011).

Ghrelin is an orexigenic hormone released from the stomach and is known as the hunger hormone as it is involved in meal initiation (Cummings et al. 2001b; Schellekens et al. 2010). We observed reductions in plasma levels of total ghrelin in the female olanzapine treated rats. The effect of antipsychotics on ghrelin has not been extensively studied, though increased levels with prolonged treatment has been found in human patients (Murashita et al. 2005; Sentissi et al. 2008). In our studies negative feedback may have occurred as a result of hyperphagia driven centrally. Intriguingly, in humans, higher basal plasma levels are associated with females (Greenman et al. 2004).

Furthermore, ghrelin levels were found to be inversely correlated with fat mass and body mass index in females but not males in humans (Makovey et al. 2007). It must be remembered that ghrelin in vivo exists as acetylated and non-acetylated forms and only the acetylated form can cross the blood-brain barrier and activate central ghrelin receptors. Also, ghrelin displays a circadian rhythm such that the time of day the animals were sacrificed (morning) may have affected ghrelin levels. Thus total plasma ghrelin levels must be interpreted carefully.

Hypothalamic ghrelin 1a receptor mRNA was increased in the male olanzapine (4 mg/kg) treated animals. Central actions of ghrelin are associated with fat deposition (Riley et al. 2005). Thus, these results imply alterations to the ghrelin system may be one mechanism by which olanzapine increases visceral fat and potentially also appetite and that these effects may be gender sensitive. Moreover, a significant inverse correlation was found between plasma ghrelin and body weight gain.

Leptin is potent anorexigenic hormone with opposing effects to those of ghrelin. Though not significantly elevated in the treatment groups, a significant correlation was found between body weight gain and plasma leptin. While changes in circulating levels of these hormones likely represent secondary rather than direct actions of olanzapine (Baptista and Beaulieu 2002), they may potentially act as important markers for those at risk for sustained weight gain following commencement of antipsychotic therapy and are important considerations in the assessment of antipsychotics metabolic impact (Sentissi et al. 2008).

The composition of the gut microbiota appeared to be considerably altered following treatment with olanzapine in the female rats and also in the male rats receiving olanzapine (4 mg/kg). In the female and male rats treated with olanzapine (4 mg/kg) the pooled samples at day 22 show a trend for increased *Firmicutes* and reduced *Bacteriodetes* compared to control animals. There was also evidence of reduced diversity at the phylum level in these olanzapine treated groups with reduced levels of *Proteobacteria* in both females and males and reduced *Actinobacteria* in the females.

The gut microbiota contributes to metabolism firstly by utilizing indigestible complex polysaccharides via fermentation for their own energy and thereby producing short-chain fatty acids (SCFA) which can then be digested and used by the host for energy (Hooper et al. 2002). The microbiota is also involved in cholesterol reduction and the biosynthesis of vitamins that can be used by the host. It is estimated that as much as 10% of our daily energy supply may be provided in this way (Flint et al. 2008).

Furthermore, in their seminal work, Gordon and colleagues showed that germ-free mice (mice devoid of any microbiota) had 40% less body fat than their conventional littermates. Furthermore, colonisation of the germ-free mice with the microbiota of lean control mice led to a significant increase in body fat while colonisation with the microbiota of genetically obese mice (*ob/ob*) led to an even greater level of weight gain (Backhed et al. 2004; Turnbaugh et al. 2006). Furthermore, germ-free mice are resistant to diet-induced obesity (Backhed et al. 2007). This series of experiments also revealed that shifts in the predominant phyla of the microbiota were associated with obesity. An increase in the relative abundance of *Firmicutes* with a concordant decrease in *Bacteriodetes* was observed (Ley et al. 2005; Turnbaugh et al. 2008). This shift was also found in a human study of obese versus lean twins and in a study of Type II diabetic patients versus non-diabetics, independent of body weight (Turnbaugh et al. 2009; Larsen et al. 2010).

Thus, our findings, while preliminary, are extremely interesting as they are closely in line with the above and other recent studies investigating the role of the microbiota in obesity and energy regulation (Cani et al. 2007b; Kalliomaki et al. 2008).

However, whether possible alterations to the gut microbiota are a direct result of olanzapine treatment or secondary to other effects is unclear. It is however, tempting to speculate that olanzapine may have influenced the gut microbiota via as yet unknown mechanisms and these changes could well contribute to, or exacerbate metabolic dysfunction induced by AAPDs, in particular fat accumulation. If this is the case modulation of the gut flora by antibiotic, prebiotic or indeed probiotic therapy may represent a useful adjunctive therapy for olanzapine-induced weight gain in the future.

In this study systemic inflammation occurred in a gender dependent fashion and was only observed in female rats, and as such likely occurred secondary to weight gain which was also only seen in the females. Importantly however, both the female and male rats did incur a number of physiologically relevant changes including increased adipose tissue, local inflammation and alterations to the gut microbiota.

Thus, this study brings into focus the need to consider the side effects of antipsychotics as a double threat involving not only weight gain, but also independent metabolic effects which may include modulation of the gut microbiota. Furthermore, appreciating differences between the sexes may have important clinical implications in not only the prescribing but also the monitoring of patients in the future (Seeman 2004).

Chapter 4

Antipsychotics and the Gut Microbiome: Olanzapine-Induced Metabolic Dysfunction is Attenuated by Antibiotic Administration in the Rat

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Abstract

Background: Atypical antipsychotics, such as olanzapine, are associated with weight gain and serious metabolic side effects, the mechanisms of which are poorly understood. The gut microbiota has been recognised in recent times as an important factor in energy regulation and metabolism. Thus, we investigated if ablation of the gut microbiota could prevent or ameliorate any of the metabolic side effects associated with chronic olanzapine treatment in female Sprague-Dawley rats.

Methods: Animals were assigned to treatment with vehicle, olanzapine (2 mg/kg/day), or olanzapine (4 mg/kg/day) for 21 days, I.P, twice daily. In addition, animals received either vehicle or an antibiotic cocktail consisting of neomycin (250 mg/kg/day), metronidazole (50 mg/kg/day) and polymyxin B (9 mg/kg/day) by oral gavage, daily, beginning 5 days prior to drug treatment. Body weight and food intake were measured daily. At the end of the study, uterine fat deposits were dissected and weighed and samples collected.

Results: As expected, treatment with the antibiotic cocktail resulted in a definitively different overall microbiota profile. The antibiotic treatment also significantly ameliorated the body weight gain and uterine fat deposition induced by olanzapine. Olanzapine-induced alterations in the expression of the liver lipogenic gene, fatty acid synthase as well circulating levels of free fatty acids were normalised by the antibiotics. Moreover antibiotic co-administration resulted in a reduced inflammatory phenotype.

Conclusions: These results suggest that the gut microbiome is an important factor in certain antipsychotic-induced metabolic side effects, and could be a novel therapeutic target for preventing antipsychotic-induced metabolic disease.

4.1 Introduction

Olanzapine and other atypical antipsychotics offer many advantages over older antipsychotics both in terms of efficacy and reduced propensity for extrapyramidal symptoms (EPS) (Jaffe and Levine 2003). However, olanzapine is associated with serious metabolic side effects, including weight gain and increased visceral fat (Newcomer 2004; Albaugh et al. 2010), which can lead to co-morbidities such as Type II diabetes mellitus and cardiovascular disease (Farwell et al. 2004; Correll et al. 2009). These metabolic side effects also contribute to poor adherence rates in schizophrenia treatment, meaning there is considerable impetus to tackle these adverse effects.

The mechanisms underlying antipsychotic-induced weight gain and metabolic dysfunction are not fully understood, and evidence from clinical and preclinical studies suggest that multiple central and peripheral mechanisms are involved; for review see (Newcomer 2005; Boyda et al. 2010). Initial increases in body weight are primarily driven by increases in appetitive drive (Davoodi et al. 2009) due to antagonism of central receptors involved in appetite regulation including 5-HT_{2c}, histamine H_1 and dopamine D_2 receptors (Matsui-Sakata et al. 2005; Kirk et al. 2009).

Olanzapine, and other antipsychotics, can however cause metabolic dysregulation independently of effects on body weight (Minet-Ringuet et al. 2006). In particular, increases in visceral fat mass, a key component in the development of metabolic disease, have been seen in the absence of overt weight gain following olanzapine treatment in both clinical and preclinical studies (Zhang et al. 2004; Victoriano et al. 2009).

The gut microbiota comprises the approximately 100 trillion bacteria (as well as fungi, archaea and viruses) which have co-evolved with the human host to live symbiotically in the gastrointestinal tract (Backhed et al. 2005b). Recently, much focus has been turned to this 'microbial organ' as technological advances have allowed more in depth analysis of our oldest ancestors (Fraher et al. 2012). Gordon and colleagues have demonstrated, using germ-free mice (mice lacking any microbiota), the critical role played by the gut microbiota in normal weight gain and fat deposition (Backhed et al. 2004; Backhed et al. 2007). Germ-free mice have 40% less total body fat than conventionally raised mice, and are resistant to diet-induced obesity (Backhed et al. 2004; Backhed et al. 2007). Moreover, some studies have found a link between obesity and the composition of the gut flora in humans (Nadal et al. 2009; Turnbaugh et al. 2009).

We recently demonstrated an altered faecal microbiota profile in rats chronically treated with olanzapine, suggesting a possible role for the gut microbiota in olanzapine-induced effects (Davey et al. 2012). To investigate this hypothesis further, we used a cocktail of broad-spectrum antibiotics to examine if ablation of the gut microbiota could prevent or ameliorate any of the metabolic side effects associated with olanzapine.

4.2 Methods

Animals

Female Sprague-Dawley rats, 6 weeks old and weighing approximately 200g were used (Harlan, UK). Female rats are used as they have been shown by us and others to better model the elevated weight gain induced by atypical antipsychotics than male animals (Arjona et al. 2004; Cooper et al. 2005; Davey et al. 2012). Animals were allowed to habituate to the facility for 10 days. Animals were housed 5 per cage (56x38x17 cm) and allowed access to standard chow and water *ad libitum.* Animals were maintained on a 12h light dark cycle, lights on 7.30 am. All experiments were approved by the Animal Experimentation Ethics Committee (AEEC) of University College Cork and carried out in accordance with the Cruelty to Animals Act 1876 and European Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

Olanzapine administration

Olanzapine (OLZ) (Discovery Fine Chemicals, UK) was dissolved in a minimal amount of glacial acetic acid (approx 0.1ml), made to volume with sterile water and pH adjusted to 6.0 with 0.1M NaOH. Animals received 2 mg/kg/d or 4 mg/kg/d. Vehicle consisted of sterile water acidified with 0.1 ml of glacial acetic acid and pH adjusted to 6.0 with 0.1M NaOH. Drug solutions were prepared fresh daily and administered via intraperitoneal injection, B.I.D., for 21 days with the first injection between 9:00 and 10:00 am and the second between 4:00 and 5:00pm. Doses of olanzapine were selected based on previous studies from our laboratory and others in which they were found to best represent the clinical setting in terms of side effects (Fell et al. 2007; Davey et al. 2012).

Antibiotic cocktail (ABX)

Neomycin (250 mg/kg), metronidazole (50 mg/kg) (Discovery Fine Chemicals, UK) and polymyxin B (9 mg/kg) (Sigma-Aldrich, Switzerland) were dissolved in sterile water and sonicated for 10 minutes to ensure complete dissolution. The antibiotic cocktail (ABX) was administered once daily *per os* in a volume of 4 ml/kg and was prepared fresh daily. Selection of antibiotics and doses was based on studies carried out to sterilise the gut of rats undergoing gastrointestinal surgery(Juno et al. 2003).

Treatment groups

Animals received either vehicle or antibiotic cocktail for 5 days prior to the commencement of olanzapine treatment and on all subsequent days. Animals received olanzapine (OLZ) (2 mg/kg/d), olanzapine (4 mg/kg/d) or vehicle (VEH) for 21 days. Hence, there were 6 treatment groups; VEH+VEH, VEH+ABX, OLZ (2 mg/kg)+VEH, OLZ (2 mg/kg)+ABX, OLZ (4 mg/k)+VEH and OLZ (4 mg/kg)+ABX. Groups were weight matched prior to study commencement. n = 9/10.

Sample collection

All animals were fasted overnight (16 hours) prior to sacrifice. Uterine fat was quickly and carefully dissected and weighed to the nearest 0.001g. Trunk blood was collected in EDTA coated tubes and centrifuged at 6000 rpm for 15 minutes at 4^{o}C. Plasma supernatant was then aliquoted and frozen. A sample of uterine fat and frontal lobe of the liver were snap-frozen. All samples were stored at -80^{o}C for later analysis.

Gut microbiota analysis

For analysis of the microbial community composition, total DNA was extracted from faecal pellets, collected directly from the rats one day prior to sacrifice (n=6), using the QIAamp DNA stool mini kit (Qiagen, West Sussex, UK), coupled with an initial bead-beating step. Universal 16SrRNA primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region, i.e. forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were used for Taq-based PCR amplification. Sequencing was performed on a Roche 454 GS-FLX using Titanium chemistry by the Teagasc454 Sequencing Platform. Resulting reads were quality trimmed, clustered, aligned and checked for chimeras using the Qiime suite of tools. A phylogenetic tree was generated using the FastTree package and principal coordinate analysis (PCoA), measuring dissimilarities at phylogenetic distances based on unweighted Unifrac analysis, was performed with Qiime.

Plasma analysis

Insulin was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (Mercodia, Sweden). Glucose was measured using a colorimetric assay (Bioassays, US). The quantitative insulin sensitivity check index (QUICKI) was calculated as the inverse log of the sum of fasting plasma insulin and fasting plasma glucose (Katz et al. 2000).

Plasma free fatty acids were measured using a commercially available colometric assay (Bioassay Systems, U.S.). All samples were analysed in duplicate.

Gene expression analysis

Total RNA was extracted using a commercially available kit (Qiagen, US). mRNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, US) in a G-Storm thermocycler (G-Storm, UK). Gene expression was analysed by qualitative real-time PCR using TaqMan Gene expression assays and the AB7300 system (Applied Biosystems). The expression of each gene was normalised to β-actin. All samples were analysed in triplicate.

Statistical analysis

Data are expressed as mean ± SEM. Body weight change and food intake were analysed using two-way repeated measures ANOVA and a Greenhouse-Geisser sphericity correction was applied. Two-way ANOVA was used for analysis of uterine fat, gene expression, QUICKI score and cytokine levels. Where a significant overall effect was observed, further analysis was carried with Fisher's Least Significant Difference test. A p value < 0.05 was considered statistically significant. LSD was selected as an appropriate post-hoc test due the relatively small N numbers and expected effect sizes.

4.3 Results

Body weight

Olanzapine (OLZ) administration had a significant effect on body weight change ($F_{(2,52)}$ = 8.32, p = 0.001). Antibiotic treatment (ABX) did not have a significant overall effect on body weight change but there was as a significant OLZ x ABX interaction ($F_{(2,52)}$ = 6.63, p < 0.05).

Animals treated with VEH+ABX had significantly greater weight gain compared to rats receiving VEH+VEH on days 8 and 12 (Fig. 4.1A). Furthermore, rats treated with OLZ (2 mg/kg)+VEH had significantly greater weight gain compared to animals receiving OLZ (2 mg/kg)+ABX on days 8-21 inclusive (p < 0.05) (Fig. 4.1B). Animals receiving OLZ (4 mg/kg)+VEH displayed greater weight gain than those receiving OLZ (4 mg/kg)+ABX on days 11-16 inclusive (p < 0.05) (Fig. 4.1C).

Area under the curve analysis demonstrated that the animals receiving OLZ (2 mg/kg)+VEH showed significantly greater weight gain overall compared to VEH+VEH (p < 0.001) as well as OLZ (2 mg/kg)+ABX treated rats (p < 0.05). Furthermore, rats treated with OLZ (4 mg/kg) + VEH had significantly greater increases in weight overall compared to VEH + VEH treated animals (p < 0.001) (Fig. 4.1D).

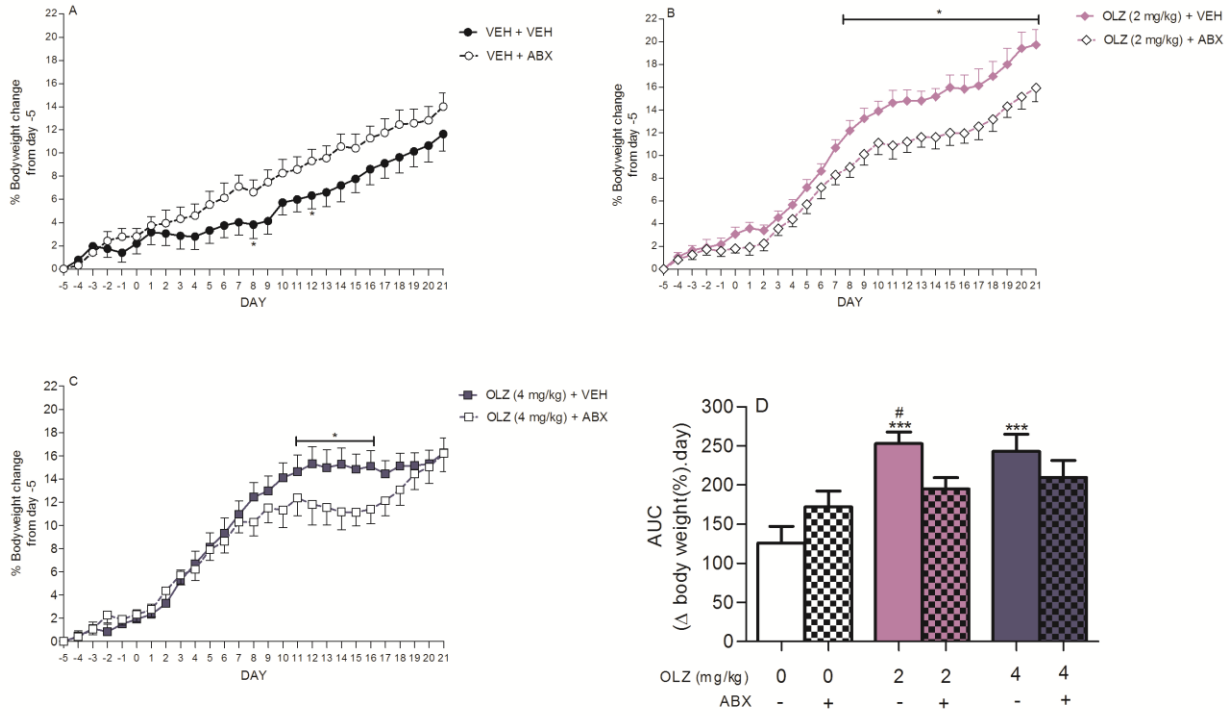


Fig. 4.1 Effect of olanzapine co-administered with an antibiotic cocktail on body weight. *Percentage body weight change from baseline following administration of (A) vehicle (VEH) + vehicle or VEH + antibiotic cocktail (ABX) (B) olanzapine (OLZ) (2 mg/kg) with or without co-administration of ABX (C) olanzapine (4 mg/kg) with and without co-administration of ABX. (D) Area under the curve values for all treatment groups. Data analysed by Two-way ANOVA as 6 groups and represented separately for graphical reasons. *p < 0.05, *** p < 0.01, #p < 0.05 versus OLZ (2 mg/kg)+ABX. Data expressed as mean ± SEM.*

Food Intake

There was significant effect of OLZ administration on food intake ($F_{(2,66)}$ = 37.25, p < 0.001). There was no overall effect of antibiotic(ABX) treatment, however, there was a significant OLZ x ABX interaction ($F_{(2,66)}$ = 18.69, p = 0.01). Food intake was also significantly affected by Time ($F_{(3,198)}$ = 53.31, p < 0.001), and there was a significant Time x OLZ interaction ($F_{(6,198)}$ = 8.96, p < 0.001), and a significant Time x ABX interaction ($F_{(3,198)}$ = 13.19, p < 0.001).

Post-hoc analysis revealed that all animals treated with ABX had significantly reduced food intake compared to animals receiving VEH+VEH between days -5 and 0($p < 0.01$) (Fig. 4.2). During the first week of olanzapine treatment (days 1-6 inclusive) animals receiving OLZ (2mg/kg)+VEH or OLZ (4 mg/kg)+VEH displayed significantly increased food intake compared to VEH + VEH treated rats ($p < 0.001$). Animals receiving olanzapine (2 mg/kg)+ABX or olanzapine (4 mg/kg)+ABX also showed increased food intake during this period compared to animals receiving VEH+ABX ($p < 0.01$ and $p < 0.001$) respectively. Animals receiving OLZ (2 mg/kg)+VEH or OLZ (4 mg/kg) + VEH further displayed increased food intake between days 7 and 13 compared to VEH + VEH treated rats($p < 0.05$,$p < 0.001$) (Fig.4.2).

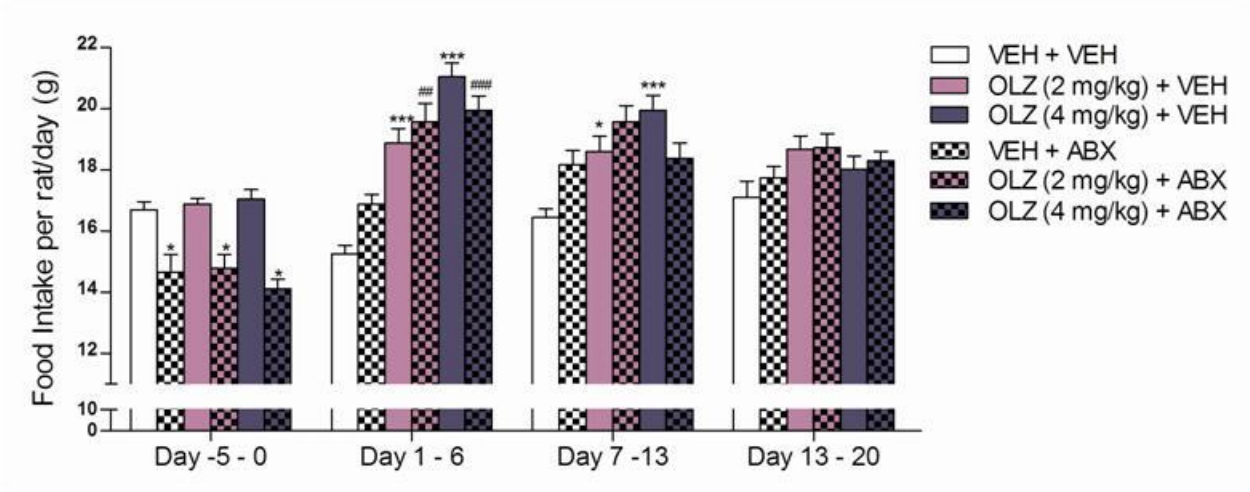


Fig. 4.2 Effect of olanzapine co-administered with an antibiotic cocktail on food intake. *Effect of vehicle (VEH), olanzapine (OLZ) (2 mg/kg) or OLZ (4 mg/kg) co-administered with vehicle (VEH) or an antibiotic cocktail (ABX) on food intake. **p<0.01, ***p< 0.001 versus VEH+VEH. ##p< 0.01 ###p < 0.001 versus VEH+ABX. Data represent mean ± SEM.*

Faecal output

None of the groups showed signs of diarrhoea, as measured by the number and consistency of pellets produced, with all groups having an average faecal output of < 0.5 pellets over a 30 minute period (data not shown).

Microbiota profile

Following total genomic DNA extraction, a total of 60,276 pyrosequencing reads from the V4-V5 region of 16S rRNA gene were generated with 454 sequencing, representing an average of 1487 reads per sample. Rarefaction analysis, using both Shannon diversity and Chao1 distances, indicated sufficient depth of sequencing (data not shown). Principal-coordinate analysis (PCoA), based on unweighted Unifrac distance revealed that all three groups receiving the antibiotic cocktail had co-clustered, showing an altered overall gut microbiota, compared with the three groups who did not receive the cocktail (Fig. 4.3). More specifically, PCoA plots demonstrate a distinct gut microbiota composition of groups receiving the antibiotic cocktail versus those who did not.

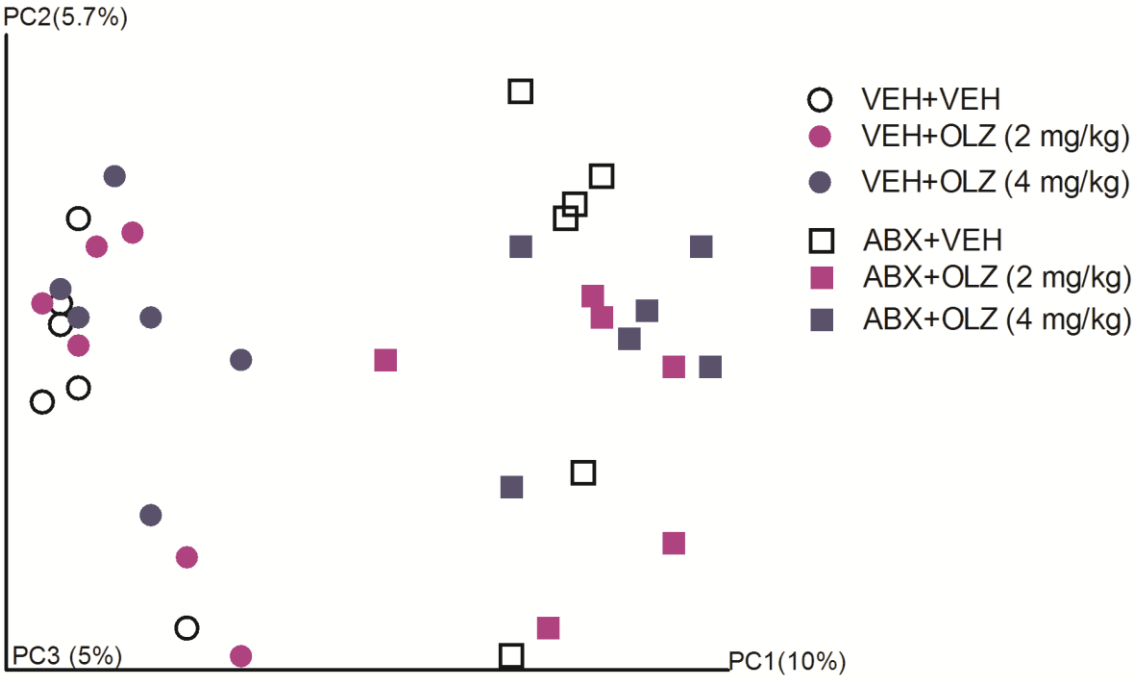


Fig 4.3. Effect of olanzapine co-administered with an antibiotic cocktail on the overall microbiota profile. *Principle coordinate analysis of each sample showing the clustering of animals receiving the antibiotic cocktail (squares) compared to those not (circles). clear= vehicle(VEH), pink = olanzapine (OLZ) (2 mg/kg) purple= OLZ (4 mg/kg)*

Further analysis of the specific microbiota composition showed that olanzapine 2 mg/kg and 4 mg/kg treated rats had a trend for increased *Firmicutes* (82.9% and 79.4%) respectively, compared to VEH+VEH treated animals (76.5%). Olanzapine treated animals also had a concomitant trend for decreased *Bacteriodetes* (10.0% and 10.6% compared to 14.3%) (Fig. 4.4).

