The action of atypical antipsychotics on body weight and associated metabolic factors

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by

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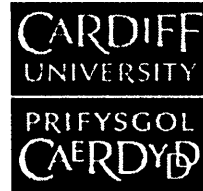
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Acknowledgements

Summary

Despite having revolutionized the treatment of psychiatric illnesses, atypical antipsychotic agents raise an increasing medical concern regarding their association with prominent body weight gain and metabolic abnormalities resulting from chronic treatment. As a consequence, the use of atypical antipsychotic medication has been linked to a substantial increase in the development of obesity, type 2 diabetes and cardiovascular diseases in patients undergoing therapy.

In this study, the primary aim was to develop a mouse model of atypical antipsychotic-induced body weight gain and adiposity. Moreover, the chosen antipsychotic agents, clozapine and olanzapine, were investigated in a fibroblast-like cell line model (7-F2) and in primary bone marrow cells, in order to test a possible direct contribution by these agents in causing adipogenesis and altered lipid metabolism at the cellular level in peripheral tissues.

It was found that the ability to produce a reliable and robust mouse model, capable of mimicking the clinical situation was obstructed by variability and inconsistency of the experimental outcomes. This prompts the suggestion that caution should be exercised in the interpretation of results from previous models and also, to question their predictive validity.

Furthermore, although a morphological study on 7-F2 cells showed that clozapine and olanzapine do not enhance the differentiation of fibroblastic cells into adipocytes, mRNA over-expression of genes involved in adipocyte formation and metabolism suggest that these antipsychotics incite *de novo* formation of fat cells in the bone marrow.

Overall, although the results are of a preliminary nature, they emphasize the need for in-depth examination of any possibility that clozapine or olanzapine might directly trigger an increase in adipocyte numbers (hyperplasia) or alter adipocyte size (hypertrophy).

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Abbreviations

AgRP/NPY	agouti-related Neuropeptide Y
AMSCs	adipose-derived mesenchymal stem cells
ANOVA	Analysis of Variance
aP2	adipocyte lipid-binding protein
APS	antipsychotic
ARP	acid ribosomal protein
BMI	body mass index
BMSCs	bone marrow-derived mesenchymal stem cells
cDNA	complementary DNA
clo	clozapine
CNS	central nervous system
CVD	cardio-vascular disease
C/EBPs	CCAAT/enhancer binding proteins
D receptors	dopamine receptors
DNA	deoxyribonucleic acid
DSM-IV	diagnostic and statistical manual
EPSEs	extrapyramidal side effects
FAS	fatty acid synthase
GABA	gamma amino-butyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT4	glucose transporter type 4
H receptors	histamine receptors
ICD-10	international classification of diseases
LPL	lipoprotein lipase
mg	milligram
ml	milliliter
mM	millimolar
μg	microgram
μM	micromolar
MP	mini pump
MRI	magnetic resonance imaging

mRNA	messenger RNA
MSCs	mesenchymal stem cells
MTS	3-(4,5-dimethylthiazol-2yl-)-5-(3-carboximethoxyphenyl)-2- (4-sulphophenyl)-2H-tetrazolium
M receptors	muscarinic receptors
NMDA	N-methyl-D-aspartic acid
ola	olanzapine
OMD	osteomodulin
PBS	phosphate buffered saline
PET	positron emission tomography
PMS	phenazine methosulfate
POMC/CART	pro-opiomelanocortin/cocaine- and amphetamine-related transcript
PPARγ	peroxisome proliferator-activated receptor gamma
qRT-PCR	quantitative Real Time-Polymerase Chain Reaction
RNA	ribonucleic acid
RQ	relative quantification
RT	reverse transcriptase
SEM	standard error of the mean
SPSS	Statistical Package for Social Science
5 HT receptors	5-hydroxytryptamine (serotonin) receptors

Chapter 1

General Introduction

1. Schizophrenia and antipsychotic drugs

1.1 The nature of schizophrenia

Schizophrenia (*skizo*-'to split' and *phrenia*-'mind') can be defined as a neuropsychiatric disorder characterized by dysfunctions in the perception of reality. It is estimated that 1% of the world population suffer from schizophrenia with the peak onset of the disorder between 10 and 25 years old for males and between 25 and 35 for women (Rajji et al., 2009).

1.1.1 Symptoms

Schizophrenia symptoms may vary from patient to patient and are often classified as positive and/or negative. Positive symptoms reflect a

pattern of behavior that is not normally present in a healthy subject (i.e

hallucinations, delusions, bizarre behavior and thoughts) while the

negative symptoms define diminished functions in comparison to a

normal subject (i.e asociality, anhedonia, avolition) (Fuller and Schultz,

2003). In addition, cognitive symptoms such as memory deficit and

attention can be present (Fuller and Schultz, 2003).

1.1.2 Diagnosis

The two most widely used sets of criteria to diagnose schizophrenia are

described in the *Diagnostic and Statistical Manual* (DSM-IV) of the

American Psychiatry Society and the *International Classification of*

Diseases (ICD-10) of the World Health Organization.

1.1.3 Causes of schizophrenia

Despite years of investigation, the origin of schizophrenia remains

uncertain. Nevertheless several risk factors were identified:

Season of birth

Some studies showed that the late onset of schizophrenia is linked to a

slight excess of births in winter and spring in the northern hemisphere,

and a slight decrease over summer and autumn (Mortensen et al.,

1999; Torrey et al., 1997).

Prenatal and birth complications

Late development of schizophrenia seems to be related to prenatal and

birth complication as several studies and meta-analyses have

confirmed. Among them: prenatal exposure to infections (Brown, 2006),

complications of pregnancy, abnormal fetal growth and development,

complications of delivery (Cannon et al., 2002).

Environment

Being born in an urban environment has been repeatedly found to

increase the risk of developing schizophrenia (Van Os et al., 2005).

Migration (i.e. foreign birth and background) (Cantor-Graae et al., 2003)

and poverty (Mueser and McGurk, 2004) also appear to have their

influence.

Genetics

It has long been recognized that schizophrenia is a disorder with a hereditary component.

Both familiar and twin studies have demonstrated the existence of genetic factors responsible for the onset of schizophrenia (O'Donovan et al., 2003), although the identification of specific genes or any involvement of genetic variations is still uncertain.

1.1.4 The neuropathology of schizophrenia

Since the introduction of modern imaging technologies such as PET and MRI, researchers have uncovered a number of small anatomical abnormalities in the brain structures of individuals affected by schizophrenia. Most of these changes appear to involve the temporal and frontal lobe (Lawrie and Abukmeil, 1998). Moreover, differences in brain size and volume have been found, total volume being slightly reduced and ventricular volume being enlarged (Steen et al., 2006).

1.1.5 The neurochemistry of schizophrenia

The dopamine and glutamate hypotheses are the two classical theories hypothesized to explain the symptoms of schizophrenia.

Dopamine hypothesis. Since early in the 1960s, a dysregulation of dopamine transmission has been suggested as an underlying aetiological factor in schizophrenia (Carlsson and Linqvist, 1963) and later on, this was followed by the observation that antipsychotic drugs were effective against the positive symptoms of schizophrenia by blocking dopaminergic D2 receptors (Creese et al., 1976). Additionally, psychotropic drugs acting as D2 agonists have been shown to enhance schizophrenia-like symptoms (Randrup and Munkvad, 1967).

In contrast, antipsychotic drugs are not as effective against negative symptoms (Keefe et al., 1999), suggesting that an up-regulation of dopamine D2 transmission could be only partially responsible for the psychiatric symptoms of schizophrenia.

Further studies concerning the role of D2, D3 and D4 subtypes in the neurochemistry of schizophrenia have been subject to conflicting results (Malmberg et al., 1993).

Glutamate hypothesis. Reduced glutamate levels in the cerebrospinal fluid of schizophrenic patients raised the question of a hypofunctionality in glutamate transmission (Kim et al., 1980), although this finding was not replicated. Moreover, studies on the NMDA receptor antagonists ketamine and phencyclidine, showed a pattern of cognitive impairment similar to the one observed in schizophrenia (Javitt and Zukin, 1991). Thus, a possible role of glutamate has been implicated in schizophrenia.

Serotonin hypothesis. Despite the close interrelationship between serotoninergic and dopaminergic systems and the fact that atypical antipsychotics show 5-HT antagonism, none of the serotonin receptor subtypes investigated have been clearly related to the pathogenesis of schizophrenia (Veenstra-VanderWeele et al., 2000)

GABA hypothesis. Data from post-mortem studies suggesting a reduction in GABAergic function in schizophrenic patients (Perry et al., 1979) led the research towards the development of a GABA agonist effective in the treatment of schizophrenia. However, no consistent results have been reported to date.

1.2 Antipsychotic drugs

Antipsychotics medications are a class of drugs mainly used for the symptomatic treatment of psychosis including schizophrenia, mania, bipolar disorder and many other conditions characterized by agitation and altered mental status. A common feature of these drugs is the antagonistic activity at dopamine D2 receptors in the central nervous system but other receptors are involved in their mechanism of action including D1 and D4 in addition to serotonin (5HT), α-adrenergic, cholinergic and histaminic receptors (Arnt and Skarsfeldt 1998).

The first generation of antipsychotic agents, also designated as 'neuroleptic' or 'typical', are potent dopaminergic D2 antagonists that have a strong propensity towards producing extrapyramidal side effects (EPSEs) arising from activity in the striatum. In contrast, the so-called newer generation of antipsychotics exhibits much less marked EPSEs and for this reason are named 'atypical'.

1.2.1 History

The history of antipsychotic medications was pioneered by the development of phenothiazine compounds as industrial dyes (i.e. methylene blue) at the end of 19th century. Subsequently, in the 1930s, one of these compounds, promethazine, was found to have antihistaminic and sedative properties and, following tests on rodents, it was found to prolong sleep induced by barbiturates. It was introduced by Laborit in the clinic for its ability to prolong and stabilize anaesthesia for surgery (Laborit et al.,1952).