Administration of the antibiotic cocktail was associated with a dramatic reduction in *Firmicutes* compared to vehicle treated rats (57.6% versus 76.5%).

This reduction was also seen between OLZ+VEH and OLZ ABX treated groups (82.9% vs. 66.7%) and (79.4% vs. 64.8%) for 2 mg/kg and 4 mg/kg doses, respectively (Fig 4.4).

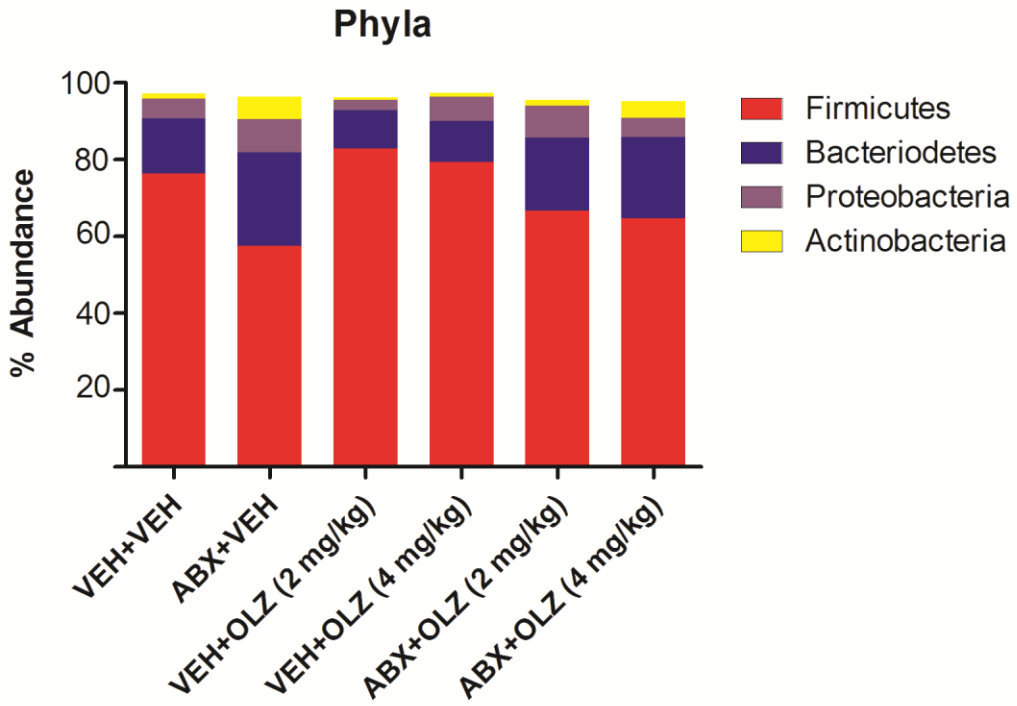


Fig. 4.4 Effect of olanzapine co-administered with an antibiotic cocktail on microbiota composition in Sprague-Dawley rats. *Percent abundance of the major phyla in the faecal microbiota of rats treated for 21 days with olanzapine(OLZ) with and without co-administration of an antibiotic cocktail (ABX).*

Adipose Tissue

Uterine Fat Weight

OLZ administration had a significant effect on uterine fat mass ($F_{(2,52)}$ = 14.553, p <0.001) and when measured as a percentage of body weight ($F_{(2,52)}$ = 13.24, p < 0.001). ABX treatment also had a significant effect on real values ($F_{(1,52)}$ = 40.753, p < 0.001) and as a percentage of body weight ($F_{(1,52)}$ = 44.93, p < 0.001).

Post-hoc analysis revealed that animals treated with VEH+ABX had significantly less uterine fat compared to those receiving VEH+VEH (real value, p < 0.05, % value p < 0.01). Animals receiving OLZ (2 mg/kg)+VEH and those receiving OLZ (4 mg/kg)+VEH displayed increased levels of uterine fat compared to VEH+VEH treated rats (p < 0.05 and p < 0.01 respectively for both real and % values). Furthermore, the OLZ (2 mg/kg)+ABX treated animals had significantly lower uterine fat than the OLZ (2 mg/kg)+VEH group (p < 0.05 for both real and % values). Similarly, the OLZ (4 mg/kg)+ABX treated animals had significantly less uterine fat compared to OLZ (4 mg/kg)+VEH treated rats (p < 0.01 for both real and % value) (Fig. 4.5A).

CD68 Expression

CD68 expression was significantly increased by OLZ ($F_{(2,52)}$ = 5.55, p , 0.01). Post-hoc analysis showed that the animals receiving OLZ (2 mg/kg)+VEH or OLZ (4 mg/kg)+VEH had significantly increased expression of CD68 compared to VEH+VEH treated rats (p < 0.05). However, the OLZ (2 mg/kg)+ABX treated rats had significantly lower expression compared to OLZ (2 mg/kg)+VEH, and there was a similar trend between the OLZ (4 mg/kg)+ABX and OLZ (4 mg/kg)+VEH groups (p = 0.06) (Fig. 4.5B).

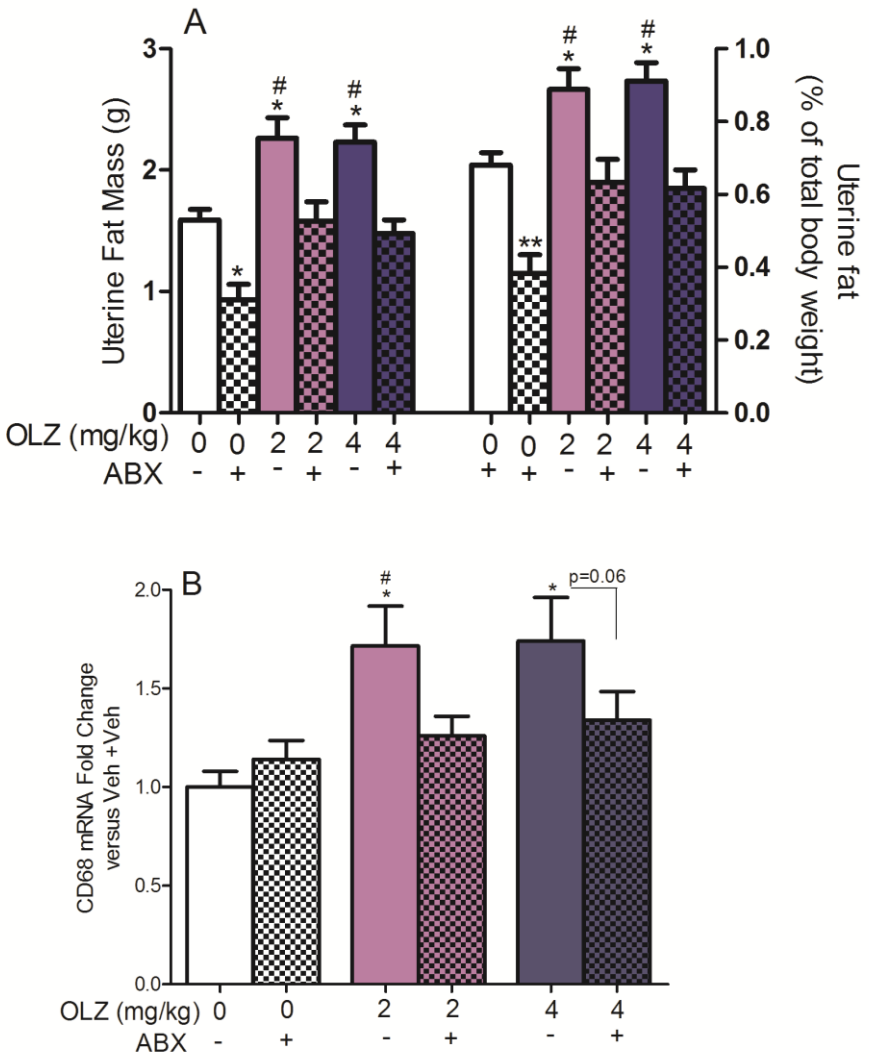


Fig. 4.5 Effect of olanzapine and antibiotic cocktail gene expression in adipose tissue. *Effect of vehicle (VEH), olanzapine (OLZ) (2 mg/kg) or OLZ (4 mg/kg) co-administered with vehicle (VEH) or an antibiotic cocktail (ABX) on (A) Uterine fat percentage and (B) CD68 mRNA expression in adipose tissue. *p < 0.05, **p < 0.01 compared to the VEH+VEH group. #p < 0.05, ##p < 0.01 versus ABX treated animals receiving same dose of olanzapine. $p < 0.05 versus VEH+ABX group. Data represent mean ± SEM.*

Liver gene expression

Sterol-regulatory element binding protein (SREBP) 1c expression in the liver was significantly affected by both OLZ ($F_{(2,52)}$ = 5.45, $p < 0.001$) and ABX treatment ($F_{(1,52)}$ = 6.79, $p < 0.05$).

Post-hoc analysis revealed that the OLZ (2 mg/kg)+ABX and OLZ (4 mg/kg)+ABX treated animals had significantly increased expression compared to the VEH+ABX treated rats ($p < 0.05$). Furthermore, the OLZ (2 mg/kg)+ABX treated group had significantly increased expression compared to OLZ (2 mg/kg)+VEH group ($p < 0.05$). The OLZ (4 mg/kg)+ABX treated group also had significantly increased expression levels compared to OLZ (4 mg/kg)+VEH ($p < 0.05$) treated rats (Fig. 4.6A).

The expression of fatty acid synthase (FAS) was significantly affected by OLZ administration ($F_{(2,52)}$ = 3.09 $p = 0.05$) and ABX treatment ($F_{(1,52)}$ = 6.72, $p < 0.05$). There was also a significant OLZ x ABX interaction, ($F_{(2,52)}$ = 4.05, $p < 0.05$). Further analysis revealed OLZ (2 mg/kg)+VEH and OLZ (4 mg/kg)+VEH treated animals had significantly increased expression compared to VEH+VEH treated rats ($p < 0.05$). The animals receiving OLZ (2 mg/kg)+VEH displayed significantly increased expression compared to OLZ (2 mg/kg)+ABX treated rats ($p < 0.05$). Similarly, the animals treated with OLZ (4 mg/kg)+VEH had increased levels compared to those receiving OLZ (4 mg/kg)+ABX ($p < 0.05$) (Fig. 4.6B).

The expression acetyl Co-A carboxylase (ACC) was also significantly affected by both OLZ administration ($F_{(2,50)}$ = 4.74, $p < 0.05$) and ABX treatment $F_{(1,50)}$ = 7.99, $p < 0.01$). The OLZ x ABX interaction did not quite reach significance however, $F_{(2,50)}$ = 2.99, $p = 0.059$).

Post-hoc analysis revealed that the animals receiving OLZ (2 mg/kg)+ABX or OLZ (4 mg/kg)+ABX had significantly increased expression of ACC compared to the VEH+ABX treated group ($p < 0.01$).

Furthermore, the OLZ (2 mg/kg)+ABX treated rats had increased levels compared to OLZ (2mg/kg)+VEH ($p < 0.05$) and similarly the OLZ (4 mg/kg) +ABX had increased levels compared to OLZ(4 mg/kg)+VEH treated rats ($p < 0.05$) (Fig. 4.6C).

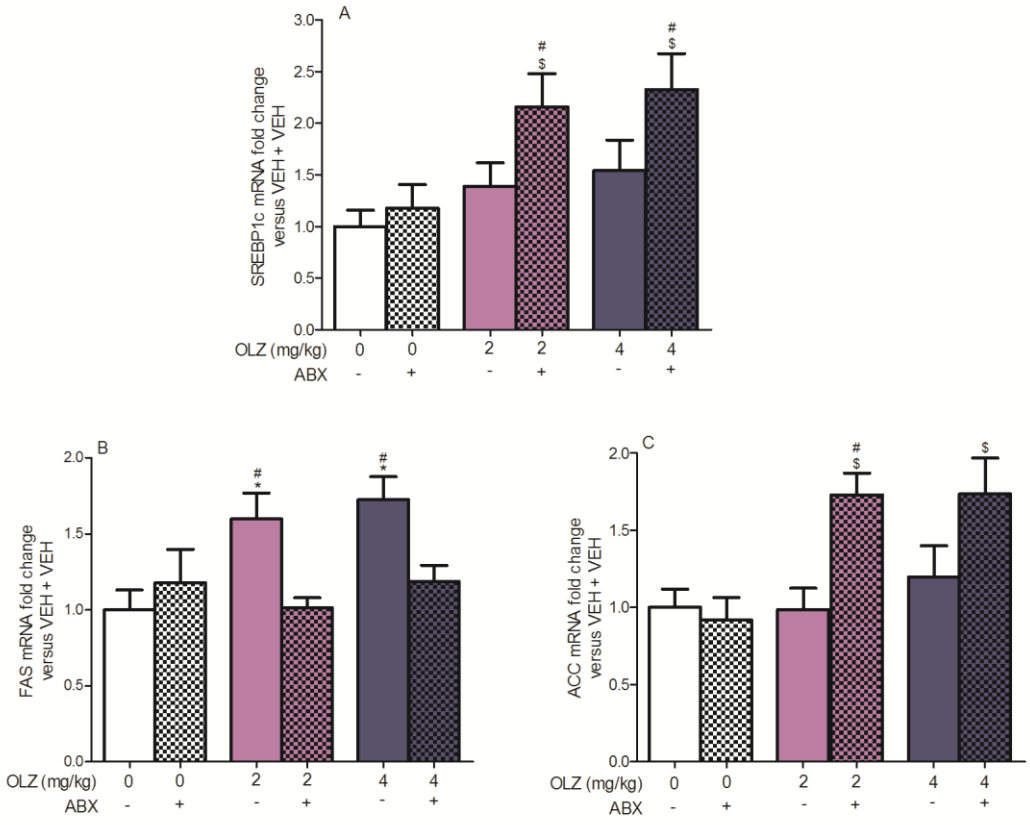


Fig. 4.6 Effect of olanzapine co-administered with an antibiotic cocktail on gene expression in the liver. *Effect of vehicle (VEH), olanzapine (OLZ) (2 mg/kg) or OLZ (4 mg/kg) co-administered with vehicle (VEH) or an antibiotic cocktail (ABX) on (A) sterol regulatory binding protein-1c (SREBP-1c) (B) fatty acid synthase (FAS) and (C) acetyl Co-A carboxylase (ACC) expression in the liver.*p< 0.05 versus VEH+VEH, $^{\$}$p< 0.05 versus VEH+ABX, $^{\#}$p < 0.05 versus same drug dose + VEH. Data represent mean ± SEM.*

Plasma Profile

Quantitative Insulin Sensitivity Check Index (QUICKI)

OLZ administration significantly affected QUICKI score (quantitative insulin sensitivity check index) ($F_{(2,49)}$ = 22.70, $p < 0.001$). Post-hoc analysis revealed that the animals receiving OLZ (2 mg/kg)+VEH or OLZ (4 mg/kg)+VEH had significantly lower QUICKI scores compared to the VEH+VEH treated rats ($p < 0.001$). Furthermore, the animals that received OLZ (2 mg/kg)+ABX or OLZ (4 mg/kg)+ABX also had reduced QUICKI scores compared to both the VEH+VEH group ($p < 0.001$, $p < 0.05$ respectively) and the VEH + ABX treated rats ($p < 0.05$) (Fig. 4.7A).

Free Fatty Acids (FFA)

OLZ treatment had a significant effect on plasma free fatty acids (FFA) ($F_{(2,50)}$ = 6.393, $p < 0.01$). Further analysis showed that animals receiving OLZ (2 mg/kg)+VEH or OLZ (4 mg/kg)+VEH had significantly elevated levels of free fatty acids compared to VEH+VEH ($p < 0.01$ and $P < 0,05$ respectively). Animals receiving OLZ (2 mg/kg)+VEH also had increased levels compared to the OLZ (2mg/kg)+ABX treatment group ($p < 0.05$) (Fig. 4.7B).

Cytokines

Plasma levels of tumour necrosis factor (TNF) were significantly affected by OLZ administration, ($F_{(2,52)}$ = 5.20, $p < 0.01$) and there was a non-significant trend for an OLZ x ABX interaction, ($F_{(2,52)}$ = 2.71, $p = 0.07$). Further analysis revealed that the animals receiving OLZ (4 mg/kg)+VEH had significantly elevated levels compared to both the VEH+VEH and the OLZ (4 mg/kg)+ABX treated rats ($p < 0.05$) (Fig. 4.7C).

Plasma interleukin (IL)-1β levels were significantly affected by ABX treatment ($F_{(1,52)}$ = 26.66, p < 0.001) and there was a significant OLZ x ABX interaction, ($F_{(2,52)}$ = 5.90, p < 0.01). Post-hoc analysis revealed that the animals receiving OLZ (4 mg/kg)+VEH had significantly elevated levels compared to both the VEH+VEH group (p < 0.05) and the OLZ (4 mg/kg)+ABX treated rats (p < 0.01) (Fig. 4.7D).

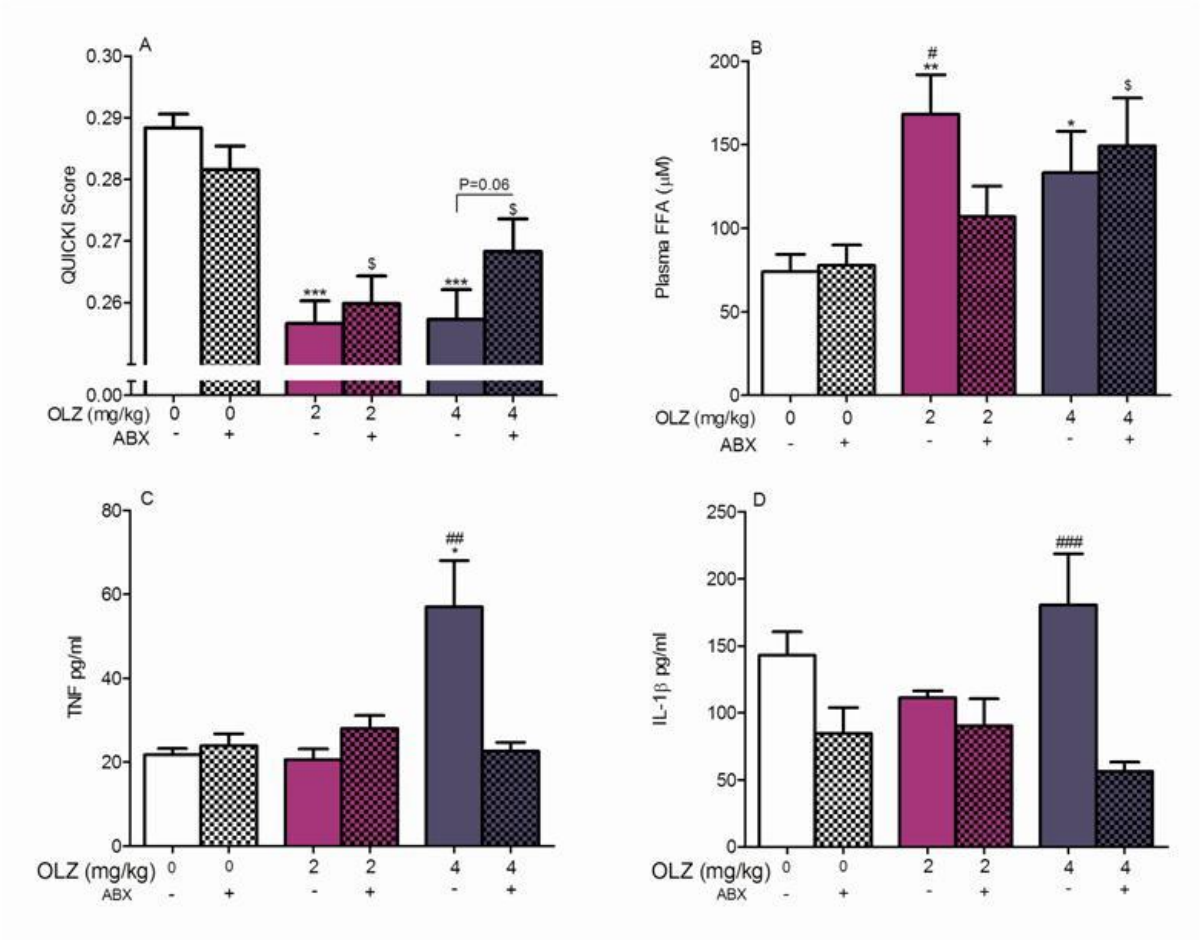


Fig.4. 7 Effect of olanzapine and antibiotic cocktail on metabolic parameters in plasma. *Effect of vehicle (VEH), olanzapine (OLZ) (2 mg/kg) or OLZ (4 mg/kg) co-administered with vehicle (VEH) or an antibiotic cocktail (ABX) on (A) Plasma free fatty acids (FFA), (B) Quantitative Insulin Sensitivity Check Index (QUICKI) scores, (C) plasma TNF levels and (D) plasma IL-β levels. *p < 0.05,***p < 0.001 compared to VEH+VEH group, $^{\#}$p < 0.05 versus OLZ (of same dose)+ABX , $^{\$}$p < 0.05 compared to VEH+ABX group. Data expressed as mean ± SEM.*

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4.4 Discussion

In the present study, olanzapine induced rapid body weight gain and significant accretion of visceral fat in line with previous reports (Fell et al. 2007; Davey et al. 2012). Herein, we show that a combination of broad spectrum antibiotics attenuates certain clinically relevant side effects of olanzapine in an animal model. This novel finding supports recent work showing antibiotic treatment can prevent weight gain in diet-induced obesity models in mice (Cani et al. 2008; Murphy et al. 2012).

Importantly, we demonstrated using 454 pyrosequencing that the antibiotic cocktail resulted in markedly different overall microbiota profile which clustered distinctly and separately from the microbiota of animals not receiving the antibiotics.

Moreover, the composition of faecal microbiota of olanzapine-treated rats showed a trend for increases in the phyla *Firmicutes* and concomitant decreases in *Bacteriodetes,* a trend observed previously (Davey et al. 2012). Whether such changes are a direct result of antipsychotic treatment or indicative of an obese phenotype is unclear, however similar shifts in the major phyla of the microbiota have been associated with weight gain in animal and human studies (Ley et al. 2005; Turnbaugh et al. 2009). Due to the diversity of the microbiota at species level, and the fact that many functions are conserved across phyla, whether these changes in our and other studies are pathogenic or merely indicative of metabolic dysfunction remains to be determined (Conterno et al. 2011). This said, these alterations to the gut flora support the theory that the microbiota has a functional role to play in metabolic complications associated with olanzapine.

In further support of this theory, the antibiotic cocktail, which ameliorated weight gain, was associated with the opposite trend with decreases *Firmicutes* and increases *Bacteriodetes* observed. Hence, with increasing evidence and impetus being focused on the microbiome-gut brain axis (Bravo et al. 2011; Cryan and O'Mahony 2011; Cryan and Dinan 2012), these data highlight the emerging potential for adjunctive therapies which more specifically target the gut microbiota to ameliorate antipsychotic-induced weight gain.

The mechanisms underlying the prevention of body weight gain observed in this study appear to be independent of food intake as the antibiotic cocktail did not reduce food consumption over the course of olanzapine treatment. The reduction in body weight was therefore most likely accounted for by a reduction in fat mass, as the antibiotic cocktail prevented increases in uterine fat. This is particular relevant to the clinical setting as increased visceral fat is a key determinant in the development of insulin resistance and the metabolic syndrome (Jensen 2008). While the uterine fat represents are relatively small proportion of the overall fat mass, it is one of the major visceral deposits and is commonly used as representative of visceral fat in rodents (Coccurello et al. 2008; Amuzie et al. 2011).

An intriguing temporal element was observed as the antibiotic cocktail resulted in amelioration of weight gain for both doses of olanzapine initially, but only the lower, 2 mg/kg, dose by the study's end. This discrepancy is possibly due to subsiding weight gain observed for the higher olanzapine dose in the final week of treatment, a phenomenon previously observed (Davey et al. 2012).

Interestingly, reduced fat mass was also seen in animals receiving antibiotics only, in line with germ-free studies (Backhed et al. 2004).

While this may seem paradoxical, as these animals also displayed a trend for increased body weight, it is worth noting this phenomenon has been utilised in the agricultural food industry for many decades, as low-dose antibiotics have been used and abused as growth promoters in to produce larger, leaner animals (Kamphues 1999).

The adipose tissue is intimately involved in the development of metabolic syndrome in part due to its ability to release a number of pro-inflammatory mediators both locally and systemically (Fantuzzi 2005). A crucial step in this inflammatory process is the recruitment of macrophages which infiltrate the fat tissue and together with the adipocytes release pro-inflammatory cytokines including IL-6 and TNF (Weisberg et al. 2003). We found increased expression of CD68, a macrophage marker, in the adipose tissue of animals receiving olanzapine but not in those receiving both olanzapine and the antibiotic cocktail. The reason for macrophage infiltration is not clear though it may be the result of adipose tissue hypertrophy, as dying adipocytes release signals that recruit macrophages (Cinti et al. 2005). Thus, by preventing increases in fat mass, antibiotics may indirectly prevent macrophage infiltration and the subsequent pro-inflammatory response.

This reduction in adipose inflammation was also reflected systemically, as the antibiotic treated rats did not display the increases in circulating TNF or IL-6 observed in response to the higher, 4 mg/kg dose of olanzapine.

The molecular mechanisms underlying the microbiota's role in fat deposition have been investigated in other models including germ-free, diet-induced and genetically obese (*ob/ob*) mice (Backhed et al. 2004; Turnbaugh et al. 2006; Murphy et al. 2010).

Some studies have indicated that the microbiota can influence the expression of a number of lipogenic genes ultimately resulting in increased triglyceride synthesis which are subsequently stored in adipocytes (Cani and Delzenne 2009).

Antibiotic treatment prevented olanzapine-induced increases in one such gene, fatty acid synthase (FAS). Increased FAS expression was recently associated with another antipsychotic, risperidone (Lauressergues et al. 2010). Increased expression of SREBP-1c and ACC-1 were only observed in animals receiving both olanzapine and antibiotics. This is contrary to what one might expect, and may be the result of a positive feedback mechanism due to a reduction in short chain fatty acids (SCFA) being absorbed in the antibiotic treated animals, or the fact the animals were fasted overnight, which is known to decrease SREBP-1c expression in the rat (Gosmain et al. 2005).