A few years later, another significant phenothiazine derivative was synthesized by Charpenter and later tested by Laborit. This compound, named chlorpromazine, was found to reduce anxiety and induce mild sedation without causing loss of consciousness, which led to its use in the treatment of psychosis in France by Delay and Deniker (Delay and Deniker, 1952) and later, chlorpromazine was licensed in the USA in 1955.

1.2.2 First generation antipsychotics (typical)

The typical antipsychotics are commonly divided into three main categories that reflect different classes of chemical structure: (a) phenothiazine, (b) thioxanthines and (c) butyrophenones.

First generation antipsychotics are also classified on the basis of their relative potency as *low, medium and high potency,* according to the dosage necessary to cause an antipsychotic effect. Aliphatic phenothiazines such as chlorpromazine are designated as 'low-potency', while piperazine phenothiazines such as fluphenazine and trifluoperazine, thioxanthines and butyrophenones are referred to as 'high potency' antipsychotics.

1.2.2.1 Pharmacology

A prominent feature of all the first generation antipsychotics is antagonism at the D2-subtype of dopamine receptors in the CNS. Although these drugs possess different affinities at D1, D4, serotonin 5HT2, histamine H1, adrenergic and cholinergic receptors, it appears

that D2 antagonism represents a major component of their antipsychotic activity (Seeman, 1980).

While dopamine is ubiquitous in the central nervous system, particularly in the nucleus caudate (nigrostriatal system), nucleus accumbens (mesocortical system), the mesolimbic system and the tuberoinfundibular pathway, dopamine D2 receptors localized in the limbic and striatal areas are implicated in antipsychotic efficacy and EPSEs.

1.2.2.2 Efficacy

Conventional first generation antipsychotics are not the current first choice for the treatment of schizophrenia and although they are effective in first-episode schizophrenia, especially in improving the positive symptoms, they are not superior to atypical agents such as clozapine (Lieberman et al., 2003). Although chlorpromazine and haloperidol reduce the risk of relapse (Thornley et al., 2003; Joy et al., 2001), they are not effective in refractory schizophrenia compared to second-generation antipsychotics (Kane et al., 1988).

At present, treatment with typical agents is only recommended for patients showing a good clinical response with minimal side effects (Sharif, 1998).

1.2.2.3 Neurological Side effects

Due to the blockade of the dopamine D2 innervations in the striatum, the conventional antipsychotics generate a range of neurological adverse effects that are much less marked or even absent in the case of the newer generation drugs. While *akathisia, dystonia, parkinsonism* and *neuroleptic malignant syndrome* represent acute reactions to these medications, *tardive diskinesia* can occur after chronic treatment.

Akathisia is a syndrome characterized by a strong feeling of physical and psychological discomfort, a classic feature is patient agitation and anxiety, restless leg and a need to maintain constant movement that is difficult to control voluntarily (Sachdev and Loneragan, 1991).

Dystonic reactions typically involve muscular contractions and spasms generally limited to the face, neck and back resulting in abnormal

posture (Van Harten et al., 1999). These are the first symptoms to appear in response to antipsychotic therapy.

The symptoms observed in drug-induced parkinsonism are virtually the same as those seen in idiopathic Parkinson disease: bradykinesia, rigidity, inability to initiate movement, tremor and a mask-like face.

The neuroleptic malignant syndrome is a rare condition compared to the other symptoms outlined previously. Symptoms include catatonia, rigidity, fever, high blood pressure and a high level of creatine kinase (Adnet et al., 2000). Given the high rate of mortality caused by this condition, immediate cessation of the antipsychotic therapy is required.

After long-term treatment with conventional antipsychotics, tardive dyskinesia may appear. Typical symptoms are exemplified by involuntary movements of the face, tongue, neck and limbs, stereotyped movements, muscular spasms and akathisia (Marsalek, 2000).

1.2.2.4 Endocrine effects

Conventional antipsychotics have long been recognized to elevate blood levels of the hormone prolactin. In fact, since the stimulation of

the D2 receptors in the tuberoinfindibular area suppresses prolactin release, all antipsychotics, particularly those acting in that brain area, cause some degree of hyperprolactinaemia *via* D2 antagonism. As a consequence, hyperprolactin-related side effects such as gynaecomastia, galactorrhoea and erectile dysfunction may occur (Haddad and Wieck, 2004).

Allied to the high levels of prolactin induced by antipsychotics, these drugs are also subject of investigation with regard to their potential contribution to the development of bone mineral density loss, which has been reported in several studies (Liu-Seifert et al., 2004; Meaney et al., 2004; Meaney and O'Keane, 2007; Kishimoto et al., 2008) and breast cancer (Harvey et al., 2008).

1.2.3 Second generation antipsychotics (atypical)

1.2.3.1 History

The history of atypical antipsychotics began with the discovery of clozapine and its antipsychotic properties.

Clozapine was introduced to the market in the 1960s and while the chemical structure first suggested that it might posses a potential effect as an antidepressant, it was soon discovered that it actually had neuroleptic properties (Hippius, 1989). However, there was an anomaly reported about this finding because clozapine yielded antipsychotic activity without causing EPSEs and this stimulated interest in the nature of this drug. In fact, at that time, the onset of EPSEs was considered an inseparable pre-requirement for antipsychotic activity (Hippius, 1989). Nevertheless, clozapine was marketed in several European countries until a study conduced in Finland in 1975 reported several cases of agranulocytosis resulting from treatment (Idaanpaan-Heikkila et al., 1975). Clozapine was then withdrawn from the clinical use, but in the following years its unique properties were extensively investigated until

the validation of its efficacy in treating resistant-schizophrenia was established, leading to the reappearance on the market in 1990.

1.2.3.2 'Atypicality'

Soon after clozapine returned to the market, a new generation of antipsychotic drugs, called 'atypical', was developed and marketed. Amongst these olanzapine, quetiapine, risperidone, ziprasidone and aripiprazole were introduced into clinical usage.

Atypical, in general terms, refers to the ability of this new drug class to produce a substantial reduction of EPSEs and high levels of prolactin compared to the first-generation antipsychotics. However, recent studies have demonstrated that atypical antipsychotics are not superior to typicals in ameliorating psychotic and negative symptoms (Carpenter and Buchanan, 2008).

1.2.3.3 Clozapine
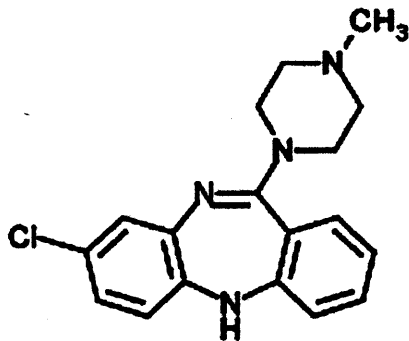
Fig 1.1 Chemical structure of clozapine

Clozapine, to date, is the only antipsychotic drug that clearly exhibits its superiority in treatment-resistant schizophrenia compared to the other antipsychotic drugs and this was demonstrated by Kane et al., in 1988. Patients who had already received treatment with three different antipsychotics and did not respond were randomly given either clozapine or chlorpromazine. 30 % of those receiving clozapine improved in terms of both positive and negative symptoms against 4 % of those who were given chlorpromazine (Kane et al.,1988).

Later studies validated the efficacy of clozapine in patients with a history of poor response to other treatments. In a meta-analysis involving 12 controlled studies (seven of them comparing clozapine to typical antipsychotics), clozapine was shown to be more effective in

treatment-resistant schizophrenia (Chakos et al., 2001). It was also more effective than haloperidol in reducing positive symptoms (Kane 2001 et al.,; Volarka et al., 2002), and also superior to risperidone (Azorin et al., 2001) in this respect.

McEnvoy et al., (2006) compared switching to clozapine with switching to olanzapine, quetiapine or risperidone, and showed that clozapine was more effective, with a longer time of discontinuation compared to the other drugs.

Moreover, in a 52-week study, clozapine was tested against chlorpromazine in 160 first-episode schizophrenia patients and the clozapine-treated patients showed faster remission (Lieberman et al., 2003). Clozapine also proved to be effective in reducing aggressive behavior in patients with schizoaffective disorder (Krakowsky et al., 2006), in childhood-onset schizophrenia (Shaw et al., 2006; Kumra et al., 2008) and in the treatment-resistant bipolar disorder and mania (Green et al., 2000).

In addition, it was also effective in reducing substance abuse and conditions associated with suicidal risk among schizophrenic patients (Iqbal et al., 2003).

Mechanism of action of clozapine

The pharmacology of clozapine is rather complex due to the interaction with a wide range of receptor families. Which of these interactions is responsible for its unique properties, particularly in treatment-resistant schizophrenia, has yet to be elucidated.

Clozapine appears to have relative low affinity for all the dopamine receptor subtypes (compared to conventional agents) with the exception of D4. Thus, radioligand binding data has shown that clozapine possesses moderate to low affinity for D1 receptors in the striatum although functional studies unveiled differing degrees of D1 receptor occupancy (Ashby and Wang, 1996).

PET studies on the occupancy of D2 receptors in the striatum of schizophrenic patients revealed that clozapine occupancy was 48%, which was significantly less than the typical antipsychotics (Farne and Nordstrom, 1992), leading to the hypothesis that this may contribute to

its atypical profile. Clozapine also possesses low affinity for D3 receptors (Ashby and Wang, 1996) but in contrast, it displays high affinity for the D4 subtype and antagonist at this site is thought to exert, at least in part, its therapeutic effect (Van Tol et al., 1991).

Furthermore, clozapine displays some $5HT_{1A}$ affinity, strong affinity for $5HT_{2c}$ and potent $5HT_{2A}$ antagonism, which coupled with low affinity for D2 receptors, has been proposed as possible explanations for its pharmacological properties (Meltzer, 1989). In addition, clozapine also displays some affinity for $5HT_6$ and $5HT_7$ receptors (Bymaster et al., 1996).