Increased levels of circulating FFA were found in association with olanzapine treatment in line with previous reports of both patients (Wang et al. 2006) and animals (Jassim et al. 2012) and moreover these increases were prevented by antibiotic treatment. Increased plasma levels FFA are correlated with obesity in humans and involved in the pathogenesis of metabolic dysfunction in part by stimulating lipogenic enzymes in the liver (Pegorier et al. 2004).

As such, effects of antibiotic administration on FFA, lipogenic genes and on visceral fat highlights that the gut microbiota can influence the full cycle of energy metabolism and thereby play a role in the spectrum of metabolic dysfunction associated with certain antipsychotics.

Additionally, we used the quantitative insulin sensitivity check index (QUICKI) as a measure of insulin sensitivity as this model is viewed as the most appropriate for animal models, especially when estimating whole-body insulin resistance (Cacho et al. 2008). Insulin resistance represents the pathogenic hallmark of metabolic syndrome and type II diabetes mellitus and glucose dysregulation and insulin resistance have been associated with olanzapine treatment in clinical and preclinical studies (Houseknecht et al. 2007; Smith et al. 2009). We found that olanzapine treatment resulted in reduced insulin sensitivity in support of previous studies (Houseknecht et al. 2007; Smith et al. 2009). While not statistically significant, there was evidence to suggest that the antibiotic treatment improved insulin sensitivity. This possibility is supported by work in which antibiotic treatment ameliorated insulin resistance (Cani et al. 2008).

There are potentially other mechanisms involved in the observed effects of the antibiotics, including pharmacokinetics as considerable alteration of the gut flora as in this potentially impacts drug absorption and metabolism. Hence, active levels of the drug may have diminished, and moreover, interventions such as this one could therefore conceivably impact on the efficacy of the antipsychotic, a concern that warrants investigation in the future.

Of course, widespread use of broad-spectrum antibiotics as an adjunctive therapy cannot be advocated due to the well-known problem of antibiotic resistance. Moreover, ablating the gut flora inevitably has other consequences that can lead to gastrointestinal problems, such as diarrhoea, resulting in weight loss. In the present report however, assessment of faecal output should no signs of any such effects (Data not shown).

Future studies, such as caecal transplant of olanzapine-treated rats to rats that have received antibiotics, may provide further elucidation of the scale of the contribution of the microbiota in antipsychotic-induced metabolic dysfunction, as similar methods have been used to demonstrate the microbiota's role in both obesity and stress (Turnbaugh et al. 2008; Bercik et al. 2012).

Clinical data supporting this hypothesis do not exist at present, this is most likely due to a lack of investigation, and antibiotic administration administered in normal clinical practice to patients receiving antipsychotics would be unlikely to produce effects such as those described herein. However, further understanding of the full metagenomic potential of specific members of the microbiota may allow for a safer and more targeted approach.

We have recently shown that olanzapine impacts on the gut microbiota and hypothesised that this may contribute to its metabolic liabilities including weight gain and increased visceral fat (Davey et al. 2012). Taken together, these findings represent an exciting proof-of-principle that manipulation of the gut flora potentially represents a new therapeutic strategy for tackling the serious clinical problem of antipsychotic-induced metabolic dysfunction.

Furthermore, the emergence of probiotic-based as potential therapies for a variety of medical conditions including obesity (Clarke et al. 2012; Mallappa et al. 2012) encourages the development of such strategies for not only antipsychotic-induced weight gain but obesity and the metabolic syndrome in general.

Chapter 5

Effects of rifaximin and vancomycin on olanzapine-induced metabolic dysfunction

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To be submitted to Gut

Abstract

Background: Olanzapine is associated with a number of detrimental metabolic side effects including weight gain and visceral fat deposition. The mechanisms of these side effects, as well as effective ways to combat them, remain mostly elusive. The gut microbiota is now recognised as a major physiological regulator of certain aspects of metabolism including fat deposition. Moreover, we have demonstrated that olanzapine is associated with an altered faecal microbial profile in rats and that the microflora is required for some aspects of olanzapine-induced metabolic dysfunction.

Methods: Female Sprague-Dawley rats were administered olanzapine (2 mg/kg/d) or vehicle by intraperitoneal injection twice daily. Animals also received one of vehicle, rifaximin (30 mg/kg) or vancomycin (50 mg/kg) *per os* for 26 days, beginning 5 days prior to the commencement of antipsychotic treatment. Body weight and food intake were monitored daily. Liver, adipose, and caecal contents were collected, frozen and later analysed.

Results: Rifaximin, but not vancomycin, attenuated olanzapine-induced weight gain. Rifaximin and vancomycin both prevented olanzapine-induced increases in uterine fat mass and liver weight. Olanzapine administration was associated with alterations in the gut flora including increases in *Actinobateria* which was reversed by rifaximin treatment, while vancomycin profoundly altered the microbiota including reductions in the abundance of *Firmicutes*.

Conclusions: These data further demonstrate the potential of the gut microbiota as a therapeutic target for antipsychotic-induced side effects.

5.1 Introduction

The gut flora, or microbiota, represents the circa 100 trillion microorganisms that inhabit the gastrointestinal tract (Lupp and Finlay 2005) and is extremely consistent across mammals (Ley et al. 2008b). The gut microbiota, comprising mainly bacteria, has co-evolved with the human host to occupy this environmental niche in a manner that is mutually beneficial (Ley et al. 2008b).

The role of the gut microbiota in energy regulation and metabolism has received much attention and produced compelling results. Studies utilising several animal models including germ-free, genetically obese (ob/ob) and diet-induced obesity have elegantly demonstrated the significant influence the microbiota can have on metabolism, fat deposition, body weight and insulin resistance (Backhed et al. 2004; Backhed et al. 2005b; Backhed et al. 2007; Turnbaugh et al. 2008; Membrez et al. 2010).

The mechanisms behind the microbiota's influence on metabolism are multiple, interlinked and not fully elucidated. They include the fermentation of otherwise indigestible polysaccharides to absorbable short chain fatty acids (scfa) (Macfarlane and Macfarlane 2003), which contribute up to 10% of ones daily energy intake (Flint et al. 2008). Furthermore, the microbiota can affect inflammatory processes (Souza et al. 2004; Maslowski et al. 2009) and expression of key genes involved in lipogenesis in the liver and adipose tissue (Backhed et al. 2007).

Atypical antipsychotics are the mainstay of treatment for schizophrenia and other schizoaffective disorders.

These drugs are associated with a myriad of metabolic complications including weight gain, increased visceral fat and insulin resistance (Allison et al. 1999; Newcomer 2007; Newcomer et al. 2009). Such side effects are a major clinical problem due to the development of co-morbidities such as Type II diabetes mellitus and cardiovascular disease, as well as contributing to patient non-adherence to treatment (Osby et al. 2000; Starrenburg and Bogers 2009). Moreover, at present, preventative or interventive therapies to tackle these side effects are lacking.

Thus, given the emerging evidence implicating the microbiota in a number of animal models of obesity, we investigated the possible role of the microbiota in a drug-induced obesity model using the atypical antipsychotic, olanzapine, which, alongside clozapine, is one of the two antipsychotics most associated with weight gain and metabolic dysfunction (Allison et al. 1999).

We recently demonstrated that olanzapine treatment in the rat is associated with an altered faecal microbiota profile (Davey et al. 2012) and that ablation of the microbiota prevents certain metabolic adverse effects induced by olanzapine (Davey et al., *submitted*).

Rifaximin is a semi-synthetic analogue of rifampacin and is known to target both Gram positive and Gram negative bacteria (Scarpignato and Pelosini 2005). Vancomcyin is a glycopeptide antibiotic and specifically targets Gram positive bacteria (Nagarajan 1991). Vancomycin was recently associated with reduced weight gain in diet-induced obese mice (Murphy et al. 2012) further supporting this approach.

In the present study, we therefore investigated if rifaximin or vancomycin, two non-absorbable antibiotics could attenuate olanzapine-induced weight gain or associated complications.

5.2 Methods

Animals

Female Sprague-Dawley rats, 6 weeks old and weighing approximately 200g were used (Harlan, UK). Animals were allowed to habituate to the facility for 10 days. Animals were housed 5 per cage (56x38x17 cm) and allowed access to standard chow and water *ad libitum.* Animals were maintained on a 12h light dark cycle, lights on 7.30 am. All experiments were approved by the Animal Experimentation Ethics Committee (AEEC) of University College Cork and carried out in accordance with the Cruelty to Animals Act 1876 and European Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

Drugs

Olanzapine (OLZ) (Discovery Fine Chemicals, UK) was dissolved in a minimal amount of glacial acetic acid (approx 0.1ml), made to volume with sterile water and pH adjusted to 6.0 with 0.1M NaOH. Animals received 2 mg/kg/d via inter-peritoneal injection, B.I.D., with the first injection at approximately 9:00 am and the second at approximately 4:30 pm. This dose regimen was selected based on previous reports by our lab and others showing its effects are in line with those observed in the clinic.(Fell et al. 2007; Weston-Green et al. 2011a; Davey et al. 2012). Vehicle (VEH) consisted of sterile water acidified with a minimal amount of glacial acetic acid and pH adjusted to 6.0 with 0.1M NaOH.

Rifaximin (RIF), 30 mg/kg (Sigma, UK) and vancomycin (VAN), 50 mg/kg (Sigma, UK) were dissolved in sterile water and briefly sonicated to ensure complete drug dissolution.

Animals received antibiotics *per os* once daily at approximately 12:00 noon. Vehicle (VEH) consisted of sterile water.

Daily Measurements

Body weight and food intake were measured daily, in the morning, to the nearest 0.01g using an electronic balance.

Sample collection

Immediately following sacrifice, liver and caecal contents were removed and weighed, with a portion of the frontal lobe being frozen along with the caecal contents. Peri-uterine fat tissue was carefully dissected and weighed, a small portion of which was then frozen.

Gut microbiota analysis

For analysis of the microbial community composition, total DNA was extracted from caecal contents, collected aseptically immediately following sacrifice, using the QIAamp DNA stool mini kit (Qiagen, West Sussex, UK), coupled with an initial bead-beating step. Universal 16SrRNA primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region, i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were used for Taq-based PCR amplification.

Sequencing was performed on a Roche 454 GS-FLX using Titanium chemistry by the Teagasc454 sequencing platform. Resulting reads were quality trimmed, clustered, aligned and checked for chimeras using the Qiime suite of tools. A phylogenetic tree was generated using the FastTree package and principal coordinate analysis (PCoA), measuring dissimilarities at phylogenetic distances based on unweighted Unifrac analysis, was performed with Qiime.

Gene analysis

RNA from adipose and liver samples was extracted for gene analysis using a commercially available kit (QIAGEN, Valencia, CA, USA). mRNA was reverse transcribed using High capacity cDNA reverse transcription Kit (Applied Biosystems) in a G-storm thermocycler (G-storm, Surrey, UK). Gene expression was analysed using TaqMan Gene Expression Assays and the AB7300 system (Applied Biosystems). The expression value of each gene was normalised to that of β-actin. All samples were analysed in triplicate.

Short chain fatty acid analysis

Approximately 300 mg caecal content was mixed with 3.0 mL Milli-Q water and, after standing for 10 min at room temperature, centrifuged at 4700 rpm for 10 min. 1.0 mM 2-ethylbutyric acid (Sigma) was added to the supernatant as internal standard and samples were then filtered. Calibration was done using standard solutions containing 10.0 mM, 8.0 mM, 4.0 mM, 2.0 mM, 1.0 mM and 0.5 mM of acetic acid, propionic acid, iso-butyric acid and butyric acid (Sigma). The concentration of S.C.F.A. was determined using a Varian 3800 GC flame ionization system, fitted with a DB-FFAP column (30 m x 0.32 mm x 0.25 µm; Phenomenex). Helium was used as the carrier gas at a flow rate of 1.3 mL/min.

The initial oven temperature was 100°C for 0.5 min, raised to 180°C at 8°C/min and held for 1 min, then increased to 200°C at 20°C/min, and finally held at 200°C for 5 min. The temperature of the detector and the injector were set at 250°C and 240°C, respectively. Peaks were integrated using the Varian Star Chromatography Workstation version 6.0 software. Standards were included in each run to maintain the calibration.

Statistical analysis

Body weight and food intake were analysed using a two-way repeated measures with olanzapine administration and antibiotic administration as factors. Gene expression and s.c.f.a. concentrations were analysed using a two-way anova. Where a significant was found, additional analysis was carried out using a Least Significant Difference (LSD) post-hoc test. Given the number of groups, N numbers and anticipated effect sizes, LSD was considered an appropriate post-hoc test. A p value < 0.05 was considered statistically significant. Data are expressed as mean ± SEM.

5.3 Results

Body weight

OLZ treatment had a significant effect on body weight change ($F_{(1,52)} = 6.493$, $p < 0.05$) as did time ($F_{(26,1352)} = 119.91$, $p < 0.001$). There was a significant time x OLZ effect ($F_{(26,1352)} = 25.794$, $p < 0.001$).

Post-hoc analysis revealed that OLZ treated rats displayed greater weight gain on days 2 to 7 and days 9 to 21 (inclusive) ($p < 0.05$ to $p < 0\ 01$) compared to VEH+VEH treated animals (Fig. 5.1A). RIF treatment alone did not affect body weight (Fig 5.1B). However, OLZ+RIF treated rats displayed significantly reduced increases in body weight compared to those receiving OLZ+VEH on days 1-9 and days 16-21 inclusive ($p < 0.05$) (Fig 5.1C). VAN+VEH treated rats did not show alterations in body weight compared to VEH+VEH treated animals, similarly, VAN+OLZ treated rats did not display differences compared to VEH+OLZ (Fig. 5.1D).

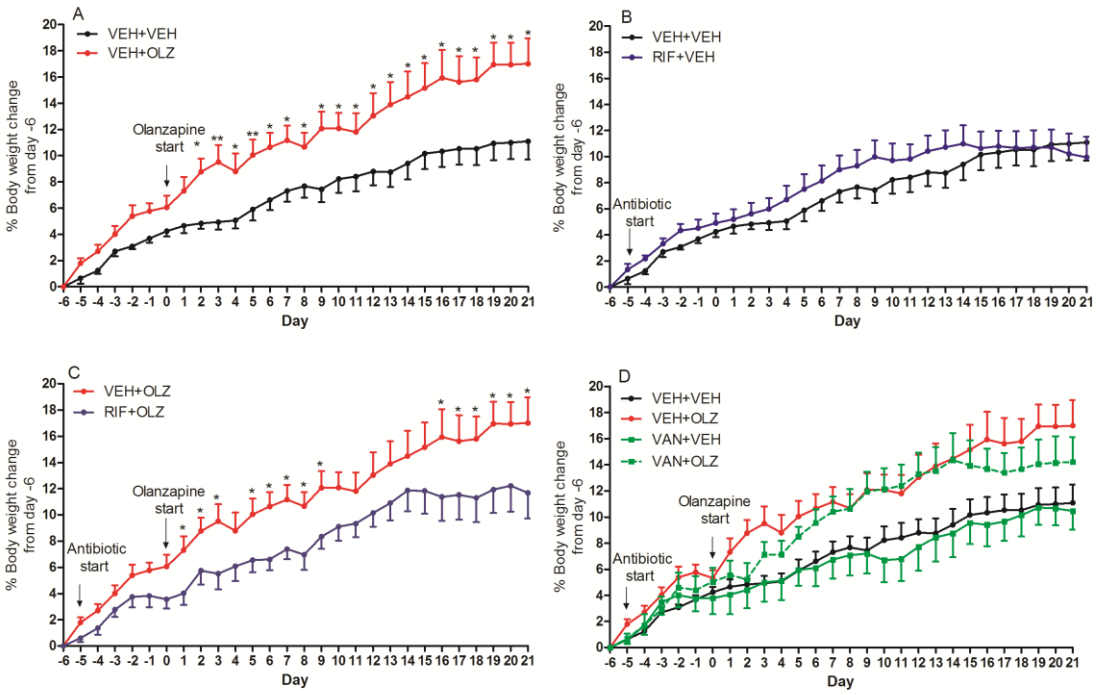


Fig. 5.1. Effect of olanzapine, rifaxamin and vancomycin on body weight. *Body weight change in female rats following treatment with (A) Olanzapine (OLZ) 2 mg/kg/d (B) Rifaximin 50 mg/kg (C) Olanzapine co-administered with or without rifaximin and (D) Olanzapine co-administered with or without vancomycin (VAN) (50 mg/kg).Data shown represents mean ± SEM. *p < 0.05, **p < 0.01 compared to bottom line on each respective graph.*

Food intake

OLZ administration had a significant overall effect on food intake ($F_{(1,66)}$ = 59.533, p < 0.001) and there was a significant OLZ x antibiotic interaction ($F_{(2,66)}$ = 3.294, p < 0.05). Time also had a significant effect ($F_{(3,198)}$ = 35.820, p < 0.001) and there was a significant time x OLZ ($F_{(3,198)}$ = 9.075, p < 0.001);time x antibiotic (F(6,198) = 12.348, p < 0.001) and time x OLZ x antibiotic interaction ($F_{(6,198)}$ = 4.23, p < 0.001).

Post-hoc analysis revealed that animals receiving VAN+VEH had reduced food intake compared to VEH+VEH over days -5-0 (p < 0.01).

Following commencement of olanzapine treatment, animals receiving OLZ+VEH displayed increased food intake compared to VEH+VEH treated rats in all subsequent time bins ($p < 005$ to $p < 0.01$) (Fig 5.2).

Animals receiving RIF+OLZ displayed increased food intake compared to RIF+VEH treated rats during days 8-14 ($p < 0.05$) but had decreased intake compared VEH+OLZ treated rats over days 15-20 ($p < 0.05$). Similarly, RIF+VEH treated rats had decreased food intake compared VEH+VEH treated animals over days 15-20 ($p < 0.05$).

VANC+OLZ treated animals had increased food intake compared to VANC+VEH treated group over days 1-7 and 8-14 ($p < 0.001$) (Fig. 5.2)

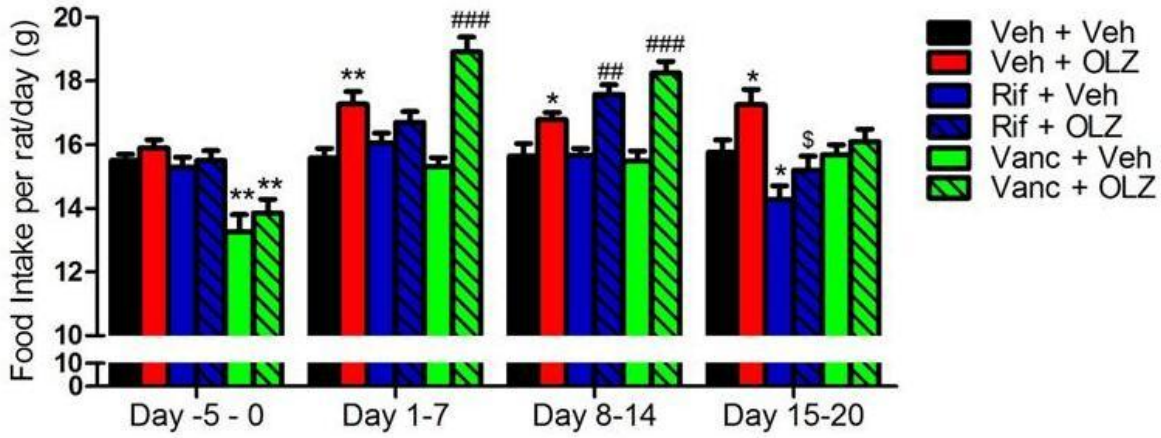


Fig. 5.2 . Effect of olanzapine, rifaximin and vancomycin on food intake. *Food intake over 5/6 day time bins following administration of vehicle (VEH)+VEH, VEH+olanzapine (OLZ), rifaximin (RIF)+VEH, RIF+OLZ, vancomycin (VAN)+VEH or VAN+OLZ in female rats. Data shown represents mean ± SEM. *p < 0.05, **p < 0.01, compared to VEH+VEH; ##p< 0.01, ###p < 0.001 versus animals receiving same antibiotic without olanzapine; $p < 0.05 versus Veh+OLZ.*

Tissue weights

Hepatic index

OLZ treatment had a significant effect on hepatic index (defined as liver weight as percentage of total body weight) ($F_{(1,52)}$ = 16.541, $p < 0.001$) as did antibiotic treatment ($F_{(2,52)}$ = 5.424, $p < 001$).

Post-hoc analysis revealed that VEH+OLZ treated rats had increased hepatic index compared to VEH+VEH treated animals ($p < 0.05$). Moreover, RIF+OLZ treated rats had reduced hepatic index compared to VEH+OLZ animals ($p < 0.05$). VAN+OLZ treated group also displayed reduced hepatic index versus VEH+OLZ treated rats while still being increased compared to VAN+VEH treated animals ($p < 0.05$) (Fig. 5.3A).

Uterine fat

OLZ treatment had a significant effect on uterine fat weight ($F_{(1,52)}$ = 9.382, $p < 0.01$) as did antibiotic treatment ($F_{(2,52)}$ = 6.354, $p < 0.01$). Further analysis revealed VEH+OLZ treatment increase uterine fat compared to VEH+VEH as well compared to RIF+OLZ and VAN+OLZ treatments ($p < 0.05$). VAN+VEH treatment also displayed reduced uterine fat mass compared to VEH+VEH (Fig 5.3B).

Caecal content mass

OLZ had a significant overall effect ($F_{(1,52)}$ = 9.232, $p < 0.01$) as did antibiotic treatment ($F_{(2,52)}$ = 157.211, $p < 0.001$). Post-hoc analysis revealed VEH+OLZ treated rats had reduced caecal content compared to VEH+VEH treated rats ($p < 0.05$) as well compared to RIF+OLZ treated animals ($p < 0.05$).

VAN+VEH treated rats had increased caecal content compared to VEH+VEH ($p < 0.001$) and VAN+OLZ had significantly increased caecal content compared to VEH+OLZ treated animals (Fig. 5.3C).

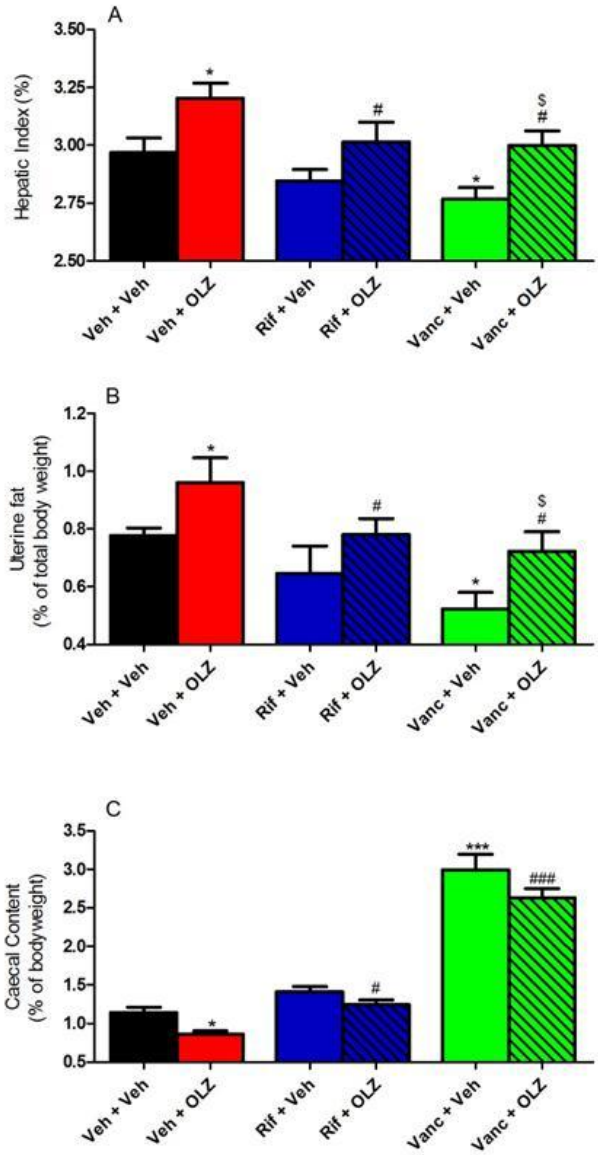


Fig. 5.3. Effect of olanzapine, rifaximin and vancomycin on specific tissue weights. *Effect of olanzapine (OLZ) co-administered with rifaximin (RIF) or vancomycin (VAN) on (A) hepatic index (B) uterine fat and (C) caecal content. Data shown represents mean ± SEM. *p < 0.05, ***p < 0.001 versus VEH+VEH; #p < 0.05, ###p < 0.001 versus Veh+OLZ; $p < 0.05 Vanc+Veh*

Short chain fatty acids (scfa)

OLZ administration did not have an overall effect on any of the short chain fatty acids measured. However antibiotic administration significantly affected acetate ($F_{(2,49)}$ = 260.535, $p < 0.001$), propionate ($F_{(2,52)}$ = 17.11, $p < 0.001$) and n-butyrate ($F_{(2,50)}$ = 75.05, $p < 0.001$). Further analysis revealed that VAN+ VEH or VAN+OLZ administration resulted in significantly reduced levels of acetate, propionate and n-butyrate compared to VEH+VEH and VEH+OLZ, respectively ($p < 0.01$)(Fig. 5.4).