Clozapine binds with high affinity to muscarinic receptors (Miller and Hiley, 1974), and it has been established as an antagonist at M1, M2, M3 and M5 subtypes and also an M4 agonist (Zorn et al., 1994). More recently however, it has been reported that clozapine behaves as a partial agonist at M1, M2 and M3 subtypes (Olianas et al., 1999).

Clozapine also binds with high affinity to histaminergic H1 receptors, while binding and functional studies indicate that it is a potent

α_1 adrenoreceptor antagonist with low to moderate affinity for α_2 adrenoreceptors (Ashby and Wang, 1996).

1.2.3.4 Olanzapine
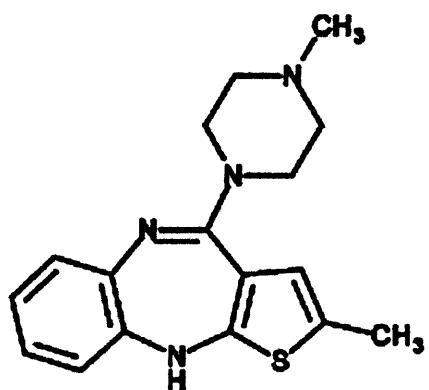
Fig 1.2 Chemical structure of olanzapine

Being similar to clozapine in both chemical structure and pharmacological profile, olanzapine also shares a similar pattern of clinical use and is one of the most common atypical antipsychotic employed in clinical practice.

In several short-term trials olanzapine exhibited superiority to haloperidol in overall symptoms improvement, safety profile and in numbers of patients who discontinued the treatment (Tollefson et

al.,1997), in first-episode schizophrenia (Sanger et al., 1999) and in poor responding patients (along with clozapine) (Volavka et al., 2002).

In contrast, results from 12 week-acute phase treatment of first-episode psychosis produced little difference in symptoms severity compared to haloperidol but better compliance to the treatment regime (Lieberman et al., 2003).

Furthermore, a review of several randomized trials indicates that olanzapine is superior to both first and second-generation antipsychotics in terms of adherence to the treatment and longer time to discontinuation (Johnsen and Jorgensen, 2008). Olanzapine also appeared to be more effective than aripiprazole, quetiapine, risperidone and ziprasidone in a recent meta-analysis (Leuch et al., 2009).

Olanzapine has been further studied in comparison with risperidone and it generated a higher general response to the treatment (Breier et al., 2005) particularly against negative symptoms (Canive et al., 2006) as well as being more effective when compared to switching to quetiapine (Deberdt et al., 2008).

Olanzapine manifests to have longer time to discontinuation (Beasley et al., 2007), and in the maintenance of response (Stauffer et al., 2009) compared to other antipsychotics. Its effectiveness was also observed in the treatment of bipolar disorder (Derry and Moore, 2007; Nabasimhan et al., 2007) and in adolescents suffering bipolar mania (Tohen et al., 2007).

Mechanism of action of olanzapine

Olanzapine exhibits a similar pharmacological profile to clozapine. Hence, it displays a potent receptor blockade at $5HT_{2A}$, $5HT_{2C}$ and $5HT_6$, muscarinic M1, H1 and α_1-adrenoceptors, although the α_1-adrenoreceptor affinity is lower than clozapine. Olanzapine also possesses higher affinity for D1 and D2 receptors but lower affinity for D4 than clozapine (Bysmaster et al., 1996).

1.2.3.5 Non-neurological side effects: weight gain and metabolic syndrome

Numerous studies have shown that schizophrenic patients have a greater propensity towards obesity than the general population and

obesity occurs more frequently in atypical antipsychotic-treated patients than those on conventional antipsychotics (Aquila, 2002). Interestingly, the incidence of weight gain among the new generation of these drugs is variable.

Despite being among the most effective antipsychotic drugs in clinical practice, clozapine and olanzapine are in fact, associated with the greatest incidence of weight gain compared to the other atypical antipsychotics.

The first meta-analysis to show that clozapine and olanzapine cause the greatest weight gain was conducted in 1999; clozapine displayed a mean weight gain of 4.45 kg during the treatment, olanzapine 4.15 kg, risperidone 2.10 kg and ziprasidone 0.04 kg (Allison et al., 1999).
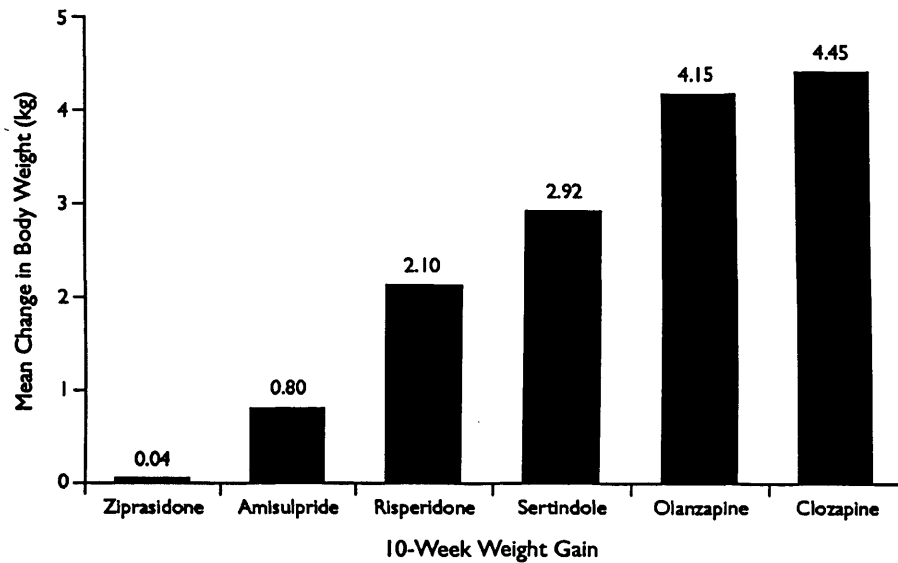
Fig 1.3 Weight change after 10 weeks on different antipsychotic drugs. (Modified from Allison et al., 1999 and Newcomer et al., 2004).

In another study, 20 % of the patients treated with clozapine gained more than 10 % of their body weight between 12 weeks and 12 months of treatment and although most the body weight increased during the first 4-12 weeks, further increases occurred following clozapine treatment. After 52 weeks, 20 % of the patients gained a further 20 % of their body weight (Iqbal et al., 2003).

In a retrospective study where 82 patients treated with clozapine were followed for up to 90 months, more than 50 % of them became overweight with a higher relative increase among patients who were underweight or normal weight at baseline (Umbricht et al., 1994).

Several studies reviewed by Nasrallah et al., (2008) showed that over one year clozapine and olanzapine caused a mean weight gain of 12 kg, against an increase of 2-3 kg for risperidone and quetiapine, and 1 kg for ziprasidone.

Likewise, olanzapine caused greater weight gain compared to quetiapine, risperidone, perphenazine and ziprasidone with a large proportion of patients gaining 7 % of their body weight or more (Lieberman et al., 2005). A similar degree of olanzapine-induced body weight increase has also being reported by Beasley (1997).

Weight gain, when associated with the development of obesity (estimated using the body mass index BMI) and abdominal fat deposition is often the cause of impaired glucose regulation, insulin resistance, hypertension and dyslipidemia, all risk factors that may result in the development of diabetes and cardiovascular disease (CVD). The presence of at least three of the above mentioned factors is known as metabolic syndrome (Van Gaal, 2006).

Furthermore, patients with mental illnesses are recognized as having a high rate of type 2 diabetes and incidence of CVDs that in turn leads to

an increased risk of mortality associated with these conditions (Casey et al., 2004). In fact, clozapine and olanzapine treatment appears to enhance plasma glucose level and evoke insulin resistance in non-diabetic patients with schizophrenia compared to healthy subjects and those treated with other antipsychotics (Newcomer, 2004). This finding has been confirmed in a subsequent meta-analysis of 14 studies examining the association between diabetes onset and treatment with atypical antipsychotics in comparison with typicals or absence of treatment (Newcomer, 2007).

Moreover, olanzapine and clozapine have been shown to increase the risk of developing hyperlipidemia in comparison with other second-generation antipsychotics (Lambert et al., 2005).

1.2.3.6 Weight gain and antipsychotic noncompliance

Despite the fact that weight gain has been recognized as a side effect of antipsychotic drug treatment for a long time, only in the past decade has its importance been appreciated. Previously, the significance of the weight gain was often underestimated because it was associated with a

largely disregarded effect of the illness itself relative to other antipsychotic side effects (Allison et al., 1999). The introduction of the second generation of these drugs and the reduction of the incidence of EPSEs allowed a focus on other side effects such as the increase in weight.

Weight gain and metabolic abnormalities are in fact, a cause of major concern in clinical practice. A number of studies have demonstrated that compliance with antipsychotic medication is generally poor and not taking medication is associated with a substantial increase in rehospitalisations and a generally poorer outcome. Noncompliance inclines patients to relapse, leading to significant psychological distress, medical morbidity and increased mortality (Fontaine et al., 2001).

In a recent study, a significant, positive association was found between the patient's increase in weight and subjective distress and medication non-compliance. Moreover, a higher baseline BMI was associated with a 2.5 fold increase in the likelihood of stopping medications (Weiden et al., 2004).

Furthermore, psychotic patients tend to smoke more than the general population and the impact of weight gain and all the metabolic abnormalities related to it may be underestimated by the fact that they are less likely to receive medical treatment for non psychiatric-related illnesses (Fontaine et al., 2001).

Over the years, clozapine and olanzapine have gained much popularity as treatment options especially in the light of the role of clozapine as the only effective treatment for refractory schizophrenia. However, they appear to have the greatest weight gain propensity and as far as clozapine is concerned, the cumulative incidence of all patients reaching 20 % overweight, representing a significant long-term health risk, has been reported to be greater than 50 % (Umbricht et al., 1994). Similarly, clozapine gives the highest incidence of diabetes and hyperlipidemia.