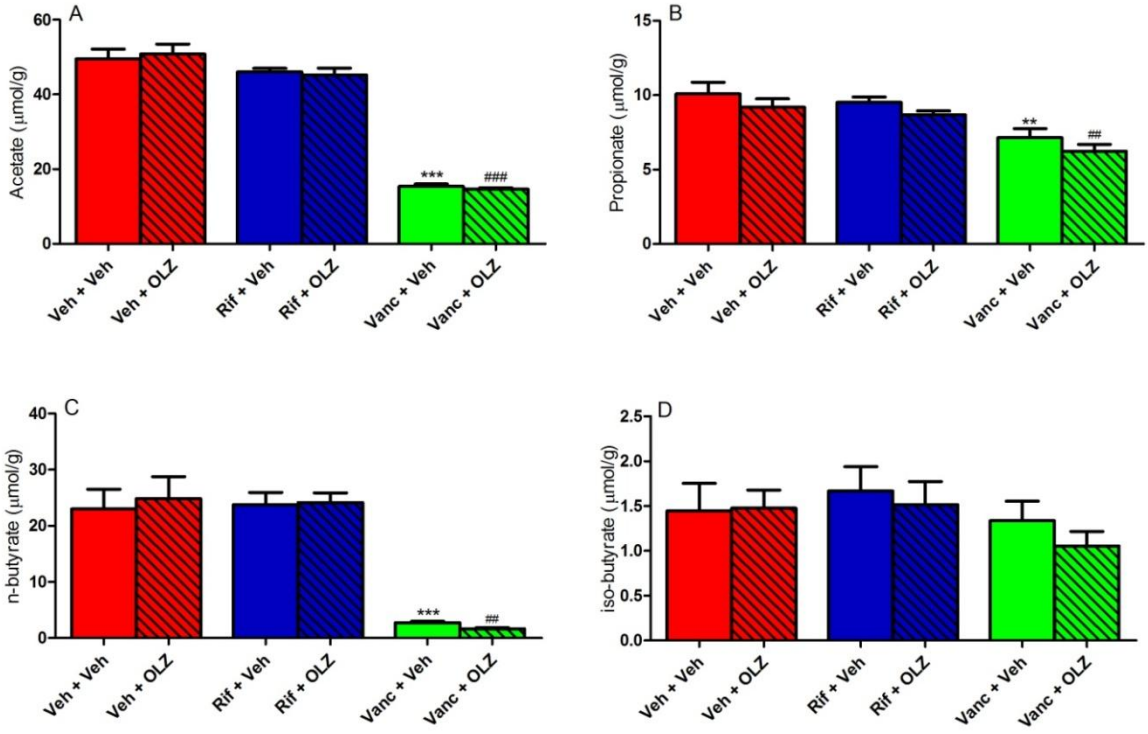


Fig. 5.4 Effect of olanzapine, rifaximin and vancomycin on short chain fatty acid concentrations. *Effect of olanzapine (OLZ) co-administered with rifaximin (RIF) or vancomycin (VAN) on (A) hepatic index (B) uterine fat and (C) caecal content. Data shown represents mean ± SEM. **p < 0.01, ***p < 0.001 versus VEH+VEH, ##p < 0.01, ###p < 0.001 versus VEH+OLZ*

Adipose Genes

OLZ significantly affected cannabinoid 1 receptor (CB1R) expression ($F_{(1,51)}$ = 7.127, p < 0.01). Post-hoc analysis showed that VEH+OLZ treated rats had significantly increased CB1R mRNA levels compared to VEH+VEH treated group (p < 0.01). However, animals receiving RIF+OLZ or VAN+OLZ did not display such increases and had significantly lower expression compared to VEH+OLZ treated animals (p < 0.05) (Fig. 5.5A).

Olanzapine also had a significant effect on fatty acid synthase expression (FAS) ($F_{(1,52)}$ = 5.653, p < 0.05). Post-hoc analysis revealed VEH+OLZ treatment led to increased FAS expression compared to that of VEH+VEH (p <0.01). Furthermore, administration of RIF+OLZ reduced FAS expression compared to VEH+OLZ (p < 0.01) while VAN+OLZ displayed a similar trend (p = 0.06) (Fig. 5.5B).

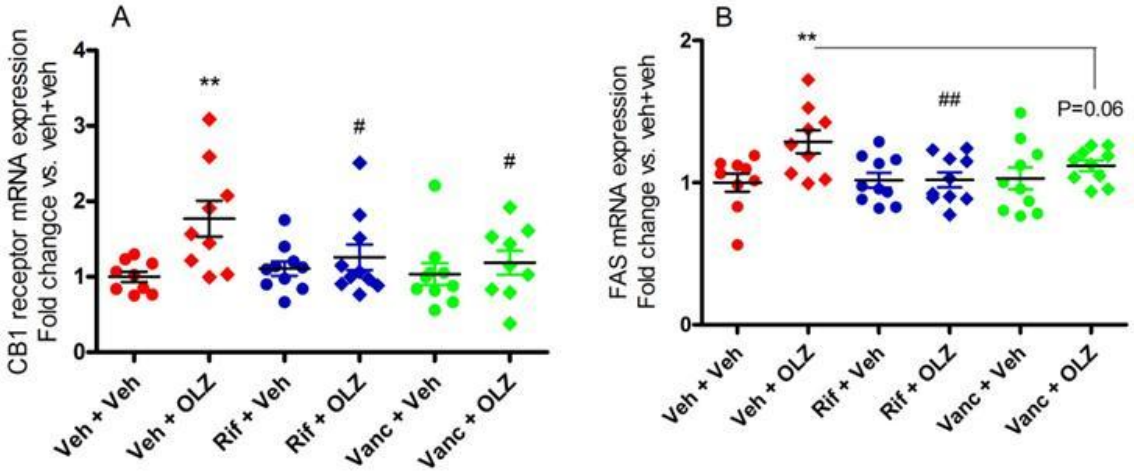


Fig. 5.5 Effect of olanzapine, rifaximin and vancomycin on gene expression in adipose tissue. *The effect of olanzapine (OLZ) or co-administered with vehicle (VEH), rifaximin (RIF) or vancomycin (VAN) on mRNA expression of (A) Cannabinoid 1 (CB1) receptor and (B) Fatty acid synthase (FAS) in adipose tissue. **p < 0.01 versus VEH+VEH, $^{\#}$p< 0.05, $^{\#\#}$p<0.01 versus VEH+OLZ.*

Liver Genes

OLZ had a significant effect on hepatic expression of fatty acid synthase (FAS) ($F_{(1,50)}$ = 5.641, $p < 0.05$). Further analysis revealed that VEH+OLZ treatment increased FAS expression compared to that of VEH+VEH ($p < 0.01$). RIF+OLZ however displayed reduced expression compared to VEH+OLZ ($p < 0.05$). VAN+OLZ treated rats also displayed increased expression compared to VAN+VEH treated animals ($p < 0.05$) (Fig. 5.6A).

Neither olanzapine treatment nor antibiotic treatment alone had an overall effect on PPARγ expression. However, there was significant olanzapine x antibiotic interaction ($F_{(2,51)}$ = 5.407, $p< 0.01$). Further analysis revealed that VEH+OLZ treatment increased PPARγ expression compared to that of VEH+VEH ($p < 0.01$). However, no such increase was observed in rats receiving either RIF+OLZ or VAN+OLZ which displayed reduced PPARγ expression compared to VEH+OLZ treated rats ($p < 0.01$) (Fig. 5.6B).

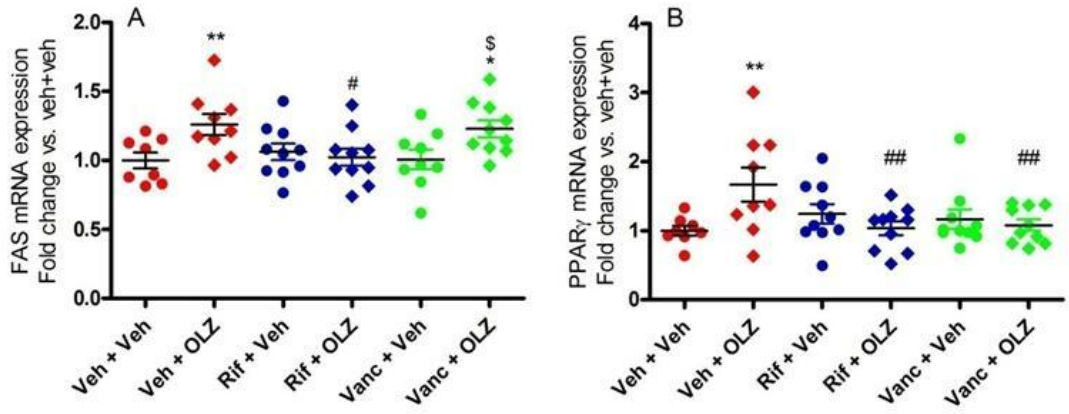


Fig. 5.6 Effect of olanzapine, rifaximin and vancomycin on hepatic gene expression. *Effect of olanzapine (OLZ) 2 mg/kg/d in female rats co-administered with rifaximin (RIF) or vancomycin (VAN) on hepatic expression of (A) fatty acid synthase (FAS) or (B) Peroxisome proliferator-activated receptor gamma (PPARγ). Data shown represents mean ± SEM. *p < 0.05, **p < 0.01 compared to VEH+VEH, #p < 0.05, ##p<0.01 versus VEH+OLZ, $p< 0.05 versus VAN+VEH*

Gut Microbiota

Pyrosequencing of the caecal flora revealed shifts in the composition of the microbiota at both the phyla and family level. As expected, antibiotic treatment resulted in an altered flora. Surprisingly, RIF+VEH treated animals had a seemingly unaltered abundance of the major phyla, *Firmicutes* and *Bacteriodetes* (Fig 5.7A). However, at the phyla level, there was an emergence of *Verrucomicrobia* (Fig 5.7B), as well as a 7-fold increase in the abundance of Actinobacteria (Fig 5.7B). Additionally, at the family level, there were increases in Bifidobacteriaceae and Rikenellaceae, albeit in lesser represent families (Fig 5.7D).

Vancomycin treatment alone (VAN+VEH) resulted in a profoundly different microbiota with changes in the predominant phyla including reductions in the abundance of Firmicutes and a bloom of Tenericutes (Fig 5.9A). At the family level, Vancomycin also induced several alterations including substantial reductions in the abundance of Lachnospiraceae and Ruminococcaceae and concomitant increases in Lactobacillaceae (Fig 5.9C) and Alcaligenaceae (Fig. 5.9D).

Olanzapine altered the caecal microflora to a lesser degree, with increases in the *Actinobacteria* (7-fold) phylum observed (Fig 5.9B). At the family level, olanzapine treatment resulted in a number of increases in the abundance of lesser represented families including Alcaligenaceae (3 fold), Bifidobacteriaceae (5-fold) and Peptostreptococcaceae (4-fold).

Intriguingly, administration of olanzapine in addition to either antibiotic resulted in alterations not observed in the VEH+VEH or respective antibiotic treatment groups. RIF+OLZ treated rats did not display increased abundance of the *Actinobacteria* phylum despite both olanzapine and rifaximin increasing levels of this phylum independently (Fig. 5.9B). Furthermore, RIF+OLZ treated animals had several alterations compared to VEH+OLZ treated animals at the family level including a reversal of the increases in Peptostreptococcaceae and Bifidobacteriaceae, as well as increased abundance of Prevotellaceae (Fig 5.7D).

Similarly, VAN+OLZ treated animals displayed a number of discrete alterations compared to the VEH+OLZ treated group. As observed in the VAN+VEH group, VAN+OLZ treatment resulted in reductions in the abundance of *Firmicutes* and a bloom in *Tenericutes* (Fig 5.7A). Additionally VAN+OLZ treatment was associated with a unique reduction on the family Prevotellaceae (Fig 5.7D).

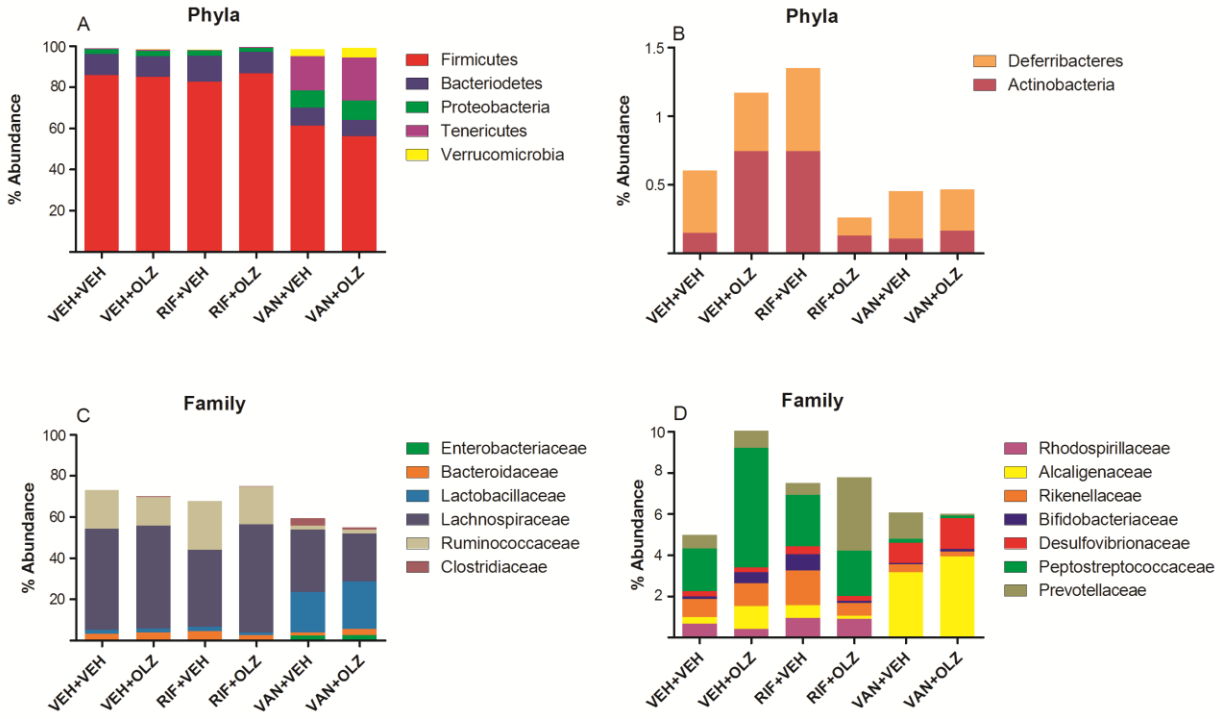


Fig. 5.7 Composition of the caecal microbiota. *The composition of the caecal microbiota in female rats treated with vehicle (VEH) + VEH, VEH + olanzapine (OLZ), rifaximin (RIF) + VEH, RIF+OLZ, vancomycin(VAN)+ VEH and VAN + VEH.(A) Abundance of major phyla (B) Abundance of lesser represented phyla (C) Abundance of major families (D) Abundance of lesser represented families.*

5.4 Discussion

In the present study, we demonstrate that olanzapine-induced weight gain in the rat is attenuated by the non-absorbable antibiotic rifaximin. Moreover, both rifaxamin and vancomycin were able to attenuate other aspects indicative of metabolic dysfunction, including, uterine fat deposition and expression of lipogenic genes. Crucially, rifaximin and vancomycin are almost entirely non-absorbable, and were administered *per os*, therefore the observed effects were almost certainly microbiota-driven. These novel findings, in conjunction with previous reports from our lab, further open the possibility that the gut microbiota represents a viable therapeutic target for antipsychotic-induced weight gain and metabolic dysfunction.

Olanzapine treatment resulted in shifts in the abundance of certain members of the gut microbiota, although primarily in less represented phyla and families. These changes at the phylum level did not mirror previously observed shifts (Davey et al. 2012). However, the current report measured caecal microbiota as opposed to faecal microbiota as previously reported, and that these two sites differ is well established (Pang et al. 2012). The alterations in the caecal microbiota observed with olanzapine were subtle but reduced caecal content observed in these rats suggests that these changes were physiologically relevant. Moreover, increased abundance of *Actinobacteria* as seen with olanzapine treatment has been reported to correlate with obesity in humans (Turnbaugh et al. 2009). This said, the alterations observed were minimal in terms of the overall composition of the flora and it remains possible that small alterations observed are secondary to other metabolic effects of olanzapine.

Regardless, manipulation of the flora that produced a beneficial therapeutic effect may be clinically relevant. It must also be remembered that the gut microbiota is a complex ecosystem and the entire metagenome acts in concert to influence the physiology of the host and hence subtle changes could potentially have knock-on and broader consequences.

As one would expect, antibiotic treatment effected the composition of the microbiota. The two antibiotics had vastly different effects on the gut flora although this is unsurprising as they act by different mechanisms and certain bacteria are susceptible to one but not the other (Lundstrom and Sobel 2004; DuPont 2011).

Intriguingly however, administration of rifaximin or vancomycin in conjunction with olanzapine resulted in a number of differences in the microbiota profile compared to olanzapine treatment alone. Moreover, the changes in microbiota observed between rifaximin and vancomycin co-administered with olanzapine were markedly different from one another, and this may explain there divergent effects on body weight gain.

Rifaxamin prevented the increased abundance in Actinobacteria that occurred with olanzapine administration suggesting this phylum may have obesogenic potential. Vancomycin did not ameliorate olanzapine-induced weight gain, despite preventing diet induced obesity in mice (Murphy et al. 2012). Reasons for this discrepancy may be species related, however, it must be noted that vancomycin did attenuate other metabolic effects of olanzapine which may be more clinically relevant than body weight gain per se.

Aside from weight gain, both antibiotics were able to prevent a number of metabolic alterations induced by olanzapine including fat deposition and expression of lipogenic genes. We investigated if differences in energy extraction may underlie such effects. While rifaxamin did not affect scfa concentrations, vancomycin had a dramatic effect on acetate, propionate and n-butyrate. Thus, it is possible that the overlapping effects of rifaximin and vancomycin occurred via different mechanisms with reduced energy extraction central to those of vancomycin but not rifaximin.

Prevention of increases in visceral fat by both antibiotics is in line with several reports showing that the microbiota can influence fat deposition (Backhed et al. 2004; Turnbaugh et al. 2006; Backhed et al. 2007; Murphy et al. 2010).

One potential mechanism linking the microbiota to adiposity previously identified is via the cannabinoid 1 receptor (Cani et al. 2010).

In support of this link, we found that both rifaximin and vancomycin prevented increases in the expression of the cannabinoid 1 receptor. The CB1 receptor is known to impact on gut permeability and blockade of the CB1 receptor prevents obesity (Gary-Bobo et al. 2007). Moreover, selective alterations in the microbiota using prebiotics have shown to alter the expression of CB1R in adipose tissue (Muccioli et al. 2010). Given CB1R's identified role in promoting lipogenesis in both liver and adipose tissue (Cota et al. 2003; Osei-Hyiaman et al. 2005; Gary-Bobo et al. 2006), it is extremely interesting that olanzapine increased CB1R mRNA in adipose tissue. Furthermore, the prevention of this increase by rifaximin and vancomycin highlight this as potential mechanistic link between the antibiotic therapy and reduced visceral fat accretion.

Additionally, FAS was found to be upregulated by olanzapine administration in both liver and adipose tissue. FAS is an enzyme that plays an integral role in lipogenesis and thus seems to be involved in increased de novo lipogenesis in hepatic tissue and the subsequent increased storage of energy as triglycerides that contributes to olanzapine-induced fat deposition. Both vancomycin and rifaximin prevented increases in the expression of FAS suggesting a direct mechanism whereby alterations of the gut microbiota can influence accumulation of visceral fat.

The expression of another lipogenic enzyme, PPARγ, was found to be increased in the liver of olanzapine treated rats. PPARγ is adipogenic and increased expression is indicative of a fatty liver and metabolic dysfunction (Gavrilova et al. 2003). However no such increases were observed when olanzapine was co-administered with vancomycin or rifaximin. This indicates that manipulation of the gut flora by either antibiotic affected lipogenic pathways that prevented or attenuated metabolic dysfunction. These effects may be secondary to effects on visceral fat as prevention of fat deposition may have prevented at the spiral of metabolic consequences associated with expanding adipose tissue.

Importantly, neither antibiotic reduced the expression of these genes when administered alone.

This suggests an interaction whereby the microbiota can affect both sides of the energy balance equation and under conditions of metabolic dysfunction act in such a way as to prevent processes that contribute to metabolic disturbances.

This view point is supported by this and other reports in which antibiotic administration can reduce body weight gain under certain conditions (Cani et al. 2008; Murphy et al. 2012) whereas the agricultural food industry has utilised antibiotics as growth promoters for generations (Kamphues 1999).

It must be remembered that the gut microbiota is a complex ecosystem and correlating shifts in individual phyla with changes in metabolic outcomes of the host may represent an oversimplified approach to understanding how dysbiosis of the gut microbiota drives physiological outcomes. However, what is clear is that alterations in the composition of the gut microbiota can have considerable consequences for the host. The precise mechanisms underpinning the microbiota's role in attenuating the effects of olanzapine as observed in this study remain to be determined, though further understanding of host-microbiota cross talk may identify pathways that allow the microbiota to influence adipose and liver tissue physiology.

More importantly, further development of prebiotic and probiotics that can affect the gut microbiota without the known drawbacks of antibiotics will open the door to utilising the therapeutic potential of the gut microbiota in a range of diseases (Clarke et al. 2012)and this may extend to antipsychotic-induced metabolic dysfunction.

These data therefore suggest that the gut microbiota is an important contributing factor in certain aspects of the metabolic dysfunction induced by olanzapine and that manipulation of this microbial organ offers a potential approach to ameliorate such dysfunction.

Chapter 6

Risperidone-induced metabolic dysfunction is attenuated by the non-absorbable antibiotic rifaximin in the rat

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Abstract

Background: Antipsychotic associated weight gain and metabolic dysfunction is a serious clinical concern. The mechanisms underlying these complications are multifactorial, with central and peripheral mechanisms involved. The gut microbiota can contribute to energy balance and body weight through a variety of direct and indirect mechanisms and we have recently demonstrated that olanzapine causes an altered microbiota in the rat, and that antibiotics can attenuate certain olanzapine-induced metabolic effects. As such, the gut microbiota is a potentially novel mechanism and therapeutic target in antipsychotic-induced weight gain.

Methods: Female Sprague-Dawley rats received risperidone (1 mg/kg/d) or vehicle via twice-daily I.P. injections for 3 weeks. Additional groups also received rifaximin (30 mg/kg) in addition to vehicle or risperidone, *per os*. Body weight and food intake were measured daily. Caecal microbiota profile was assessed by 454 pyrosequencing.

Results: Risperidone administration caused rapid weight gain and was also associated with increased visceral fat and elevations in mRNA expression in adipose and liver tissues indicative of inflammation and metabolic dysfunction. Co-administration of the antibiotic rifaximin did not attenuate risperidone-induced weight gain but did prevent other aspects of metabolic dysfunction including fat deposition.

Conclusions: These results further strengthen the link between the gut microbiota and metabolic dysfunction and more specifically that the flora may be a viable therapeutic target for antipsychotic-induced metabolic dysfunction.

6.1 Introduction

Atypical antipsychotics are the mainstay treatment for schizophrenia. However, several atypical antipsychotics, including risperidone, are associated with several metabolic side effects including weight gain, increased visceral fat and insulin resistance (Bobes et al. 2003b; Chintoh et al. 2009b; Albaugh et al. 2012). These side effects are a significant clinical problem due to their contribution to co-morbidities such as Type II diabetes and cardiovascular disease in a patient population already at increased risk of such disorders (Allison et al. 2009; Starrenburg and Bogers 2009; Li et al. 2011). Furthermore, these side effects contribute to the alarmingly high rate of non-adherence to treatment observed in schizophrenic patients (Ascher-Svanum et al. 2008).

The mechanisms underpinning these side effects are several and converging, comprising both central and peripheral effects that produce increases in food intake, body weight, adiposity and glucose dysregulation leading to insulin resistance (Minet-Ringuet et al. 2006; Fell et al. 2007; Scheen and De Hert 2007; Cooper et al. 2008b; Chintoh et al. 2009a; Albaugh et al. 2011a). To date, there has been very limited success in attempts to prevent or attenuate antipsychotic-induced metabolic effects.

The gut microbiota has been recognised in recent years as an important contributor to metabolism in both healthy and disease states (Uribe et al. 1994; Souza et al. 2004; Abt and Artis 2009; Turnbaugh et al. 2009). Indeed, the gut microbiota can affect both sides of the energy balance equation (Turnbaugh et al. 2006; Cani and Delzenne 2009).

As such, the gut microbiota has received increasing attention in investigations of both the pathogenesis and potential treatment of several metabolic related disorders including obesity and Type II Diabetes (Cani et al. 2007a; Membrez et al. 2010; Rabot et al. 2010).

We therefore recently investigated the potential contribution of the microbiota to antipsychotic-induced weight gain and metabolic dysfunction, and demonstrated that olanzapine alters the faecal microbiota of rats (Davey et al. 2012) and antibiotic manipulation of the flora can attenuate certain aspects of olanzapine-induced metabolic dysfunction (Davey et al., submitted; chapter 5).

The gut microbiota has the potential therefore to be a novel therapeutic target for the prevention or amelioration of antipsychotic-induced metabolic dysfunction and weight gain. However, whether this is case for antipsychotics other than olanzapine needs to be established.

We therefore investigated if risperidone, an atypical antipsychotic associated with metabolic dysfunction and weight gain (Wirshing et al. 2001; Farwell et al. 2004; Lin et al. 2006) affects the gut microbiota in rats. Moreover, we investigated if rifaximin, a non-absorbable, semi-synthetic antibiotic (Scarpignato and Pelosini 2005) could attenuate any observed metabolic dysfunction.

6.2 Methods

Animals

Female Sprague-Dawley rats, 6 weeks old and weighing approximately 200g were used (Harlan, UK). Animals were allowed to habituate to the facility for 10 days. Animals were housed 5 per cage (56x38x17 cm) and allowed access to standard chow and water *ad libitum.* Animals were maintained on a 12h light dark cycle, lights on 7.30 am. All experiments were approved by the Animal Experimentation Ethics Committee (AEEC) of University College Cork and carried out in accordance with the Cruelty to Animals Act 1876 and European Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

Drug Administration

Risperidone (RISP) (Discovery Fine Chemicals, UK) was dissolved in a minimal amount of glacial acetic acid (approx 0.1ml), made to volume with sterile water and pH adjusted to 6.0 with 0.1M NaOH. Animals received risperidone, 1 mg/kg/d, or vehicle (VEH) via interperitoneal injection, B.I.D. Vehicle consisted of sterile water acidified with 0.1 ml of glacial acetic acid and pH adjusted to 6.0 with 0.1M NaOH. Drug solutions were prepared fresh daily and injections took place between 9:00 and 10:00 am and between 4:00 and 5:00 pm. Doses of risperidone and gender of animals were selected based on previous studies in which they were found to best represent the clinical setting in terms of side effects (Fell et al. 2007).

Rifaximin (RIF), 30 mg/kg, was administered daily, *per os* in a volume of 4 ml/kg beginning 5 days prior to vehicle or risperidone treatment. The dose of rifaximin was selected based on studies in our laboratory in which this dose attenuated certain metabolic effects of olanzapine (Davey et al., in preparation).

Locomotor Activity Test

To assess possible levels of sedation, rats were placed in clear plastic boxes (60x50x40 cm) for 30 minutes and recorded using tracking software (Ethovision, Noldus, Netherlands). The test was carried out two days prior to the end of the study and animals were moved to the testing room one hour before the start of the test.