Interestingly, while treatment with clozapine reduced the risk of suicide by 80-85 % in treatment resistant-schizophrenic patients (Meltzer, 1999), a recent estimate of the consequences of drug-induced weight gain and diabetes concluded that its benefit in preventing suicide "may

essentially be offset by the deaths due to weight gain" (Fontaine et al.,

2001).

The above effects on compliance coupled with the morbidity and

mortality associated with antipsychotic induced obesity and

hyperglycemia clearly highlights the need for adjunctive intervention to

improve the health of patients treated with atypical antipsychotic drugs.

1.3 Aims of the project

Given the relevance of atypical antipsychotic-induced side effects in the clinic, it is vital to study the biological basis underlying metabolic side effects using animal models and *in-vitro* and *ex-vivo* cell models.

Thus, the aims of the project are:

1. Initially, to develop a reliable and consistent mouse model of olanzapine and clozapine induced-weight gain and augmented adiposity which will mimic the increase in body weight and fat deposition seen in the clinic. The development of such a model would not only facilitate the testing of possible adjunctive treatments to counteract atypical antipsychotic-induced side effects but would also prompt further research to uncover the mechanisms responsible for them.

2. In addition, to correlate with the above investigation, cultured fibroblastic-like cells 7-F2 and primary bone marrow cells will be employed as a model to study adipogenesis. The principal goal of this study will be to probe the likelihood that clozapine and olanzapine might

produce a direct peripheral effect on adipocyte formation and/or perturb

lipid metabolism.

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Chapter 2

Materials and Methods

2. Materials and methods

2.1 Animal model

2.1.1 Animal husbandry

Female mice C57BL/6J, aged 4-5 weeks, purchased from B&K (UK), were used in the animal studies. On the arrival animals were housed as two per cage to avoid social isolation. They were kept under standard conditions of husbandry receiving pellet mouse food and water *ad libitum*.

Room temperature was maintained at 20.0 ± 1.5 °C in humidity-controlled conditions and the lighting was on a 12 h/12h light/dark cycle. Animals were acclimatized for a week before commencement of experiments, during which time they were handled daily for habituation.

The use of animals in these studies was carried out in accord with UK Home Office licensing, as prescribed by The Animals (Scientific Procedures) Act 1986.

2.1.2 Drug treatments

Clozapine and olanzapine were purchased from Kemprotec Ltd, (Middlesbrough, UK).

The drug was offered orally to the animals once a day at the stated doses in the morning. The vehicle consisted of pure honey (Tesco, UK).

2.1.3 Methodology

Animals were self-administered with honey (as the drug vehicle) or the drug mixed with honey placed inn a small plastic Petri dish (2.5 cm diameter) on a trial basis for 15 minutes daily over a 28-day period (plus a 5-day period of habituation).

During drug self-administration, animals were housed individually. Each particular animal was checked to ensure consumption of the drug or vehicle.

Treatment 1: Effect of olanzapine 10 mg/kg on body weight and fat deposition.

Two groups of mice (n=12 per group) were food deprived for 5 hours, then given 0.1 ml of honey mixture with 0 (control) or 10 mg/kg of olanzapine once a day orally for 28 days.

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Treatment 2: Effect of olanzapine 5 and 15 mg/kg on body weight and fat deposition.

Three groups of mice (n=10) were given 0.1 ml of honey mixture with 0 (control), 5 or 15 mg/kg of olanzapine once a day orally for 28 days.

Treatment 3: Effect of clozapine 4 and 8 mg/kg on body weight and fat deposition.

Three groups of mice (n=10) were given 0.1 ml of honey mixture with 0 (control), 4 or 8 mg/kg of clozapine once a day orally for 28 days.

Treatment 4: Effect of clozapine 15 and 25 mg/kg on body weight and fat deposition.

Three groups of mice (n=10) were given 0.1 ml of honey mixture with 0 (control), 15 or 25 mg/kg of clozapine once a day orally for 28 days.

Treatment 5: Effect of clozapine 15 mg/kg, metformin 500 mg/kg, and clozapine 15 mg/kg + metformin 500 mg/kg on body weight and fat deposition.

Four groups of mice (n=9) were given 0.1 ml of honey mixture containing 0 (control), 15 mg/kg of clozapine, 500 mg/kg of metformin or clozapine 15 mg/kg + metformin 500mg/kg once a day orally for 28 days.

Treatment 6: Effect of clozapine 15 and 25 mg/kg and olanzapine 10 and 12.5mg/kg on body weight and fat deposition.

Five groups of mice (n=7) were given 0.1 ml of honey mixture with 0 (control), 15 or 25 mg/kg of clozapine and 10 or 12.5 mg/kg of olanzapine once a day orally for 28 days.

2.1.4 Measurements

Body weight was recorded every morning prior to the start of treatment. At the end of each 28-day experiment, animals were killed under terminal gaseous anaesthesia.

White adipose tissue from perirenal, periuterine and intraabdominal areas were dissected out, pooled and weighted.