Sample Collection

Following sacrifice, uterine fat was quickly and carefully dissected and weighed to the nearest 0.001g. Caecal contents and the liver were removed and weighed. Trunk blood was collected in EDTA coated tubes and centrifuged at 6000 rpm for 15 minutes at 4^{o}C. Plasma supernatant was then aliquoted and frozen. A sample of uterine fat, frontal lobe of the liver, caecal contents and a portion of the soleus muscle were collected and snap-frozen. All samples were stored at -80^{o}C for later analysis.

Microbiota Analysis

For analysis of the microbial community composition, total DNA was extracted from caecal contents, collected aseptically immediately following sacrifice, using the QIAamp DNA stool mini kit (Qiagen, West Sussex, UK), coupled with an initial bead-beating step.

Universal 16SrRNA primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region, ie the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were used for Taq-based PCR amplification. Sequencing was performed on a Roche 454 GS-FLX using Titanium chemistry by the Teagasc454 sequencing platform. Resulting reads were quality trimmed, clustered, aligned and checked for chimeras using the Qiime suite of tools. A phylogenetic tree was generated using the FastTree package and principal coordinate analysis (PCoA), measuring dissimilarities at phylogenetic distances based on unweighted Unifrac analysis, was performed with Qiime.

Plasma analysis

Endotoxin was measured using the commercially available ToxinSensor chromogenic LAL assay (Genscript, U.S) following manufacturer's instructions. Briefly, plasma samples were diluted and heated in pyrogen-free tubes before adding of limulus amebocyte lysate (LAL) and incubated for 45 minutes at 37^{O}C. Chromogenic substrate is then added and samples incubated at 37^{O}C for a further 6 minutes. Stop solution is then added and absorbance measured at 535 nm. Samples were analysed in duplicate.

Gene expression analysis

Total RNA was extracted using a commercially available kit (Qiagen, US). mRNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, US) in a G-Storm thermocycler (G-Storm, UK).

Gene expression was analysed by qualitative real-time PCR using TaqMan Gene expression assays and the AB7300 system (Applied Biosystems). The expression of each gene was normalised to β-actin. All samples were analysed in triplicate.

Statistical analysis

Data are expressed as mean ± SEM. Body weight change and food intake were analysed using two-way repeated measures ANOVA. Two-way ANOVA was used for analysis of uterine fat, gene expression. Where a significant overall effect was observed, further analysis was carried with Fisher's Least Significant Difference test. Given the number of groups, N numbers and anticipated effect sizes, LSD was considered an appropriate post-hoc test.

6.3 Results

Body weight and food Intake

RISP treatment significantly increased body weight ($F_{(1,31)}$ = 13.422, p =0.01). There was no significant overall effect of RIF treatment. RISP only treated animals displayed greater weight gain compared to vehicle treated animals on days 4-11 and days 13-22 (inclusive) ($p < 0.05$-$p < 0.001$) (Fig.6.1A). The RISP+RIF treated group displayed greater weight gain compared to RIF+VEH treated rats on days 5-22 (inclusive) ($p < 0.05$-$p < 0.01$) (Fig.6.1B).

RISP also significantly increased food intake ($F_{(1,44)}$ = 70.73, $p < 0.001$). This hyperphagia was evident compared to VEH+VEH treated animals during all weeks of risperidone administration ($p<0.05$-$p < 0.01$) (Fig. 1C). Similarly, RISP+RIF also caused increases in food intake compared with RIF+VEH treated animals ($p < 0.05$-$p < 0.001$) (Fig.6.1C).

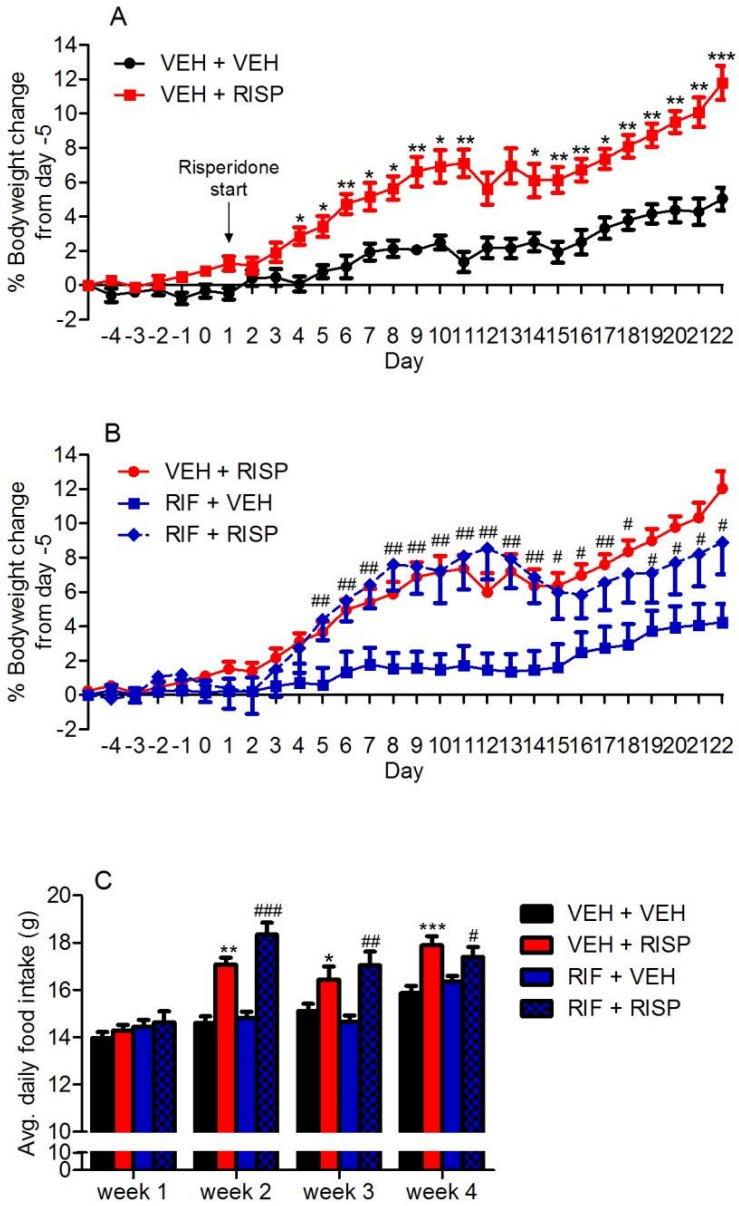


Fig. 6.1 Effect of risperidone and rifaximin on body weight and food intake. *Effect of chronic risperidone (RISP) (1 mg/kg) on body weight gain in female rats (A) Effect of risperidone co-administered with rifaximin (RIF) (30 mg/kg) on body weight gain (B) and effects of risperidone co-administered with rifaximin (RIF) (30mg/kg) or vehicle (VEH) on food intake (C). Data expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to VEH + VEH. $^{\#}$p< 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p<0.001 to RIF + VEH.*

Locomotor activity

RISP treatment had a significant effect on locomotor activity ($F_{(1,35)}$ = 4.098, p = 0.05). Overall, RISP+VEH treated rats had significantly reduced locomotion compared to VEH+VEH treated animals (p < 0.05) (Fig 6.2A). Further analysis revealed that the RSIP+VEH treated rats had reduced locomotor activity between 0-5, 15-20 and 25-30 minutes respectively (p < 0.05) (Fig 6.2B).

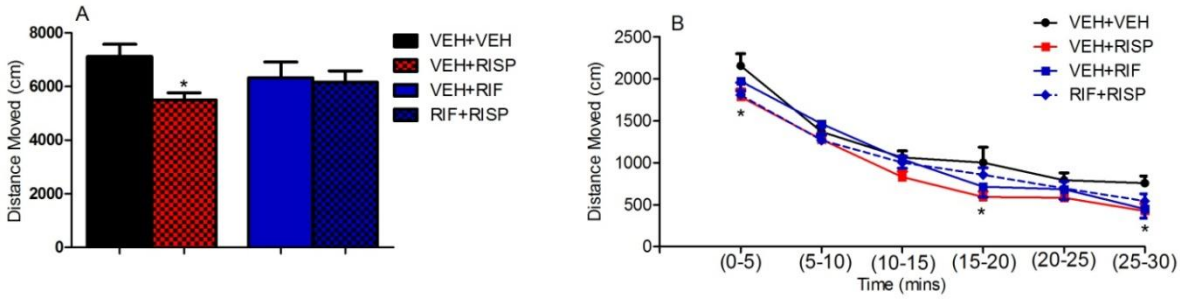


Fig. 6.2 Effect of risperidone and rifaximin on locomotor activity. *(A) Total distance moved over a 30 minute time period following administration of risperidone (RISP) (1 mg/kg/d) co-administered with rifaximin (RIF) (30mg/kg/d) or vehicle (VEH)(B) Distance moved over 5-minute time bins following administration of risperidone (1 mg/kg/d) with or without co-administration of rifaximin (30mg/kg/d). Data represent mean ± SEM.*P < 0.05 versus VEH+VEH group.*

Tissue weights

Uterine fat

RISP significantly increased uterine fat mass ($F_{(1,35)}$ = 27.087, p < 0.001) while RIF treatment was associated with a significant reduction in uterine fat ($F_{(1,35)}$ = 4.417, p < 0.05). Moreover, there was a significant RISP x RIF interaction ($F_{(1,35)}$ = 7.259, p < 0.05). RISP treated animals had significantly greater uterine fat mass compared to vehicle treated animals (p < 0.01) as well as those receiving RIF+RISP (p < 0.05) (Fig. 6.3A).

Liver

RISP treatment resulted in significantly increased liver weight ($F_{(1,35)}$ = 9.177, $p < 0.01$) whereas RIF administration was associated with an overall significant reduction in liver mass ($F_{(1,35)}$ = 10.952, p < 001). RISP+VEH treated rats had increased liver weight compared to both the VEH+VEH ($p < 0.01$) and RIF+RISP ($p < 0.01$) groups (Fig. 6.3B).

Caecal contents

RISP administration was not associated with a significant effect on caecal content mass, however, RIF treatment was associated with a significant overall increase ($F_{(1,35)}$ = 10.269, $p < 0.01$). RIF+RISP treated animals had greater caecal mass compared to risperidone only treated group (p <.01) (Fig. 6.3C).

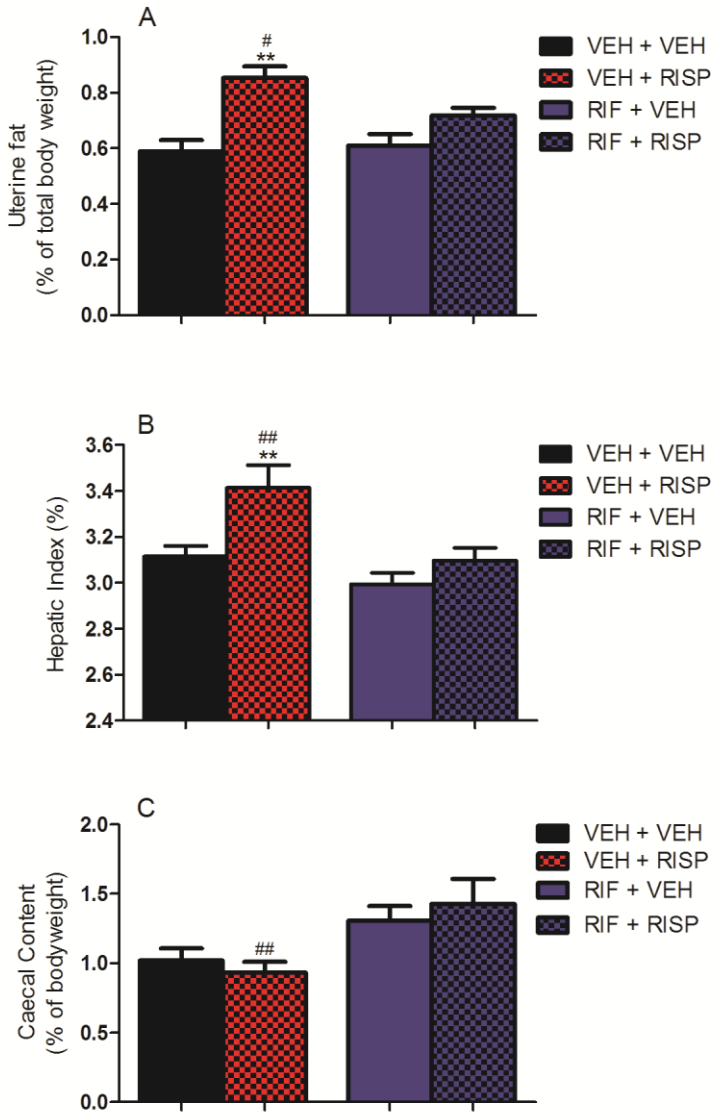


Fig. 6.3 Effect of risperidone and rifaximin on specific tissue weights. *Effect of risperidone (RISP) (1 mg/kg) co-administered with rifaximin (RIF) (30 mg/kg) or vehicle (VEH) on (A) uterine fat mass percentage (B) hepatic index (liver weight percentage) and (C) caecal content mass. Data expressed as mean ± S.E.M. **p < 0.01 versus VEH+VE, $^{\#}$p< 0.05, $^{\#\#}$p < 0.01 versus RIF + RISP.*

Inflammatory markers

Plasma endotoxin

RIF had a significant impact on plasma endotoxin levels ($F_{(1,14)}$ = 5.043, p < 0.05) (Fig. 6.4A). A number of samples in the VEH+RIF and RIF+RISP groups were found to be below the limit of detection. Post-hoc analysis revealed that the RIF+RISP treated animals had reduced levels of circulating endotoxin compared with RISP+VEH treated animals.

Adipose gene expression

RISP treatment had a significant overall effect on CD68 mRNA expression in adipose tissue ($F_{(1,34)}$ = 7.832, p < 0.001). Post hoc analysis revealed that VEH+RISP treatment resulted in increased expression compared to VEH+VEH treated rats (p< 0.05) and moreover this was significantly reduced by co-administration of rifaximin (Fig. 6.4B). No significant difference was observed in the expression of IL-6 mRNA (Fig. 6.4C).

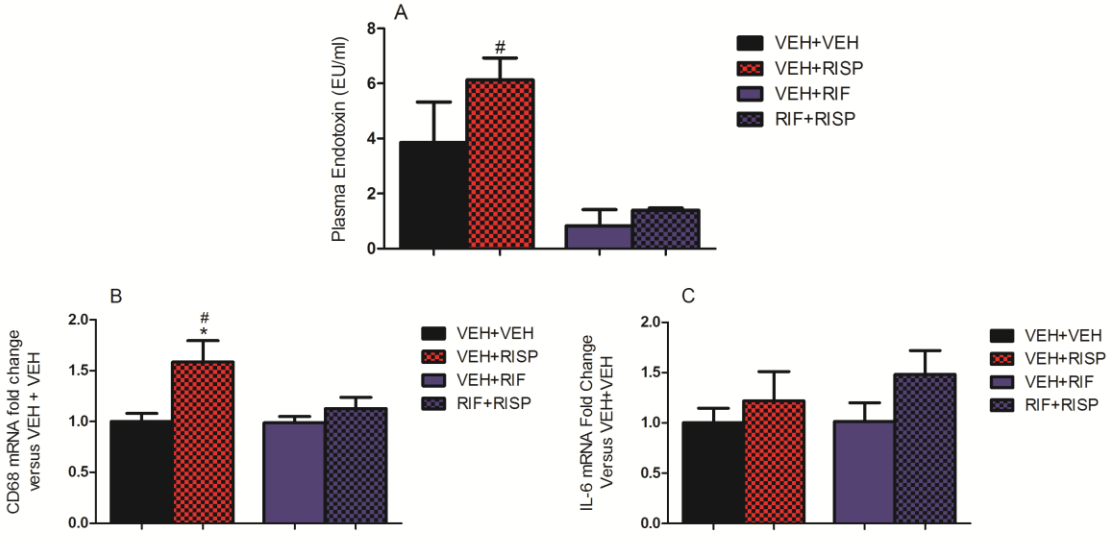


Fig. 6.4 Effect of risperidone and rifaximin on inflammatory markers. *Effect of risperidone (RISP) (1 mg/kg/d) co-administered with rifaximin (RIF) (30 mg/kg) or vehicle (VEH) on (A) plasma endotoxin (B) Adipose tissue expression of CD68 and (C) Adipose tissue expression of IL-6. Data expressed as mean ± SEM. *p < 0.05 versus VEH + VEH, $^{\#}$p < 0.05 versus RIF+RISP.*

Toll-like receptor 4 (TLR 4) Expression

Adipose Tissue

RISP treatment was associated with a significant effect on toll-like receptor (TLR)-4 expression in adipose tissue ($F_{(1,34)}$ = 4.24, $p < 0.05$). Post-hoc analysis revealed that risperidone treated animals had reduced TLR-4 expression compared to both VEH+VEH treated rats and those receiving RIF+RISP (Fig. 6.5A).

Skeletal Muscle

RISP treatment was not associated with an overall effect on TLR-4 expression in muscle tissue, however, rifaximin treatment resulted in increased expression levels ($F_{(1,34)}$ = 9.412, $p < 0.01$). RIF+RISP treated animals displayed increased expression compared to those receiving VEH+RISP ($p < 0.05$) (Fig.6.5B).

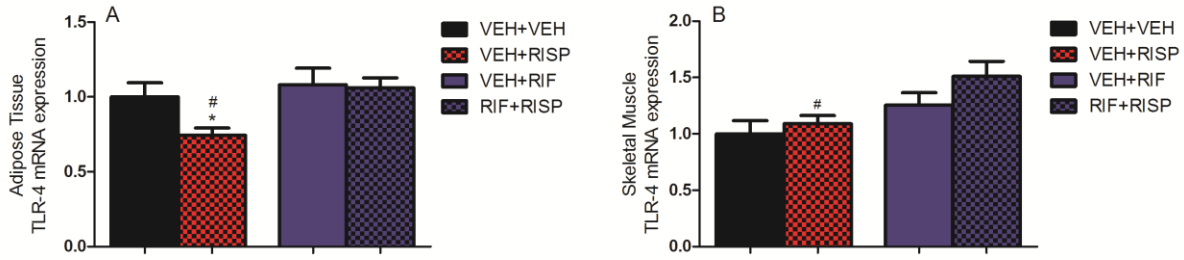


Fig. 6.5 Effect of risperidone and rifaximin on TLR-4 expression. *Effect of chronic risperidone (RISP) co-administered with rifaximin (RIF) (30 mg/kg) or vehicle (VEH) on TLR-4 mRNA expression in (A) Adipose tissue and (B) Skeletal Muscle tissue. Data expressed as mean ± SEM. *p < 0.05 compared to VEH + VEH, #p< 0.05 compared to RIF+RISP.*

Gut microbiota

Pyrosequencing revealed that risperidone treatment caused subtle alterations in composition of the caecal microbiota. Risperidone administration did not affect the major phyla (Fig. 6.6A) but was associated with increased levels of the phyla *Actinobacteria* (3.5 fold) (Fig. 6.6B). Additionally, at the family level risperidone treatment was associated with reduced abundance of Lachnospiraceae compared with vehicle treatment (52% versus 40%, respectively) (Fig. 6.6C). Animals receiving rifaxamin co-administered with risperidone treatment did not display either of these alterations and also had increased levels of *Terenicutes* (5 fold) and an absence of *Verrucomicrobia* (Fig. 6.6B).

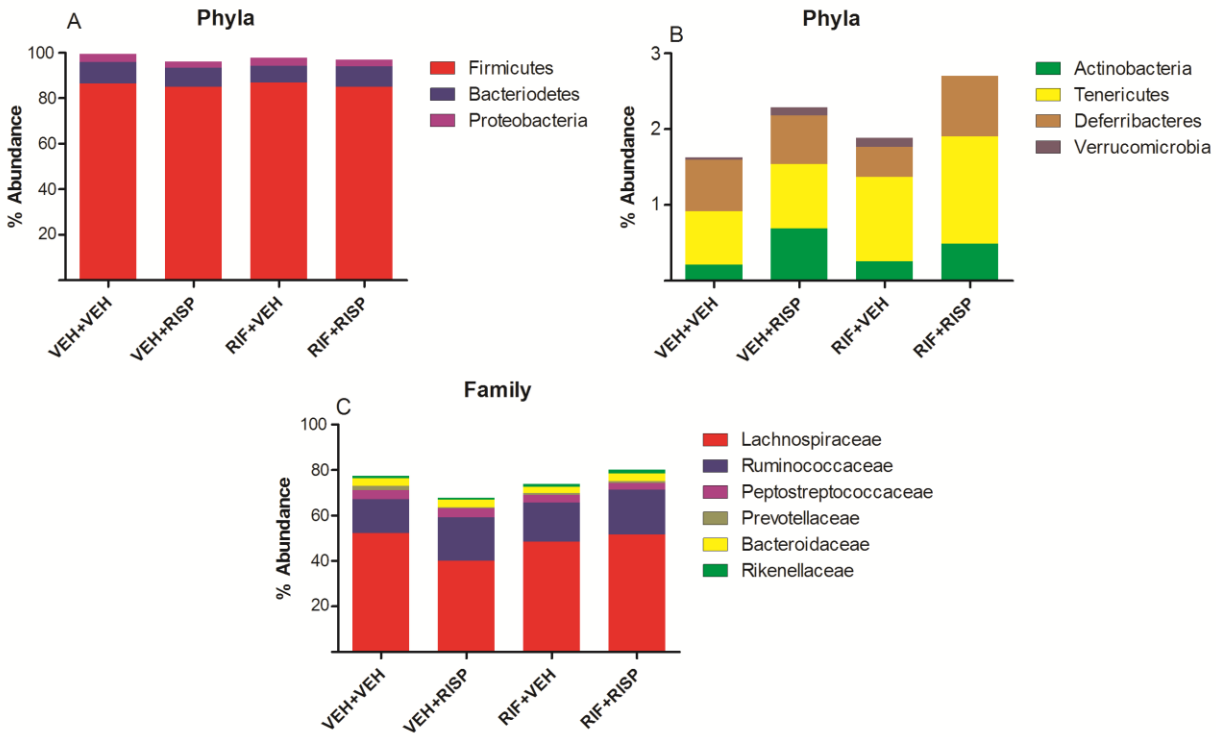


Fig. 6.6 Composition of the caecal microbiota. *Effect of risperidone (RISP) co-administered with rifaximin (RIF) (30 mg/kg) or vehicle (VEH) on (A) Major phyla (B) Less represented phyla and (C) Major families of bacteria.*

6.4 Discussion

In the present study, risperidone induced raid weight gain and hyperphagia in female rats in line with previous reports (Fell et al. 2007). This weight gain was associated with increases in both adipose tissue and liver weight signifying metabolic dysfunction. Rifaximin, a non-absorbable antibiotic, was able to attenuate several metabolic abnormalities induced by risperidone including accretion of visceral fat and macrophage infiltration. This further strengthens the evidence that the microbiota may be an important factor contributing to the metabolic effects of atypical antipsychotics and furthermore that it may be a novel therapeutic target for ameliorating such effects.

Rifaximin did not, however, attenuate risperidone-induced weight gain as was previously observed for the antipsychotic olanzapine (Davey et al., submitted; chapter 5). The lack of effect of rifaximin on risperidone-induced weight gain may reflect differences in the mechanisms underlying metabolic-dysfunction between olanzapine and risperidone. Risperidone and olanzapine have previously been shown to have divergent effects on metabolic parameters including triglycerides, adiponectin and cholesterol, potentially explaining the difference observed in this study compared to the previous study of olanzapine (Scheen et al. 2010; Wampers et al. 2012).

However, as in our previous reports with olanzapine (Davey et al, *in preparation; chapter 5*), rifaximin did lead to a reduction in both uterine fat mass and liver weight compared to drug treated animals. This supports the hypothesis that the microbiota contributes to the dysfunction in energy regulation observed with antipsychotic treatment.

These findings are in line with data from other animal models of metabolic dysfunction such as diet induced obesity and genetic obesity in which the gut microbiota has been shown to be crucial for the development of obesity and that antibiotic treatment can improve metabolic health in such models (Backhed et al. 2004; Cani et al. 2008)

In the present report, the non-absorbable antibiotic rifaximin was able to attenuate risperidone induced increases in uterine fat.

Expansion of visceral fat is a central step in the development of the metabolic syndrome through its role as an endocrine organ (Mohamed-Ali et al. 1998; Galic et al. 2010). Inflammatory processes are well established as being central in obesity and metabolic dysfunction (Dandona et al. 2004; Bastard et al. 2006b) and visceral adiposity is central to this increased inflammatory tone observed in obesity. Hence, reductions in visceral fat seen with antibiotic treatment may have important clinical implications, especially given that rifaximin also reduced expression of the macrophage marker CD68. Macrophage infiltration of adipose tissue is central to the proinflammatory response of expanding adipose tissue. Thus, reductions in CD68 suggest that rifaximin was able to prevent macrophage infiltration, an effect that was likely secondary to preventing increased fat mass.

Therefore, manipulation of the gut flora may be able to reduce visceral fat accretion, thereby ameliorating the proinflammatory response associated with risperidone and preventing ensuing metabolic anomalies. Support of this is also seen in the liver, where risperidone was associated with increased liver weight, indicative of fatty liver which was not the case in animals co-administered rifaximin.

Though not statistically significant, there was a trend for increased endotoxin levels in risperidone treated rats, suggesting metabolic endotoxemia may have contributed to the observed metabolic dysfunction. Further evidence of a reduced inflammatory phenotype was evidenced by reduced levels of circulating endotoxin which were associated with rifaximin treatment. Endotoxin is known to be associated with metabolic dysfunction in rodents and reduced levels have been associated with improvements in metabolic phenotype of mice (Cani et al. 2007a; Cani et al. 2008; Cani and Delzenne 2009)

LPS, the endotoxin, is a ligand for the TLR-4 receptor and is proposed to be involved in the relationship between inflammation and metabolic dysfunction (Shi et al. 2006). Contradictory to this theory, we found reduced expression of TLR-4 in adipose tissue in response to risperidone. This down-regulation may reflect negative feedback over the course of chronic administration. Interestingly, no such reduction was seen in animals receiving risperidone co-administered with rifaximin, suggesting that antibiotic treatment was able to prevent the metabolic disturbance that led to decreased levels following risperidone administration.

Risperidone alone was associated with subtle alterations in the microbiota. Intriguingly one such change was increased abundance of the phyla Actinobacteria, which was previously associated with obesity in humans (Turnbaugh et al. 2009) and was also found to be increased following olanzapine administration (Davey et al. in preparation; chapter 5).

The specific contribution of changes in a single phylum or family is extremely difficult to discern as the microbiota represents a complex ecosystem. The entire population ultimately makes up the metagenome of the microbiota which impacts on the health of the host and several functions are conserved across the different phyla. However, these findings highlight the possibility that dysbiosis of the gut microbiota may be a clinically relevant feature of antipsychotic therapy and that the microbiota may therefore represent a viable therapeutic target for addressing metabolic effects of these drugs.

Of course, whether shifts in the microbiota are secondary to other metabolic effects of antipsychotic compounds remains to be determined. Regardless, manipulation of the gut flora may be beneficial due to effects on factors involved in the mechanisms of antipsychotic-induced metabolic dysfunction.

Of course, in humans targeting the flora is made more difficult by the heterogeneous diets of patients as well as effects of sex, location and age, all of which impact on the make-up of the gut microbiota (Wu et al. 2011; O'Toole 2012). However, advancements in development of probiotics and prebiotics for a range of disorders (Clarke et al. 2012) may one day allow for targeted and effective use of gut flora as tool to tackle not only antipsychotic-induced metabolic dysfunction but the obesity epidemic in general.

Chapter 7

General Discussion

7.1 Overview and summary

In this thesis, we have demonstrated that antipsychotic-induced metabolic adverse effects are associated with alterations to the gut microbiota composition. Moreover, we have demonstrated that modulation of the gut microbiota can prevent or attenuate certain aspects of the metabolic dysfunction associated with these compounds, including body weight gain. This research has therefore opened the door to the possibility of microbiota-targeted therapeutics as adjunctive therapies for antipsychotic-induced metabolic side effects in the future, and also strengthens the evidence for such approaches in the treatment and prevention of obesity in general.

Summary of discussion topics	
Key topic	**Section**
Why are atypical antipsychotics still used given their known metabolic liabilities?	**7.2**
Can clinically relevant side effects of antipsychotics be modelled in animals?	**7.3**
What metabolic effects of olanzapine and risperidone were observed in our studies?	**7.4**
What alterations to the microbiota were observed in response to antipsychotics and what is their potential relevance	**7.5**
What effect does alteration of the gut microbiota have on antipsychotic-induced metabolic dysfunction and what mechanisms potential underlie any such effects	**7.6-7.8**
Does the microbiota represent a viable therapeutic target of the future for metabolic disorders and antipsychotic-induced side effects	**7.9**

Table 7.1. Summary of central discussion topics.

Antipsychotics represent the most effective tool available to tackle the devastating condition of schizophrenia. As such, they are set to remain the mainstay of treatment for

psychiatric disorders such as schizophrenia and bipolar disorder. However, the therapeutic benefits of these drugs come at a considerable metabolic cost to the patient.

Patients with schizophrenia have three times the mortality risk of the general population, and their life expectancy is reduced by 16-25 years (Brown et al. 2010; Tiihonen et al. 2011). As with any pharmacological intervention, the rewards must out-weigh the risks and antipsychotic medication reduces this mortality risk compared to untreated patients, as well as improving quality of life (Foley and Morley 2011). However, treated patients maintain a much greater risk of mortality compared to the general population, and while a large increase in the rate of suicide is a considerable contributor to this (Brown et al. 2010), in fact, cardiometabolic disease is the major cause of morbidity and mortality in patients with schizophrenia (Osby et al. 2000; Brown et al. 2010; Foley and Morley 2011). Indeed, up to 60% of deaths in schizophrenia are linked to metabolic disease (Osby et al. 2000). Moreover, the survival gap between schizophrenia patients and the general population is ever widening, making the physical health of the mentally ill an increasingly important issue (Capasso et al. 2008; Tiihonen et al. 2011).

There are several factors that contribute to the high rate of metabolic dysfunction in schizophrenia patients including a high rate of smoking, poor diet and physical inactivity. However, antipsychotics are associated with several aspects metabolic dysfunction including weight gain, increases in abdominal fat as well as lipid and glucose abnormalities (Farwell et al. 2004; Correll et al. 2009; Citrome et al. 2011b). Hence, the detrimental metabolic effects of antipsychotics makes this already vulnerable group at

even greater risk of developing co-morbidities such as type II diabetes mellitus and cardiovascular disease (Farwell et al. 2004; Correll et al. 2009; Citrome et al. 2011b).

Thus, it is has become apparent that tackling the causes of metabolic disease in schizophrenia patients is a clinical imperative, and ways to ameliorate the metabolic effects of antipsychotics is a top priority in this regard (Foley and Morley 2011; Tiihonen et al. 2011).

The gut microbiota plays a role in energy regulation and metabolism in normal physiology but also in pathological states such as obesity and type II diabetes (Backhed et al. 2004; Backhed 2011; Burcelin et al. 2011). Investigations into the involvement of the gut microbiota in body weight, fat deposition and related diseases have increased rapidly in recent years owing to technological advances in measuring and monitoring the gut microbiota (Fraher et al. 2012).

This increased research has also been fuelled by growing evidence supporting a substantial effect of the gut microbiota on aspects of metabolism in both animal models and humans (Backhed et al. 2007; Cani et al. 2007a; DiBaise et al. 2008; Armougom et al. 2009; Turnbaugh et al. 2009; Ding et al. 2010; Schwiertz et al. 2010).

Given the link between the gut microbiota and metabolic function and the documented metabolic dysfunction associated with antipsychotics, we set out to investigate if the gut microbiota may represent a novel mechanism involved in the metabolic side effects of certain atypical antipsychotics, and moreover, if the microbiota could potentially represent a therapeutic target for ameliorating such effects.

To this end, we observed that both olanzapine and risperidone alter the composition of the gut microbiota in rats. This is intriguing, especially given the lack of confounding factors such as diet or environment in our experiments, that are encountered in clinical studies. These changes in gut flora occurred in concert with other aspects of metabolic dysfunction commonly associated with these antipsychotics including weight gain, increased abdominal fat and evidence of abnormalities in lipid handling, glucose regulation and inflammatory processes. It is not currently possible to say whether these changes are cause or consequence of the ensuing metabolic dysfunction caused by the antipsychotics.

Furthermore, the changes in microbiota composition observed were relatively subtle and thus the true clinical relevance of the observed alterations remains to be determined.

This said, growing evidence suggests that increases and/or decreases in specific bacterial groups correlate with metabolic alterations and the diversity of the microbiota means differences across studies is to be expected.

The contribution of specific bacterial groups compared with the impact of the overall composition of the flora on metabolic systems remains unclear and understanding the link between microbial dysbiosis and metabolic phenotype of the host remains an ongoing challenge.

However, we also demonstrate that ablation of the gut flora using a cocktail of broad-spectrum antibiotics attenuates olanzapine-induced body weight gain as well as other

metabolic abnormalities. This provides evidence that the gut microbiota can influence, either directly or indirectly, antipsychotic associated metabolic dysfunction.

In further support of this, we show that a non-absorbable antibiotic, rifaximin, also ameliorates olanzapine-induced weight gain in the rat, albeit to a modest degree. Moreover, rifaximin, and another non-absorbable antibiotic vancomycin, attenuated metabolic dysfunction associated with olanzapine administration including increases in visceral fat, pro-inflammatory responses and alterations in the expression of lipogenic enzymes.

We have highlighted therefore that the gut microbiota is another factor to be considered when evaluating the milieu of metabolic complications induced by atypical antipsychotics.

More importantly, we have shown that the microbiota can be targeted therapeutically to ameliorate the ensuing weight gain and metabolic dysfunction, especially visceral fat deposition. Hence, this work opens the door for further studies utilising agents such as probiotics and prebiotics to help prevent or reduce the metabolic liability of antipsychotics and therefore improve patients' physical health and ultimately result in better long-term outcomes.

These findings have broader implications beyond antipsychotic-induced effects as obesity reaches epidemic proportions worldwide (Ogden et al. 2006; Berghoefer et al. 2008; de Onis et al. 2010). The complex interactions of metabolic and inflammatory pathways underpinning antipsychotic-induced metabolic dysfunction represent the same physiological pathways involved in obesity and associated co-morbidities in the general

population. Thus, our data strengthen the evidence for a critical role played by the gut microbiota in energy balance and support the development of microbiota based therapeutics for obesity prevention and treatment in the future

7.2 Advantages of atypical antipsychotics

Atypical antipsychotics are indicated for the treatment of first-episode schizophrenic patients (Mortimer '03). Thus, a large increase in the prescription of atypical over typical antipsychotics has taken place in recent times (Verdoux et al. '10). Given the adverse events associated with atypical antipsychotics, one could wonder why they continue to be the drugs of choice in first line treatment of schizophrenia. However, as is the case for any pharmacological intervention, clinicians must weigh all benefits against costs of any medication before assigning an antipsychotic (Meltzer 2001; Meltzer et al. 2002).

As previously mentioned, atypical antipsychotics were heralded as a breakthrough in the treatment of schizophrenia due to their reduced propensity to cause extra- pyramidal symptoms (EPS), and were even defined as a drug class based on this property. However, atypical antipsychotics seem to offer further advantages over their typical counterparts.

While not conclusive, studies indicate that atypical antipsychotics, including olanzapine, offer greater efficacy in terms of rate to respond and overall efficacy (Bobes et al. 2003a; Lieberman et al. 2003).

The prescribing of an antipsychotic agent is extremely patient specific based on the clinician’s assessment of a wide range of factors including medical history, age, sex and symptom profile. Thus, while a preference for the prescription of an antipsychotic with a

better metabolic-side effect profile, such as aripiprazole (Pae 2009), can be made, patient differences in response to alternative drugs means such alternatives may not be a suitable option in many cases. Hence, drugs such as olanzapine, clozapine and risperidone are set to remain valuable agents in the treatment of schizophrenia (Leucht et al. '09).

Several studies have indicated that olanzapine and other atypical antipsychotics offer benefits in improving cognitive deficits associated with schizophrenia (Keefe et al. 1999; Woodward et al. 2005). Cognitive impairment is a serious complication of schizophrenia and is inter-related with traditional negative symptoms (Sharma and Antonova 2003). Indeed, cognitive impairments remain a major barrier for societal integration, even when positive symptoms are under control. Atypical antipsychotics have recently been shown to be superior to typical antipsychotic in improving psychosocial aspects of cognition (Fujimaki et al. 2012).

In particular, atypical drugs have shown an ability to improve verbal fluency, fine motor functions and executive functions (Keefe et al. 1999). Thus, an ability of atypical antipsychotics to improve aspects of cognitive functions may be highly beneficial to the patient.

The mechanisms underlying such effects of atypical antipsychotics are unclear, though their diverse receptor pharmacology seems to be at the core. In particular, effects on the serotonergic system in discrete brain regions appears to be involved (Martyn 2005).

It should also be noted that not all studies have found beneficial effects of atypicals on cognition, although, given the complex nature of the disease and the heterogeniet of both patients and treatment regimens, this is perhaps unsurprising (Keefe et al. 2007).

Schizophrenia patients have an alarmingly high rate of non-adherence to treatment. Atypical antipsychotics are associated with better treatment adherence and persistence compared to typical drugs (Ascher-Svanum et al. 2006; Ascher-Svanum et al. 2008). Dropout rates from clinical studies as a result of adverse effects have also been found to be reduced with atypical compounds compared to typical counterparts (Martin et al. 2006).

Atypical antipsychotics, in particular olanzapine, clozapine and amisulpirde, are also purported to reduce negative symptoms in schizophrenia patients, a major advantage over typical drugs which do not treat, or even worsen, negative symptoms (Collaborative Working Group On Clinical Trial 1998; King 1998; Murphy et al. 2006; Curtis et al. 2008).

Mechanisms underlying these effects seem to include preferential effects of atypical drugs in the different dopamine pathways, in particular, increasing dopamine release in the pre-frontal cortex relative to subcortical areas which occurs with olanzapine and clozapine but not haloperidol (Li et al. '98).

At present, atypical antipsychotics therefore remain the preferred choice over typical drugs in many patients on the basis of not only reduced EPS liability but also the aforementioned advantages. Hence, drugs such as olanzapine, clozapine and risperidone

are set to continue to dominate the treatment of schizophrenia, emphasising the need to understand and prevent the metabolic side effects of these drugs.

7.3 Modelling antipsychotic-induced metabolic dysfunction in rodents

In order to reliably produce clinically relevant animal studies investigating the effects of antipsychotics, a number of challenges must be overcome. One such difficulty encountered with atypical antipsychotics is half-life. Antipsychotics have considerably shorter half-lives in rodents than in humans, reducing from between 24-48 hours in humans (Callaghan et al. 1999) to between 2-4 hours in rodents (Aravagiri et al. 1999). Hence, this issue must be addressed and the route of administration as well as dosing regimen must be carefully considered when designing preclinical studies using antipsychotics, and also when interpreting the results of such studies.

One potential solution that has been employed in some studies is the use of osmotic-mini pumps which are implanted subcutaneously at the start of a study, and steadily release a drug over a period of time.

While this may seem ideal at first, a number of antipsychotics, including olanzapine, are unstable in solution such that the potency of these drugs after the first few days becomes questionable (van der Zwaal et al. 2008).

Administration of antipsychotics via food or water has been employed in order to try and ensure consistent dosing (Albaugh et al. 2006). However, as a main effect of these drugs is increased food and indeed water intake, the treated animals would presumably

increase their dose automatically, possibly influencing experimental outcomes in studies interested in metabolic parameters.

In our studies, we administered the drugs in more than one dose each day i.e. twice-daily injection (B.I.D) in order to off-set the effects of the decreased half life. While this option was preferable to a single injection, pharmacokinetic studies have suggested that 4-daily injections would be required to match receptor occupancy profiles observed in humans, which would be logistically problematic to say the least (Kapur et al. 2003).

Overall, the use of rodents remains critical to investigating the side effects of antipsychotics and ways to counteract them. While not perfect, the methods employed in our investigations reflected the clinical situation as closely as possible in terms of dosing and administration protocols allowing for potentially clinically- relevant conclusions to be drawn.

7.3.1 Species differences in susceptibility to antipsychotic side effects

Of course, preclinical work must be translational in order to draw any clinically relevant conclusions. We have investigated the impact of antipsychotics in both mice and rats and found that mice were resistant to antipsychotic-induced weight gain.

This phenomenon has been observed previously (Arjona et al. 2004; Albaugh et al. 2006). This may have been due to inadequate dosing or duration of treatment, as, in some cases, mice have shown a propensity to gain weight when treated with atypical antipsychotics at considerably higher doses than those used in our studies (Cope et al. 2005). However,

evidence of metabolic dysfunction was observed in mice, including alterations in fat deposition.

Importantly, as mice failed to display a propensity for weight gain in response to olanzapine as is seen clinically, we subsequently focused our work in rats. This precluded the use of germ-free mice, a common model for investigating the influence of the gut microbiota in disease states.

Furthermore, germ-free rats facilities are an extremely rare commodity for several practical and logistical reasons, thus alternative approaches to germ-free were utilised in our investigations.

We subsequently found that rats incur more robust and more reproducible responses to antipsychotic administration in terms of weight gain and metabolic dysfunction in line with several other reports (Cooper et al. 2005; Fell et al. 2007; Fell et al. 2008).

The reason for this species difference remains unclear, although the important factor is which most reliably provides a template for the clinical setting. Olanzapine and risperidone both produced rapid hyperphagia and increases in body weight reflecting the clinical situation.

Thus rats were used in further studies, allowing for clinical relevant investigations of ways to ameliorate certain metabolic effects associated with atypical antipsychotics.

7.3.2 Sex-specific responses to antipsychotic treatment

In addition to species differences, we observed a definitive effect of sex on olanzapine-induced weight gain in rats and also fat deposition in mice. This has previously been observed (Albaugh et al. 2006; Albaugh et al. 2010) though the reasons and implications of this has not been fully explored. This anomaly in relation to sex effects have led some authors to suggest that the present animal models of antipsychotic induced weight gain are not relevant to the clinical situation, hence rendering any conclusions invalid (Pouzet et al. 2003). However, additional metabolic effects associated with these drugs in the absence of weight gain, mean that weight gain *per se* is not essential for a valid animal model of antipsychotic-induced side effects.

These effects include increased fat accumulation, dyslipidemia and hyperglycemia (Coccurello et al. '06; Minet-Ringuet et al. '06; Lykkegaard et al. '08; Albaugh et al. '10), which correlate with clinical setting demonstrating that rodent models are appropriate for studying the mechanisms of these side effects (Weston-Green et al. 2011b).

In line with this, we found that female mice and male rats both showed signs of metabolic dysfunction without the occurrence weight gain, in line with recent reports (Victoriano et al. 2009; Albaugh et al. 2010).

Sex differences in the side effects incurred by patients remain somewhat unclear. A review by Aichhorn et al 2007, found the incidence of weight gain and the metabolic syndrome to be more frequent in females and that this correlated with higher drug plasma levels in women for olanzapine and clozapine. This strongly indicates the existence of a sex difference; however another study of olanzapine found that males had

a higher frequency of weight gain (Basson et al. 2001a) and a number of clinical studies have found been inconclusive (Haack et al. 2009).

Several other factors have been found to be associated with increased risk of antipsychotic-induced weight gain. These include baseline weight and genetic factors which may therefore mask, or indeed emphasise, a sex bias (Basson et al. 2001a).

A number of mechanisms potentially underlie the sex bias observed in preclinical studies. Male rats display a much steeper growth curve compared to females and this may therefore represent a ceiling effect on body weight gain at in rats of this age.

Male gender, smoking and gene variants are all associated with lower corrected plasma concentrations, meaning females have higher circulating plasma levels for a given dose of an antipsychotic (Callaghan et al. 1999; Kelly et al. 1999; Seeman 2004). This is likely due to sex differences in metabolic enzyme systems such as cytochrome enzymes CYP3A4 and CYP1A2 (Parkinson et al. 2004).

CYP1A2 is the main metabolising enzyme for olanzapine, and is less active in women than men (Kelly et al. 1999; Gex-Fabry et al. 2003). Thus, the male rats in our experiments may have had lower circulating levels of olanzapine which may have accounted for their lack of weight gain in this and other studies. Moreover, sex differences in hepatic drug metabolism are more robust in rodents than humans (Waxman and Celenza 2003). Thus, the sex-specific differences in drug pharmacokinetics and pharmacodynamics may impact on side effect susceptibility in humans, and it is possible that these are reflected more clearly when translated to animals.

Of course, notable gender differences in hormones may influence the propensity to incur metabolic effects of antipsychotics, especially as olanzapine has known and considerable effects on the reproductive function in female rats (Fell et al. 2005). However, ovarectomised rats gain excessive weight when treated with olanzapine (Park et al. 2010), suggesting hormonal differences do not play a direct role in the observed sex-differences of antipsychotic-induced weight gain.

It should be noted that male rats can be induced to gain excessive weight when given high doses for long periods and in addition to a high-fat diet (Shobo et al. 2011).

Interestingly, Kluge et al, found a delayed onset of weight gain in male patients compared to females (Kluge et al. 2009) which is in someway reflected in the longer protocol required for weight gain in the recent study by Shobo and colleagues <Shobo, 2011 #225>. Therefore, we cannot rule out that the male rats may have shown increases in weight if treated for a longer period or given a higher dose.

In support of a clinically relevant effect of sex, we identified a number of physiological differences between the male and female rats that may have contributed to the divergence in response to antipsychotic treatment. These included baseline differences in circulating levels of the orexigenic hormone ghrelin, a difference that is mirrored in humans (Greenman et al. 2004).

These results therefore highlight the need for greater attention to be placed on appropriate prescribing of antipsychotics, taking sex into account (Seeman 2004). Patient monitoring following prescription of certain antipsychotics could also be tailored taking

sex into consideration, with women receiving more regular clinical assessment for the presence of metabolic dysfunction.

7.4 Metabolic effects of olanzapine and risperidone

Olanzapine and risperidone both induced metabolic effects in the rat reminiscent of those observed clinically (Table 7.2). These effects allowed us to assess whether novel potential mechanisms contributing to this metabolic dysfunction i.e. the gut microbiota

Parameter	Olanzapine	Risperidone
Body weight	↑↑↑	↑↑
Food intake	↑↑↑	↑↑
Locomotor activity	↓	↓
Liver weight	↑	↑
Visceral adiposity	↑↑	↑
Macrophage infiltration	↑	↑
Inflammation[#]	↑	↑
Insulin sensitivity	↓	-*
Microbiota	↑↓	↑↓

Table 7.2. Summary of the effects of olanzapine and risperidone in the rat. *[*]Measured as mRNA expression, cytokine levels or circulating endotoxin, [#]not measured in our studies but previously reported to be reduced (Chintoh et al. 2009a). Number of arrow indicates scale of change.*

As can be seen from Table 7.1, both drugs induced weight gain in rats, which was slightly greater for olanzapine, as is the case clinically (Allison et al. 1999; Foley and Morley 2011). Furthermore, both compounds induced evidence of metabolic dysfunction in line with

previous studies in rats and also reflecting the clinical situation (Lin et al. 2006; Smith et al. 2009; Lauressergues et al. 2010; Citrome et al. 2011b; Zugno et al. 2012). Hence, in our experiments, a valid template was established that allowed us to examine whether such dysfunction was also associated with dysbiosis of the gut microbiota.

7.5 Antipsychotic-associated alteration in the gut microbiota

Administration of olanzapine and risperidone was associated with shifts in certain microbial constituents of the gut microbiota. Initial investigations of the faecal microbiota of olanzapine treated rats highlighted a trend for shifts in the major phyla with increases in *Firmicutes* and concomitant decreases in *Bacteriodetes.* This finding was particularly exciting as this trend was first described as the 'signature' of an obese microbiome in seminal studies by Gordon and colleagues (Ley et al. 2005).

A similar trend was subsequently observed in a follow-up experiment. Strikingly, in this experiment, co-administration of the antibiotic cocktail resulted in a reversal of this shift, with decreased *Firmicutes* and increased *Bacteriodetes*. Thus, as the ratio of these phyla is thought to be indicative of an obese phenotype, this trend supports the hypothesis that an aberrant microbiota is a feature of antipsychotic induced weight gain.

Other studies have found a similar trend in humans (Turnbaugh et al. 2009). However, several recent reports have not identified this shift in ether animals or humans, and in some cases the opposite has been observed (Murphy et al. 2010; Schwiertz et al. 2010). Thus, it still remains to be determined if such a phylogenetic switch has a role to play in obesity in certain cases.

A lack of consistency across studies does not necessarily rule out an important influence of such shifts in phyla, as the direct effect of various shifts in phyla on the overall metabolic functioning of the gut microbiota is yet to be elucidated and studies to date have been primarily descriptive.

This means that the alterations in microbiota observed in different cohorts, while different from one another, could produce the same ultimate effects that impact on host energy balance. Hence, when trying to correlate a phenotypic trait as globally pervasive as energy metabolism, it may not be surprising that different studies show different results in terms of microbial populations correlating with obesity. This is particularly plausible as different phyla contain a large and heterogeneous population of bacteria and metabolic functions are conserved across diverse species. Hence, shifts in the major phyla may represent important indicators of obesity, or may indeed play an etiological role in a non-specific fashion (Cummings et al. 2004)

Perhaps unsurprisingly therefore, we did not observe the same shifts in the caecal microbiota of olanzapine treated rats. In fact, the caecal microbiota is known to differ considerably from the faecal microbiota (Pang et al. 2012).

We did however find more subtle changes in the caecal microbiota in the less represented phyla, including increased *Actinobacteria.*

Increases in *Actinobacteria* have been described previously in obese humans (Turnbaugh et al. 2009). Intriguingly, risperidone treatment resulted in similar increases in *Actinobacteria.*

Additional, albeit minor, changes in the gut flora were observed for both antipsychotics at the family level. Differences between control animals, as well as antipsychotics treated animals, were observed across studies.

This arguably makes interpretation of the results somewhat difficult. However, this in itself is not surprising considering the complex interplay between the intestinal microbiota and the immune system, endocrine system, energy homeostasis and lipid metabolism (Conterno et al. 2011).

Thus, it remains to be determined if antipsychotics such as olanzapine and risperidone directly affect the composition of the gut microbiota or whether such changes are a result of other metabolic abnormalities that then feed into the cycle of dysfunction associated with these drugs. Either way, these results suggest that antipsychotic therapy causes dysbiosis of the gut microbiota in conjunction with many other metabolic disturbances implying that the microbiota represents another aspect of metabolic liability associated with these compounds that requires investigation as a therapeutic target.

7.6 Ameliorating the metabolic side effects of antipsychotics

A number of drugs have been investigated as adjunctive therapies aimed at ameliorating the metabolic side effects of atypical antipsychotics. Of these, metformin, an anti-diabetic drug, is the only widely used clinically as it has been shown to be the most consistent (Maayan et al. '10). Reboxetine, a selective noradrenaline reuptake inhibitor, has also shown promise in attenuating olanzapine induced weight gain (Poyurovsky et al. '07).

A number of other co-therapies have been have shown promise recently as potential treatments to counteract the metabolic liabilities of these compounds (Table 7.3). None of the current therapies work in all patients and none completely attenuate weight gain (Maayan et al. '10). Thus, while these interventions may be useful tools for individual patients, they are not a long-term clinical solution at present.

Intervention	Mode of Action	Species	Main Finding	Reference
Metformin	Hepatic-insulin sensitizer	Human	Reduced olanzapine-induced weight gain in patients	(Wu et al. 2008)
Reboxetine	selective noradrenaline reuptake inhibitor	Human	Reduced olanzapine-induced increases in appetite and body weight	(Poyurovsky et al. 2007)
Naltrexone	Opioid antagonist	Rat	Ameliorated olanzapine-induced increases in body weight and food intake	(Kurbanov et al. 2012)
Betahistine	H1 agonists/H3 antagonists	Rat	Attenuated olanzapine-induced weight gain and reduced feeding efficiency	(Deng et al. 2012)
Omega-3 fatty acid	Effects on TG synthesis	Rat	Omega-3 deficiency augmented risperidone-induced metabolic effects	(McNamara et al. 2012)
Sibutramine	5-HT-NA reuptake inhibitor	Rat	Prevented olanzapine-induced motivation for palatable food	(van der Zwaal et al. 2012)
BGP-15	Heat shock protein co-inducer	Rat	Prevented olanzapine-induced weight gain insulin resistance	(Literati-Nagy et al. 2012)
Melatonin	Increases circulating levels of melatonin	Rat	Attenuated olanzapine-induced weight gain	(Raskind et al. 2007)
Mifepristone	Glucocrticod antagonist	Humans	Reduced olanzapine and risperidone- induced weight gain	(Gross et al. 2009; Gross et al. 2010)

Table 7.3 Therapies investigated as adjunctive therapies to antipsychotic to prevent metabolic side effects. *TG, triglycride, 5-HT, serotonin, NA, noradrenaline*

Co administration of other atypical drugs with little or no weight-inducing tendencies, such as ziprasidone or aripiprazole, have even been shown to attenuate the hyperphagic effects of olanzapine in rats, presumably via competitive effects on central receptors such as the serotonergic system (Snigdha et al. 2008).

Importantly, trends in developing newer antipsychotics are focused on narrowing the pharmacological profile of atypical compounds without altering efficacy and at the same time avoiding a return to EPS-like side effects seen with first-generation drugs. One such drug is lurasidone, which has no affinity for histamine H_1 or muscarinic M_1 receptors, but has high affinity for D_2 and 5-HT_{2A} receptors. Thus far, in clinical trials, lurasidone has shown comparable efficacy to ziprasidone with a seemingly negligible side effect profile (Meyer et al. '09). Asenapine has also been recently approved for acute schizophrenia and bipolar disorder, and while it does not seem to offer superior efficacy, it has displayed a promising side effect profile (Tarazi and Shahid '09). Aripriprazole is the first 'third generation' antipsychotic to be approved and has minimal effects on metabolism (Pae 2009).

Only time will tell if these drugs are suitable replacements for the current ensemble of atypical compounds, in particular in relation to long-term efficacy and tolerability.

Interestingly, nutritive therapy i.e. dietary changes, alongside exercise, can effectively attenuate antipsychotic-induced weight (Skouroliakou et al. 2009; Ball et al. 2011). As already mentioned, schizophrenia patients have poorer metabolic health regardless of

antipsychotic therapy and hence this form of intervention could be beneficial to the patients on more than one front. Increased exercise and improvements in diet could not only attenuate drug-induced side effects but allow the patient to have a healthier lifestyle additionally improving their long term outcomes in terms of morbidity and mortality.

Unfortunately however, as we have learned from the obesity epidemic in the general population, the use of exercise and good diet as a long term tool is easier said than done, and this is likely to prove even more difficult in a psychiatric patient population.

Whether therefore lifestyle intervention is a viable option in most cases is questionable, but of course should always be encouraged.

7.6.1 Microbial approaches to attenuate antipsychotic-induced metabolic side effects

In the past number of years, modulating the gut microbiota with antibiotics has been shown to reduce weight gain, improve insulin resistance and reduce inflammation in a number of experimental models including ob/ob and diet-induced obese mice (Cani et al. 2008; Membrez et al. 2008; Carvalho et al. 2012; Murphy et al. 2012). However, whether such effects extend to antipsychotic-induced weight gain and metabolic dysfunction such had not been explored.

In the studies described herein, we therefore employed three antibiotic based approached to investigate if the gut microbiota could be targeted therapeutically to attenuate the effects of the atypical antipsychotics, olanzapine or risperidone. The nature and effects of these approaches is summarised in Table 7.4.

Intervention	Bacterial target	Main findings
Antibiotic cocktail: neomycin, metronidazole, polymyxin B	Broad coverage of Gram-positive and negative anaerobes and aerobes	Attenuated olanzapine-induced weight gain & fat deposition Non-significant improvement in insulin resistance
Rifaximin	Gram positive and negative anaerobes	Attenuated olanzapine but not risperidone-induced weight gain Normalised expression of key lipogenic enzymes
Vancomycin	Gram positive, mainly Bacilli	Reduced scfa production, prevention of increased adiposity

Table 7.4 Antibiotic approaches to attenuate antipsychotic-induced metabolic dysfunction. *scfa, short-chain fatty acids*

We first used a cocktail of broad-spectrum antibiotics to ablate the gut flora and investigated if this reduced the metabolic liability of olanzapine. The cocktail of antibiotics employed are known to effectively destroy the gut flora of rats (Juno et al. 2003). The antibiotic cocktail not only altered the microbiota but also reversed the changes in the faecal microbiota associated with olanzapine treatment.

To ensure that any benefits of microbiota manipulation were in fact microbiota-driven, we subsequently used two non-absorbable antibiotics, administered *per os*, to further test our hypothesis.

Rifaximin and vancomycin produced vastly different effects on the gut flora, with vancomycin having much more profound effects. This is to be expected as the two compounds act via differing mechanisms and target different bacteria.

Vancomycin reduced the abundance of the major phyla *Firmicutes*, were as rifaximin did not. The more subtle effects of rifaximin most likely reflect its bacterial coverage in relation to the commensal flora. Nevertheless, both antibiotics caused significant alterations in the overall make-up of the microbiota as evidenced by principle co-ordinate analysis (data not shown).

As discussed earlier, correlating the specific effects of these antibiotics, or indeed ones used in other studies, with changes in host metabolism is likely an over-simplistic view of the complex interactions within the microbiota environment and the subsequent equally complex interaction of the microbiota with the host via immune endocrine and hormonal systems.

Indeed, as recent reports from the human microbiome project (http://www.hmpdacc.org/) indicates, what bacteria are there may not be as important as what genetic potential is present, i.e. the metagenome (Turnbaugh et al. 2007; Arumugam et al. 2011). As mentioned, several metabolic functions are conserved across bacterial groups making changes in the composition of the flora difficult to interpret. Studies of the metagenome have revealed that diseases such as type II diabetes correlate with not only changes in the composition of the flora but also with specific function such as reduced butyrate production and increased sulphate reduction (Qin et al. 2012).

7.7 Effects of antibiotic administration on antipsychotic-induced weight gain

In our experiments, antibiotic treatment attenuated or prevented several aspects of clinically-relevant metabolic dysfunction induced by the atypical antipsychotics, olanzapine and risperidone. This included the attenuation of olanzapine–induced weight gain by both a cocktail of antibiotics and the antibiotic rifaximin. Whether the scale of the reduction in weight gain observed reflects a clinically significant change is debatable, however these findings represent an exciting proof-of-principle highlighting that modulation of the gut flora can impact on olanzapine-induced weight gain. Other issues not fully resolved included the temporal effects on weight of the co-administration of antibiotics and the differences observed at different doses of olanzapine.

Atypical antipsychotics undoubtedly act centrally to increase appetite leading to hyperphagia and rapid weight gain, as has been seen several times in both animals, healthy volunteers and patients (Davoodi et al. 2009; Fountaine et al. 2010). Thus, the contribution of the gut microbiota through effects on peripheral mechanisms may be unable to influence these initial, central effects. This may explain the lack of an effect of antibiotics on body weight in the first week of olanzapine administration. In line with this possibility, the antibiotics did not ameliorate olanzapine-induced hyperphagia. Hence, it may be the case that the contribution of the microbiota cannot be used therapeutically until additional metabolic anomalies, stemming in part from weight gain, begin to occur.

The antibiotic cocktail had a lesser effect on body weight gain in rats receiving the higher dose (4 mg/kg) of olanzapine, compared to the lower dose (2 mg/kg).

While the reasons for this remain obscure, it is worth noting that in our studies, like others, a dose dependent effect of olanzapine was not observed in relation to weight gain (Citrome et al. 2009a). Indeed, similar to the clinical setting, olanzapine-induced weight gain plateaued after approximately two weeks. The antibiotic administration showed little effect on body weight when administered alone and in fact showed a trend for increasing body weight. Thus, it would seem that the contribution of the microbiota to normal energy homeostasis becomes apparent under situations of metabolic dysfunction. Hence, as the plateau effect was more evident for the higher dose, this may go some way to explain why the effects of the antibiotics were less robust at the 4 mg/kg dose.

Rifaximin attenuated olanzapine-induced weight gain while this was not the case for risperidone treatment. While initially surprising, this is perhaps not too unexpected as these drugs differ pharmacologically and this difference in pharmacology likely corresponds to differences in the peripheral mechanisms belying their respective metabolic effects. Indeed, a number of studies have found differential effects of risperidone and olanzapine on metabolic parameters in including triglycerides, adiponectin and cholesterol, suggesting divergent effects, particularly on adipose tissue physiology (Smith et al. 2009; Scheen et al. 2010; Smith et al. 2010; Wampers et al. 2012).

Unlike rifaximin, the antibiotic vancomycin did not reduce olanzapine-induced weight gain. This is in contrast to a recent report in which vancomycin attenuated weight gain in diet-induced obesity in mice (Murphy et al. 2012).

The reasons for this are unclear as the mechanisms involved in vancomycin's effects in the mouse model remain unclear. However, as will be discussed below, the microbiota can impact on host metabolism and energy balance in several ways, and the phenotypic outcome of this interaction is likely to be different under different paradigms of metabolic disease.

Crucially, it has been realised in recent times that antipsychotics such as olanzapine can have profound negative metabolic effects in the absence of over weight gain. This includes effects on adiposity as well as lipid and glucose regulation which contribute substantially to the cardiometabolic threat posed by these drugs. Hence, tackling these metabolic threats, in conjunction with, or even aside from, tackling body weight gain is extremely important.

7.8 Potential mechanisms of microbiota attenuation of antipsychotic-induced metabolic dysfunction

Our investigations have revealed that the gut microbiota can influence the metabolic liabilities of antipsychotics via several overlapping and converging mechanisms (Fig. 7.1). These mechanisms include nutrient handling, energy storage and inflammation (Fig 1.10).

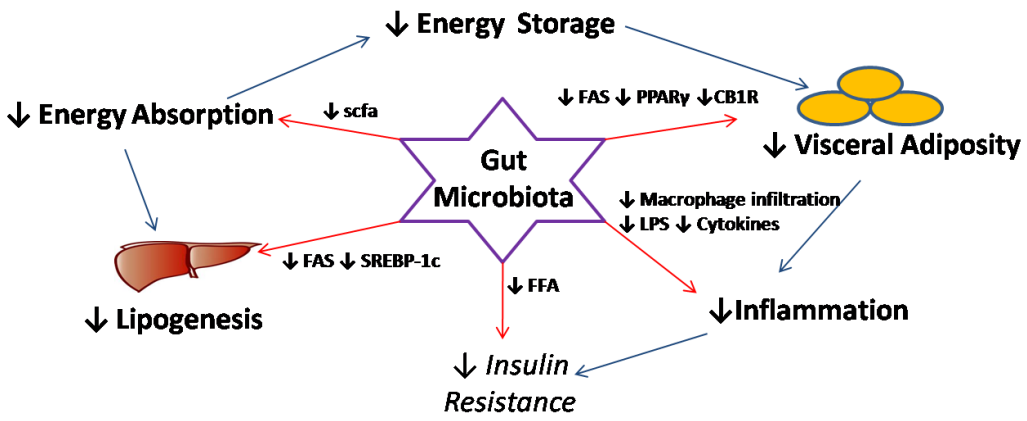


Fig. 7.1 Working model in which the gut microbiota may attenuate the metabolic dysfunction associated with antipsychotics based on our studies

7.8.1 Visceral fat accumulation

All three antibiotics used in our studies significantly attenuated or even prevented olanzapine-induced increases in peri-uterine fat, and rifaximin did the same for risperidone. This finding was remarkably consistent across studies and reinforces the potential of microbiota-targeted interventions have for antipsychotic-induced metabolic dysfunction.

The peri-uterine fat pad, along with the mesenteric and peri-renal fat, makes up the visceral fat of rats. Visceral fat is now recognised as much more than an energy repository and can be viewed as an endocrine organ as it is capable of producing and releasing hormones and cytokines, especially proinflammatory mediators (Mohamed-Ali et al. 1998; Trayhurn and Beattie 2001; Trayhurn and Wood 2004).

Increased release of proinflammatory mediators as well as free fatty acids from an expanding adipose mass put visceral fat at the centre of the spiral of metabolic dysfunction linking increased weight, inflammation and insulin resistance (Trayhurn et al. 2011).

A number of possible mechanisms have been shown to link the gut microbiota to adipose tissue development in different experimental models. In a series of elegant experiments, Backhed and colleagues showed that germ-free mice are resistant to diet-induced obesity and that this was due to two seemingly independent mechanisms (Backhed et al. 2007). Firstly, fasting-induced adipose factor (Fiaf) was reduced following conventionalisation of germ-free mice, leading to increased lipoprotein lipase (LPL) activity and subsequent triglyceride accumulation in adipocytes (Backhed et al. 2004). Secondly, inactivation of AMP-activated kinase (AMPK), a key cellular fuel gauge, was found to be reduced in the presence of the gut microbiota thereby indirectly promoting energy storage over utilisation (Backhed et al. 2007).

7.8.2 Lipogenesis

Central to the mechanisms of fat deposition is the process of lipogenesis, i.e. de novo synthesis of lipids which occurs primarily in the liver but also in adipose tissue itself. Sterol regulatory element binding protein-1c (SREBP-1c) is an insulin-sensitive, master transcription factor which, in concert with other genes, controls lipogenesis. SREBP-1c exerts this control via activation of lipogenic enzymes, in particular, fatty acid synthase (FAS) and acetyl-coA carboxylase (ACC) (Ferre and Foufelle 2007).

In our studies, we found that olanzapine increased the expression of FAS in both hepatic and adipose tissues. Moreover, antibiotic administration prevented these increases. Thus, the microbiota either directly or indirectly inhibited olanzapine's ability to drive lipogenesis, thereby reducing subsequent fat deposition.

One of the ways olanzapine leads to increased lipogenesis is via centrally driven increases in food intake. Increased food intake leads to increased delivery of carbohydrates to the liver where they are converted to lipids (lipogenesis) and subsequently stored as fat. Thus, given the microbiota's known role in energy extraction, altering the microbiota may have led to reduced nutrient absorption such that increased food did not equate to increased carbohydrate absorption. This is therefore one mechanism whereby manipulation of the microbiota could reduce fat deposition. This hypothesis is supported by the fact that antibiotic treatment alone did not alter FAS expression. In further support of a role of FAS, increased FAS expression has previously been implicated in risperidone induced metabolic dysfunction (Lauressergues et al. 2010)

Intriguingly, in the case of ACC the opposite phenomenon was observed, as only animals receiving olanzapine and the antibiotic cocktail had increased hepatic expression levels. While initially surprising, this also suggests that the impact of the microbiota was only apparent in the context of metabolic dysfunction. As in the case of FAS, effects on energy extraction could have impacted on ACC expression levels, and while reduced levels would be expected, in the presence of olanzapine-induced changes there may be competing or compensatory mechanisms that led to the observed increases.

In support of this theory, we also found increased expression of peroxisomal proliferator-activated receptor gamma (PPARγ) in the adipose tissue of olanzapine treated rats but unchanged levels in those also given an antibiotic.

PPARγ is the master regulator of adipogenesis (Bays et al. 2004), thus this finding is consistent with a microbiota-driven mechanism whereby reduced hepatic lipogenesis results in decreased delivery of triglycerides to the adipose tissue and no expansion of adipose tissue (adipogenesis).

7.8.3 Energy absorption

A major way in which the microbiota contributes to energy balance is via the fermentation of indigestible dietary fibres into short-chain fatty acids (scfa). The primary scfa produced are acetate, propionate and butyrate which account for approximately 80% of all scfa in the gut (Cummings et al. 1987). Butyrate is used primarily as the preferred energy substrate of the colonic epithelium (Roediger 1980). Acetate and propionate on the other hand are delivered to the liver where they affect hepatic lipid processing in opposite ways, with acetate a lipogenic substrate and propionate inhibiting lipogenesis (Conterno et al. 2011). Interestingly, these affects are mediated, at least in part, via affects on the expression of FAS (Agheli et al. 1998).

We therefore investigated if the antibiotics, rifaximin or vancomycin, impacted on the production of scfa by measuring the levels acetate, propionate and butyrate in the caecal content of treated rats. Surprisingly, vancomycin, but not rifaximin, dramatically reduced the concentration of scfa, especially acetate and butyrate.

Thus, this suggests that vancomycin administration through alterations in the gut flora resulted in not only reduced acetate, but also an increased propionate/acetate ratio thereby inhibiting hepatic de novo lipogenesis in olanzapine-treated animals and preventing increased fat storage.

This does however suggest that vancomycin and rifaximin prevented olanzapine-induced effects via separate mechanism. This is entirely possible and highlights the diverse ways in which the microbiota can influence host physiology. However, is should also be noted that while rifaximin did not alter the caecal concentrations of scfa, it could conceivably, have altered their absorption (Tremaroli and Backhed 2012).

Thus, while not fully elucidated, the microbiota's impact on energy absorption and subsequent effects on lipogenic enzymes appears strong enough to significantly attenuate olanzapine-induced lipogenesis and fat storage.

7.8.4 Insulin resistance

We also found that the co-administration of an antibiotic cocktail reduced the plasma levels of free fatty acids (FFA). Circulating free fatty acids are released from the adipose tissue as the storage capacity of adipocytes becomes reduced in the face of continued energy storage (overflow hypothesis).

Plasma free fatty acids are then stored in ectopic sites such as liver and muscle, exacerbating insulin resistance (Yki-Jarvinen 2002; Eckel et al. 2005). In addition, we found that antipsychotic treatment resulted in increased liver weight as a proportion of body weight, indicative of a fatty liver. Antibiotic treatment prevented such increases in line with the finding of reduced FFA.

It should be noted that the role of FFA in metabolic dysfunction is not entirely clear and the current concept is challenged by the fact the adipose tissue release of FFA mainly stems form subcutaneous fat not visceral fat (Karpe et al. 2011). This said, antipsychotics such as olanzapine effect lipid handling in both visceral and subcutaneous adipose tissue (Albaugh et al. 2010), such that our findings may also reflect similar dysfunction lipid handling in subcutaneous tissue though further studies would be required to confirm this

7.8.5 Inflammation

The microbiota's role in inflammation and metabolism also involves endotoxin, also known as lipopolysaccharide (LPS). LPS is a component of the cell wall in gram-neagative bacteria and increased plasma levels of LPS has been found to correlate with type II diabetes in humans (Creely et al. 2007). LPS is potently proinflammatory and increased levels contributing to metabolic dysfunction has been coined 'metabolic endotoxemia' (Cani et al. 2007a).

As already mentioned, the adipose tissue is now viewed as an endocrine organ which can play an active role in metabolic dysfunction. Thus, the prevention of antipsychotic-induced increases in visceral fat could have indirectly produced other beneficial effects observed.

The adipose tissue is responsible for the release of proinflammatory cytokines but does so in concert with, and in response to, macrophages, which infiltrate the adipose tissue in conditions of obesity or metabolic dysfunction (Xu et al. 2003). This infiltration is seen as a key step in development of obesity-related inflammation which contributes to ensuing insulin resistance and metabolic dysfunction (Xu et al. 2003).

The infiltration of macrophages into adipose tissue is thought to be a response to signals released from dying adipocytes as the adipose tissue hypertrophies (Cinti et al. 2005).

Thus, it is highly likely that the reduction in macrophage infiltration observed in animals co-administered an antibiotic was an indirect result of preventing the expansion in adipose mass.

In addition, we observed that antibiotic treatment attenuated systemic inflammation induced by olanzapine, in particular TNF. This too was most likely a result of preventing visceral fat expansion and subsequent lack of macrophage infiltration. Over the course of our studies, we did however observe inconsistent effects of olanzapine on circulating cytokines. A key difference between these studies, however, was that in one case the animals were fasted and in another they were not. Fasting is known to have acute effects on the levels of cytokines, in particular in cases of underlying metabolic dysfunction such as type II diabetes (Esposito et al. 2002). Hence, the effects of fasting may explain the variability between our studies in this parameter.

We found evidence of increased levels of LPS following risperidone treatment though this did not reach statistical significance, possibly due to a number of samples falling below the level of detection, especially in the control groups. Intriguingly, in line with a reduced microbial load expected with antibiotic treatment, rifaximin treated animals had reduced levels circulating LPS. This suggests another distinct mechanism whereby manipulation of the flora could conceivably attenuate the proinflammatory effects of antipsychotics. Of course, further research is needed to discern whether antipsychotics do result in increased levels of LPS and if this is due to increased gut permeability.

Increased gut permeability is associated with obesity and increases in visceral fat (de La Serre et al. 2010; Gummesson et al. 2011).

It is still unclear whether systemic inflammation leads to epithelial barrier dysfunction which results in increased LPS, or whether increased permeability via other mechanisms such as stimulating the secretion of the chemokine Ccl5 stimulate adipose tissue inflammation (Tremaroli and Backhed 2012).

Increased levels of LPS nevertheless contribute to the cycle of metabolic abnormalities leading to insulin resistance. LPS stimulates Toll-like receptor (TLR) 4 receptors on macrophages, adipose tissue and other tissues causing further release of inflammatory mediators, such as TNF, which induce insulin resistance. We investigated this link by examining TLR 4 mRNA expression in adipose and muscle tissue. We observed reduced TLR 4 expression in adipose tissue of risperidone treated animals, but not in animals also receiving rifaximin. Reduced expression following risperidone treatment may have been a result of receptor internalization due to chronic stimulation although this cannot be assumed and further investigation of the contribution of an LPS-TLR 4 mechanism in antipsychotic-induced insulin resistance is warranted.

Several studies in mice have highlighted that reducing levels of LPS impacts positively on the metabolic health of animals in a number of paradigms (Cani et al. 2007a; Cani and Delzenne 2009). This therefore lends weight to the possibility that the reductions in LPS observed in response to rifaximin may have reduced the proinflammatory tone in risperidone treated rats, though whether this was via TLR4, potentially in other tissues, remains to be elucidated.

7.8.6 Endocannabinoid system

Another mechanism shown to be involved in the link between the microbiota, gut permeability and obesity is the endocannabinoid (eCB)system (Cani et al. 2010). Research found that obesity in mice is associated with a dysregulated eCB tone. Increased eCB tone i.e. increased receptor levels of cannabinoid receptor 1 (CB_1) as well as increased tissue levels of endocannabinoids, were associated with increased adipogenesis and the development of visceral fat mass (Muccioli et al. 2010). Moreover, these investigators demonstrated that the gut microbiota influences eCB tone in such a way as to control gut permeability and consequently low grade inflammation and fat deposition.

We therefore investigated in our model, if olanzapine-induced increases in fat mass could involve the endocannabinoid system and found that the mRNA expression of CB_1 was up-regulated in olanzapine treated rats. Moreover, antibiotic treated animals did not show any such increases in response to olanzapine, strongly supporting a link between the gut microbiota the endocannabinoid system and fat deposition. Indeed, a combination of antibiotics, or indeed prebiotics, was previously shown to reduce CB_1 expression and fat mass development in genetically obese mice (Cani et al. 2010). It must be noted however, that contrary to our observations, these authors also found increased expression of FAS and PPARγ in conjunction with increased CB1 expression. Given the complex regulation of adipogenesis, this difference may reflect the different models of metabolic dysfunction under investigation.

It is therefore clear from our studies, that the gut microbiota is an important factor in the cycle of metabolic dysfunction via a number of mechanisms both direct and indirect, involving energy absorption, inflammation and metabolism. Moreover, we have demonstrated that these mechanisms also extend to antipsychotic-induced metabolic dysfunction, at least in rats.

The converging effects of this metabolic dysregulation lead to insulin resistance which ultimately lead to type II diabetes and increased cardiovascular risk in patients. We found that the antibiotic cocktail, while not normalising it, did appear to improve insulin sensitivity in olanzapine-treated rats. Thus the beneficial effects observed throughout our investigations, if reproduced in humans, may well act to decrease the risk of morbidity and mortality in patients receiving atypical antipsychotics.

The considerable complexity and indeed implications of this trialogue between the immune system, metabolism and the gut microbiota is only beginning to be unravelled (Shulzhenko et al. 2011), and further understanding of these interactions will no doubt lead to greater understanding of the underlying mechanisms as well opportunities for intervention in several disease states, including metabolic disease, such as that caused by certain antipsychotics.

7.9 Microbiota as a therapeutic target

As has been outlined in the previous section, we have utilised different antibiotics to manipulate the gut microbiota and thereby attenuate certain effects of atypical antipsychotics.

This potentially opens the door for therapeutics that target the gut flora as means to prevent or ameliorate the side effects of these drugs thereby improving patient outcomes by reducing morbidity and mortality as well as improving compliance.

While we have shown that antibiotics can influence the microbiota in such a way as to produce beneficial effects in rats, the use of such agents in humans is complicated by issues of antibiotic resistance as well as potential gastrointestinal side effects of their own.

This said, our studies have provided an exciting proof-of-principle that the microbiota is a viable drug target in antipsychotic-induced metabolic dysfunction, supporting similar findings in other paradigms (Cani et al. 2007b; Cani et al. 2010; Muccioli et al. 2010; Murphy et al. 2012).

In addition to the mechanisms already discussed, manipulation of the gut microbiota has the potential to improve metabolic health by a number of mechanisms (Table 7.5). A number of these mechanisms are secondary to effects already mentioned. For instance, scfa are ligands for G-protein receptors, GPR41 and GPR43, which when activated stimulate the release of PYY (Musso et al. 2010b) which not only slows gastric transit but also contributes to short-term satiety following a meal (Beglinger and Degen 2006).

Mechanism	Mode of action	Ref
↑ CLA	Synthesis of CLA	(Wall et al. 2009)
Neurotransmitter synthesis	Release of GABA	(Barrett et al. 2012)
	↑ Dopamine	(Asano et al. 2012)
	↑ 5-HT	(Wikoff et al. 2009)
↑ Gut peptide release	↑ PYY	(Musso et al. 2010b)
	↑ GLP-1	(Cani et al. 2009a)
	↑ GLP-2	(Cani et al. 2009b)
↓ Inflammation	↑ IL-10production	(Ueda et al. 2010)
	↑ IL-12 production	(Takeda et al. 2006)
	↓ Levels of LPS	(Cani et al. 2008)
Improved gut barrier function	↑ Butyric acid production	(Van Immerseel et al. 2010)
	↑ Defensins	(Wehkamp et al. 2004)

Table 7.5 Mechanisms linking the modulation of the gut microbiota to improvements in metabolic health. *CLA, conjugated linoleic acid; GABA, gamma-amino butyric acid; 5-HT, serotonin; PYY, peptide YY; GLP-1, glucagon-like pepetide-1; GLP-2 glucagon-like pepetide-2; IL-10, interleukin-10; IL-12, interleukin 12; LPS, lipopolysaccharide*

Importantly, a number of probiotics/prebiotics have been shown to propagate the above mechanisms in animal studies (Grangette et al. 2005; Cani et al. 2009a; Burcelin et al. 2010; Van Immerseel et al. 2010). These mechanisms allow the microbiota to influence both sides of energy balance, affecting appetite, nutrient absorption and energy storage.

7.9.1 Probiotics

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit to the host. Probiotics therefore consist of bacterial strains perceived to be beneficial, such as *bifidobacterium* and *lactobacillus*. Such probiotics have shown promise in a range of conditions, especially inflammatory bowel disease and irritable bowel syndrome (Clarke et al. 2012; Veerappan et al. 2012).

In humans, probiotics have shown both positive and negative results in trials of obesity (Gobel et al. 2012; Million et al. 2012). *Lactobacillus gasseri* (LG 2055) reduced body weight and adiposity in adults (Kadooka et al. 2010).

The use of probiotics as therapeutics is in its infancy, and to this end there is currently no FDA or EFSA approved probiotic health claim. This is likely due in part to the complex task of categorising a probiotic as either a drug, food supplement, medical food or biologic (Degnan 2008).

In the coming years, more robust data will become available for probiotics already in use and increased understanding of specific probiotic-host and probiotic-disease interactions will no doubt allow the development of more targeted and efficacious probiotics which can then be judged fairly as tools against diseases such as obesity.

7.9.2 Prebiotics

Prebiotics are selectively fermented ingredients that result in specific changes to the composition and/or activity of the gut microbiota thus conferring benefit(s) to the host. Prebiotics most commonly include inulin and fructose oligosaccharides.

As with probiotics, these products have thus far been primarily focused on promoting the growth of *bifidobacterium* or *lactobacillus*, and studies in humans have provided mixed results (Brownawell et al. 2012). Hence, also like probiotics, no prebiotic health claim has so far been approved by the FDA or ESFA, as debate over what constitutes a normal microbiota and proof of cause and effect in response to such treatments continues (Brownawell et al. 2012).

7.9.3 Faecal microbiota transplantation (FMT)

Another intriguing intervention that offers an opportunity for manipulation of the gut microbiota is faecal microbiota transplantation (FMT). FMT has in fact been used in selected cases for over 50 years (Eiseman et al. 1958). FMT involves the transferring of a donor microbiota to a recipient via filtered faecal content, generally administered by transcolonoscopic infusion (Borody and Khoruts 2012). Unlike probiotic or prebiotics, FMT aims to completely replace or repair the entire microbiota of the individual.

At present, FMT is used primarily for *Clostridium difficile* infection that is recalcitrant to antibiotic therapy. However, exciting case studies suggesting efficacy of FMT in a range of diseases including ulcerative colitis and even multiple sclerosis are emerging (Borody et al. 2011). Moreover, FMT has also sown promise in metabolic disorders with improved insulin resistance reported in obese subjects receiving FMT from lean donors (Vrieze et al. 2010) replicating findings in mice (Membrez et al. 2010). A recent support also demonstrated diverging microbiota between diabetes-prone and diabetes-resistant rats (Serino et al. 2012) emphasising the possibility that microbiota transfer could be a viable therapeutic strategy for diseases such as type II diabetes in the future.

Whilst the evidence for FMT in a range of diseases is gathering (Aroniadis and Brandt 2012), a number of potential hurdles are yet to be fully addressed including issues over safety, donor selection and administration. This said, fears of the 'yuck' factor have perhaps caused the greatest delay in the area of research (Zipursky et al. 2012) though research suggests that despite its unappealing nature, patients are indeed willing to consider FMT if it potentially offers disease relief.

Thus, further studies investigating the potential use of FMT in metabolic disease and indeed in antipsychotic-induced metabolic side effects may prove revealing. Characterisation of an obese-microbiome or even an antipsychotic-related microbiome as reported in several previous reports and continued herein, will prove essential to designing and carrying out such investigations.

It is now apparent therefore that the gut microbiota does indeed represent a therapeutic target of immense, thus far untapped, potential (Jia et al. 2008). Considerable advancements in recent years have accelerated this area of research and will no doubt continue to do so. It seems therefore only a matter of time until probiotic and prebiotic products can be utilised in such a way as to be of benefit to patients with, or at risk of, a range of conditions including obesity and metabolic dysfunction.

Given the ever growing appreciation of the complexity of the gut microbiota ecology and interaction with the host, such treatments of the future may rely on prior analysis of an individuals' microbiota.

Of course, given the microbiota's susceptibility to changes in diet, stress, age and location; targeting the microbial organ on a global scale, as seen with conventional pharmaceuticals is going to be a considerable challenge over the next number of years.

7.10 Overall conclusions and future perspectives

We have investigated whether the gut microbiota contributes to the metabolic dysfunction associated with certain atypical antipsychotics, and whether it therefore represents a novel therapeutic target.

We have demonstrated that olanzapine and risperidone when administered chronically induce alterations in the gut microbiota of rats, in conjunction with several other metabolic abnormalities including weight gain, increased visceral fat, and a proinflammatory phenotype. Thus, this novel finding highlights yet another paradigm in which altered microbiota has been observed and adds to the ever growing list of human pathologies in which the microbiota has been implicated.

Whether changes in the gut flora in response to antipsychotics are a cause or effect of weight gain and/or other metabolic effects remains unknown. However, we undertook a proof-of-principle approach using a cocktail of broad-spectrum antibiotics to investigate if gut microbiota has a substantive role in the side effects of olanzapine. Indeed, co-administration of the antibiotic cocktail did attenuate olanzapine induced weight gain to a limited degree. Moreover, and arguably more importantly, the antibiotic cocktail produced dramatic effects on adiposity; preventing olanzapine-induced increases in peri-uterine fat and associated macrophage infiltration.

This novel finding supports previous work of others in which antibiotics had similar beneficial effects in other paradigms of obesity and metabolic dysfunction (Cani et al. 2008; Muccioli et al. 2010).

One question that remained following the use of the antibiotic cocktail was to show that the effects were indeed driven by the microbiota, as systemic effects of antibiotics on metabolism have been reported (Takamoto et al. 2003). We therefore investigated if two, non-absorbable antibiotics could, when delivered per os, reproduce any of the effects observed with antibiotic cocktail.

Both rifaximin and vancomycin prevented olanzapine-induced increases in visceral fat, while rifaximin also attenuated olanzapine-induced weight gain to a modest but significant degree. Several other features of antipsychotic related metabolic dysfunction were also normalised showing that the gut microbiota does indeed feed into the complex cycle of metabolic derangement observed following antipsychotic treatment.

We have also shown that the involvement of the gut microbiota also extends to the atypical antipsychotic, risperidone, with similar effect observed in response to rifaximin on adiposity and inflammation.

Given the recognised importance of visceral fat accumulation and low grade inflammation in the myriad of complications observed in the metabolic syndrome, our findings are of considerable clinical interest.

Thus our studies add impetus to research investigating the microbiota as a therapeutic target in obesity and metabolic disease by opening an entirely new avenue for the use of such treatments i.e. antipsychotic-induced metabolic dysfunction.

Much work is yet to be done, and translating promising animal data into humans is a considerable and crucial challenge yet to be met. What is emerging, however, is that the gut microbiota appears to be altered in obesity (including antipsychotic-induced) or on obese-type diets and that this aberrant microbiota can impact on various physiological mechanisms that regulate energy metabolism, lipid homeostasis, and immune function of the host. Moreover, dietary components can be used to modulate this aberrant microbiota and their interactions with the host. However, despite strong data from animal studies, the ability to modulate the gut microbiota for improved human energy homeostasis remains to be confirmed in well-powered human intervention studies

Moving forward therefore a number of challenges remain. Specifically, whether an aberrant microbiota exists in patients receiving antipsychotics should be established. However, as studies in humans have already shown, assigning a correlation between faecal microflora and host phenotype may not be as easy as once hoped.

However, showing that dysbiosis exists in patients is the first step in exploring if the microbiota can be targeted in the more complex situation presented by humans.

Our research also adds further support to the growing work linking the microbiota to the development, exacerbation and potential treatment of obesity and the metabolic syndrome. Obesity is an ever growing global epidemic and new approaches are desperately needed to curb its ever increasing burden on public health.

Probiotics and prebiotics may offer an exciting new weapons in combating obesity in the near future, and as newer probiotics and prebiotics are developed, investigating if these have use in antipsychotic-induced metabolic side effects should be a high priority.

Thus, this thesis has added to the ever burgeoning realisation that our oldest ancestor, the gut microbiota, is much more than an invisible tenant. The potential for microbiota targeted interventions in antipsychotic-induced side effects, in addition to many other metabolic related diseases, suggests that with future research, the microbiota is set to become a major part of the pharmacological landscape.

Appendix I

Pilot study investigating the effects of haloperidol and olanzapine on body weight in mice

Abstract

Background: Atypical antipsychotics are called such due to their reduced liability for extra pyramidal side effects (EPS) which occur commonly with the older, typical antipsychotics. However, just as atypical drugs can cause EPS, typical antipsychotics have been associated with metabolic dysfunction. Thus, efforts are needed to understand both the liability and potential mechanistic differences between the alternative drug classes in causing metabolic dysfunction.

Methods: Male C57Bl/6 mice received either vehicle, olanzapine (2 mg/kg) or haloperidol (1 mg/kg) via intra-peritoneal injection (I.P.) for 34 days. Bodyweight was measured daily and food intake was measured for 2 days each week. Activity was monitored using activity monitoring suites and body composition was analysed at the end of the study.

Results: Haloperidol treatment was associated with reduced locomotor activity, but no differences in body weight or body composition. Olanzapine showed evidence of increased food intake but a trend for a reduction in bodyweight.

Conclusions: This experiment highlighted that male mice may not be suitable for investigating the metabolic effects of antipsychotics and also highlight potential role of sedation, in particular in the case of haloperidol.

I.1 Introduction

Early, typical antipsychotics revolutionised the treatment of schizophrenia. However, these compounds are dogged by serious extra pyramidal symptoms (EPS) which comprise a range of motor disorders such as dyskinesia and Parkinsonism (Dayalu and Chou 2008).

Subsequent atypical antipsychotics were defined as a group of compounds based on reduced EPS liability despite their pharmacological heterogeneity. Importantly, atypical antipsychotics can and do cause EPS when administered at high (usually super-therapeutic) doses (Tarsy et al. 2002). Equally, while EPS understandably was the main focus for typical antipsychotics in terms of adverse events, haloperidol and other typical drugs have been associated with metabolic dysfunction (Bobes et al. 2003b; Perez-Iglesias et al. 2008).

Hence, it is important to understand the differing propensities and mechanisms underlying metabolic dysfunction seen between the major classes of antipsychotic drug. Recent evidence suggests that peripheral mechanisms such as increases in fat mass play a central role in detrimental metabolic effects of antipsychotics and in obesity in general. It is therefore important to understand if the effects of both typical and atypical compounds on factors such as accretion of visceral fat mass differ.

Therefore, we investigated the effects of both a commonly prescribed typical and atypical drug on body weight and body composition in mice.

I.2 Methods

Animals

Male C57Bl/6 mice (Harlan, UK) of approximately six weeks of age were used and were singly housed. Animals were maintained on a 12 hour light/dark cycle with lights on at 7:30 am. Animals had access to standard chow and water *ad libitum*. All experiments were approved by the Animal Experimentation Ethics Committee (AEEC) of University College Cork and carried out in accordance with the Cruelty to Animals Act 1876 and European Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

Drug preparation

Olanzapine and haloperidol (Discovery Fine Chemicals, Ireland) solutions were prepared fresh daily. Olanzapine and haloperidol were dissolved in a minimal amount of glacial acetic acid (approx 0.1 ml) and then made to volume and pH adjusted to approximately 6 with 1M NaOH. Vehicle consisted of distilled water acidified with glacial acetic acid and pH adjusted to 6 with 1M NaOH.

Drug treatment

Mice (n=6) received either vehicle, olanzapine (2 mg/kg) or haloperidol (1 mg/kg) daily via intra-peritoneal (I.P) injection for 34 days. The injection took place between 9:00 a.m. and 10:00 a.m. Doses were chosen based on previously published literature (Cooper et al. 2005; Fell et al. 2005; Hagan and Jones 2005).

Body weight

Body weight was measured daily to the nearest 0.01g using an electronic balance.

Food intake

Food intake was measured on two consecutive days each week. Food was weighed using an electronic balance to the nearest 0.01g at the same time on each respective day.

Body composition

Body composition was analysed in conscious animals using a minispec nuclear magnetic resonance body composition analyser (Bruker Optics, MA,U.S.). Briefly, mice were placed carefully into a clear plastic tube which was then inserted horizontally into the minispec machine. The analysis takes 20-30 seconds before the mouse was removed from the tube, the tube cleaned and the next animal placed inside.

Locomotor activity

Locomotor activity was monitored for two consecutive days (48 hours) per week using home-cage activity monitoring suites (Medscape). Locomotion was monitored by an overhead infrared camera.

Statistical analysis

Food intake and locomotor activity were analysed using repeated measures ANOVA. Body composition and body weight change were analysed using one way ANOVA. LSD post-hoc test used for further analysis where appropriate.

I.3 Results

Body weight

As expected, time had a significant effect on body weight ($F_{(33,495)}$ = 42.116, $p < 0.001$). Drug treatment did not have an overall significant effect ($F_{(2,15)}$ = 2.88, $p = 0.087$). However, there was a significant time x drug interaction, ($F_{(66,495)}$ = 2.124, $p < 0.001$). No significant differences in body weight were observed between treatment groups over the course of the experiment (Fig. I.1).

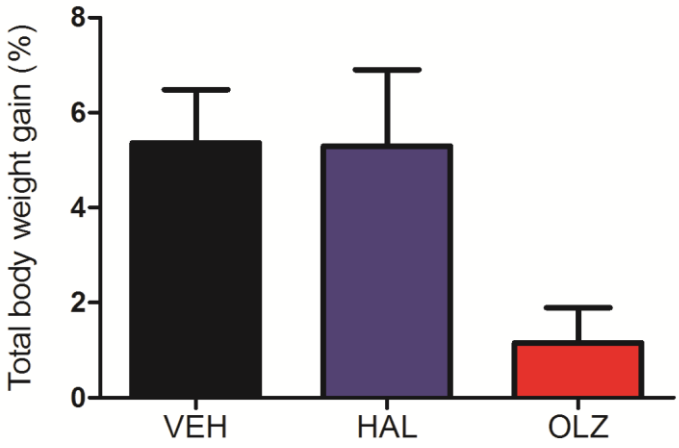


Fig I.1. Effect of haloperidol and olanzapine on body weight gain in C57/Bl/6 mice. *Effect of vehicle (VEH), haloperidol (HAL) (1 mg/kg) or olanzapine (OLZ) (2 mg/kg) in male C57Bl/6 mice following 34 days administration. Data expressed as mean ± SEM.*

Food intake

Time had a significant effect on food intake ($F_{(3,45)}$ = 12.174, $p < 0.001$), as did drug treatment ($F_{(2,15)}$ = 4.436, $p < 0.05$). Post-hoc analysis showed that olanzapine treated mice had significantly increased food intake compared to haloperidol treated mice on days 7-8 and 14-15 (inclusive) ($p < 0.05$) (Fig. I.2).

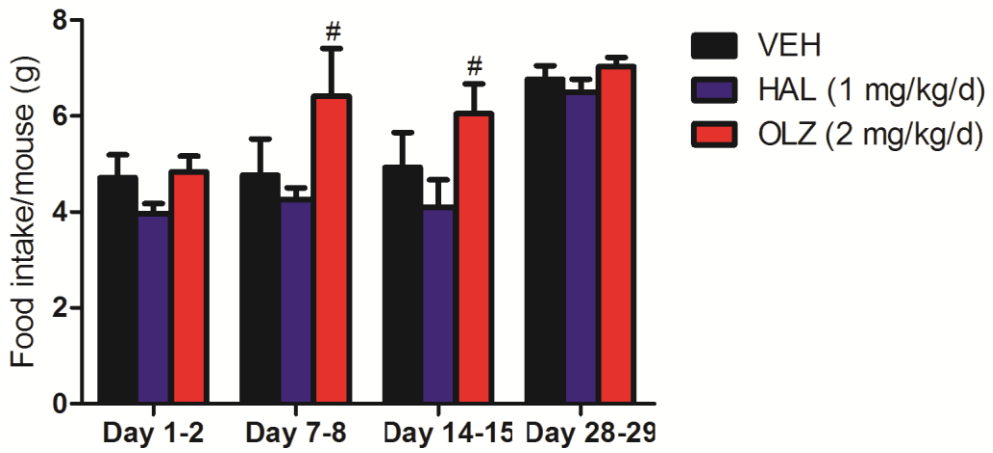


Fig I.2 Effect of haloperidol and olanzapine on food intake in C57Bl/6 mice. *Effect of vehicle (VEH), haloperidol (HAL) (1 mg/kg) or olanzapine (OLZ) (2 mg/kg) administration on food intake on two consecutive days during each week of treatment over 34 days* $^{\#}p < 0.05$ *versus haloperidol treated mice. Data represent mean ± SEM*

Locomotion

Locomotor activity was significantly affected by time ($F_{(2,30)}$ = 12.244, $p < 0.001$). Drug treatment also had a significant effect ($F_{(2,15)}$ = 3.695 $p < 0.05$), though there was not a significant interaction ($F_{(4,30)}$ = 1.126, $p = 0.363$). Post-hoc analysis showed that on days 14 and 15 mice treated with olanzapine or haloperidol had significantly reduced locomotor activity compared to mice receiving vehicle ($p < 0.05$) (Fig. I.3).

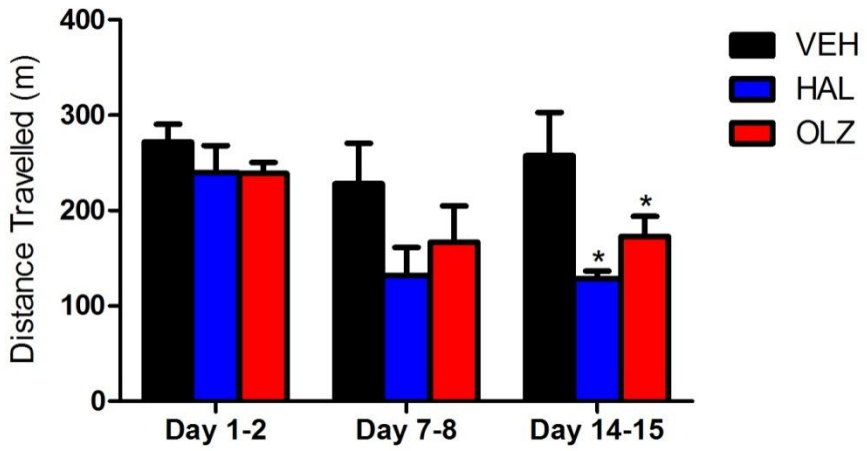


Fig. I.3 Effect of olanzapine and haloperidol on locomotor activity. *Effect of vehicle (VEH), haloperidol (HAL) (1 mg/kg) or olanzapine (OLZ) (2 mg/kg) administration on locomotor activity on two consecutive days during each week of treatment in C57Bl/6 mice. Data expressed as mean ± SEM. *p < 0.05 versus vehicle treated mice.*

Body composition

Drug treatment had a significant effect on body fat percentage ($F_{(2,15)} = 6.968$, $p < 0.01$). Drug treatment also had a significant effect on body lean percentage ($F_{(2,15)} = 4.045$, $p < 0.05$).

Post-hoc analysis revealed that mice treated with olanzapine had significantly reduced body fat compared to both vehicle treated ($p < 0.05$) and haloperidol treated mice ($p < 0.01$) (Fig. i.4A). The mice receiving olanzapine also had increased lean mass percentage compared to the haloperidol treated mice ($p < 0.05$) (Fig. I.4B).

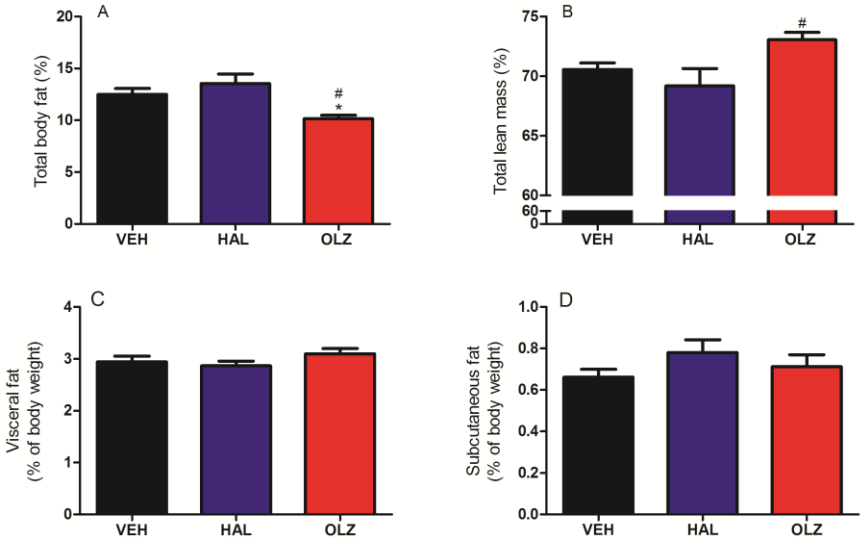


Fig I.4 Effect of haloperidol and olanzapine on body composition in C57Bl/6 mice. *Effect of vehicle (VEH), haloperidol (HAL) (1 mg/kg) or olanzapine (OLZ) (2 mg/kg) administration for 34 days on (A) body fat mass, (B) body lean mass (C) visceral fat and (D) subcutaneous fat in male C57Bl/6 mice. Data expressed as mean ± SEM *p < 0.5 versus vehicle treated animals, $^{\#}p < 0.05$ versus haloperidol treated mice.*

I.4 Discussion

In this experiment, neither haloperidol nor olanzapine produced increases in body weight in male mice. Contrary, to its clinical effects, olanzapine caused a trend for reduced body weight over the course of treatment. These findings are in line with other reports suggesting mice are resistant to the weight inducing liabilities of antipsychotics (Albaugh et al. 2006).

Olanzapine did however cause subtle increases in food intake further supporting increased appetite as a driving force in olanzapine induced weight gain (Davoodi et al. 2009).

Sedative effects of antipsychotics are often mooted as a potential explanation for their weight gain liabilities, particularly in preclinical studies. To investigate this possibility, and to compare a typical with an atypical drug, we used activity-monitoring cages to assess locomotion at various stages of the treatment. The mice receiving either haloperidol or olanzapine showed reduced locomotor activity which was significant during days 14 and 15 of treatment. This effect was most pronounced in haloperidol treated mice, which is expected given it's sedative properties at higher doses, a property utilised clinically to tackle extreme agitation (MacDonald et al. 2010). However, as mentioned, weight gain was not observed during the course of treatment suggesting observed reductions in activity in other studies is not a primary cause of weight gain.

Body composition analysis showed that neither haloperidol nor olanzapine caused increases in overall fat mass, but rather, olanzapine treated mice had decreased fat mass with a concomitant increase in lean mass.

This is somewhat unsurprising given the body weight findings though it is important to remember that overall fat mass may not be representative of important changes in fat mass in specific regions. Indeed increases in visceral fat have been associated with antipsychotic treatment even in the absence of weight gain (Victoriano et al. 2010; Albaugh et al. 2011a).

However, in the present report, no changes in visceral or subcutaneous fat were observed suggesting that the protocol employed was insufficient to produce clinically relevant changes in metabolic processes. Potential reasons for this include the drug dose selected, dosing schedule and/or species. Moreover, use of the home cage activity monitoring suite appeared to impact on food intake which was lower than one would expect, and thus this likely was a confounding factor in the study.

Overall, the potential confounding factor of sedation was highlighted and should be an important consideration for future investigations, although it is unlikely to be a factor in weight gain observed in other murine studies.

This experiment also highlights the need for careful consideration in carrying experimental models in order to ensure any outcomes and conclusions are valid to the clinical setting

Appendix II

Assessment of microbiota

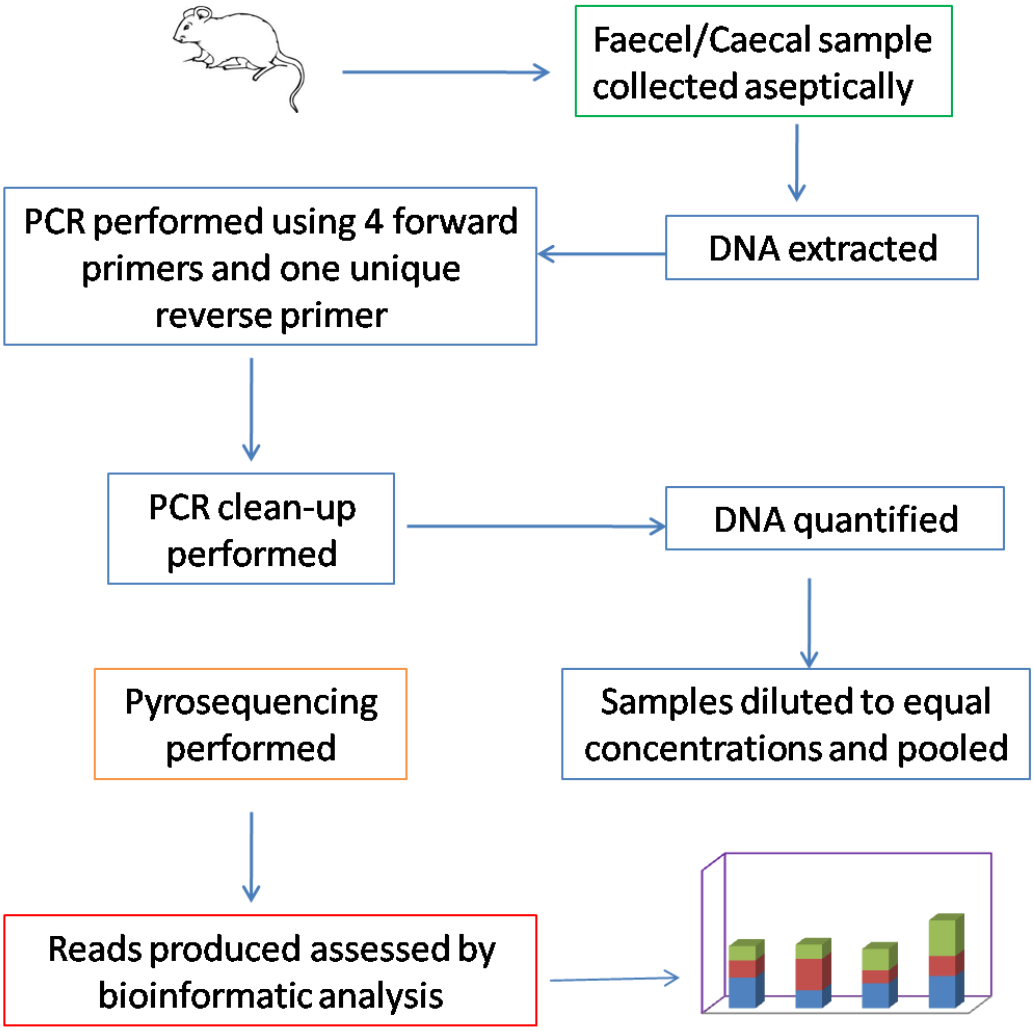


Fig II.1 Work flow of how the microbiota composition is analysed.

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