Bone marrow was collected from femur bones. The femur bones were dissected out, separated from their attached muscle and connective tissue, then cut at both ends and bone marrow flushed with RNAse-free water (Ambion, UK), then stored in RNA *later* (Ambion, UK) at -20 C until required for qRT-PCR analysis.

2.1.5 Quantitative Real Time-PCR

RNA from bone marrow was extracted using Trizol reagent, then cDNA was reverse transcribed from total RNA samples using random primers and the PCR products were subsequently synthesized from cDNA samples using the PCR mastermix.

2.1.5.1 RNA extraction and quantification

Total RNA was extracted with Trizol according to manufacturers instructions. This reagent isolates high quality RNA from the biological material; it combines phenol and guanidine thiocyanate in a mono-phase solution to facilitate the immediate and most effective inhibition of RNAse activity. The biological sample was homogenized in Trizol then separated into aqueous and organic phases by chloroform addition followed by centrifugation (RNA remains in the aqueous phase, DNA in the interphase and proteins in the organic phase). RNA was precipitated by addition of isopropanol, then washed with ethanol and subsequently solubilised.

The frozen tissues were homogenized in 1.0 ml of Trizol at room temperature using a PowerGen 125 Tissue Homogenizer (Fisher Scientific). The homogenates were then transferred to clean tubes and left at room temperature for 5 minutes. Chloroform (0.2 ml) was added and the tubes vigorously shaken for 15 seconds and incubated at room

temperature for 2 minutes. The samples were then centrifuged at 12,000 x *g* for 15 minutes at 4 °C.

After centrifugation the colourless upper aqueous phase was transferred to clean tubes and mixed with 0.5 ml of isopropanol and stored overnight at -20 °C.

The samples were then centrifuged at 12,000 x *g* for 15 minutes at 4 °C. The RNA precipitate forms a white pellet on the side and bottom of the tube. Pellets were then washed twice with 1.0 ml of 75% ethanol and centrifuged at 12,000 *g* for 10 minutes at 4 °C.

Ethanol was then removed and RNA pellets air-dried for 5 minutes.

Pellets were resuspended in 20 µl in RNAse-free water and stored at -80 °C.

RNA quantity was determined on a Beckman UV-DU64 spectrophotometer by measuring optical density at 260 nm wavelength. The ratio between the absorbance values at 260 and 280 nm gave a measure of RNA purity. A 260/280 ratio between 1.6 and 2.0 indicated a sample of sufficient purity.

The integrity of each RNA sample was confirmed using Agilent BioAnalyzer.

2.1.5.2 Reverse Transcription Reaction (RT)

cDNA was reverse transcribed from 1 µg of total RNA using random primers (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, UK).

The following reaction was set up in individual tubes:

RT mastermix	Volume/reaction
10X RT Buffer	2 µl
25X dNTP mix (100mM)	0.8 µl
10X RT random primers	2 µl
Multiscribe Reverse Transcriptase	1 µl
RNAse Inhibitor	1 µl
Nuclease-free Water	3.2 µl

Table 2.1a Volumes of regents used for RT

Tubes were placed on ice and mixed with the following mix:

RNA sample	Total amount	Volume/reaction
RNA	1 µg	X µl
Nuclease-free water	–	Up to 10 µl

Table 2.1b Concentrations and volumes of reagents used for RT

Negative controls, containing water instead of RNA, were also prepared to verify that none of the kit reagents was contaminated with DNA.

The RT reaction was carried out at the following thermal profile: 25 °C × 10 minutes / 37 °C × 120 minutes / 85 °C × 5 minutes.

The cDNA was then stored at -20 °C.

2.1.5.3 qRT-PCR procedure

Quantitative Real Time-PCR was performed using the 7900HT Fast Real-Time PCR system and TaqMan gene expression assay (AB Applied Biosystems, UK).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeper gene. Pre-designed and labeled primers/probe sets were purchased from AB Applied Biosystems.

For each gene, the following qPCR mix was prepared in 96-well PCR plate:

qPCR mix	Volume/reaction
TaqMan Universal PCR master mix	10 μl
dH$_2$O	5 μl
Gene expression assay solution	1 μl

Table 2.2 Volumes of regents used for qPCR

cDNA (4 μl) was added to the qPCR mix (4 μl of dH$_2$O for non-template control). The plate was then centrifuged briefly and the reaction was carried out at the following thermal profile: 95 °C x 10 minutes/ 95 °C x 15 seconds + 60 °C x 1 minute (40 cycles).

All the reactions were performed in triplicate and results were normalized relative to GAPDH expression control. The results were expressed in terms of relative quantification (RQ). RQ determines the changes in expression of a target sequence in a test sample relative to the same sequence in a calibrator sample.

The RQ expression of the genes of interest was analyzed using SDS software, Sequence Detection System (Applied Biosystems).

2.1.6 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) version 12. Data from weight gain experiments were analyzed employing statistical comparison between means and repeated measures analysis of variance (ANOVA), followed by Dunnett's post-hoc. If comparison was made between two groups Mann-Whitney test was used. For the qRT-PCR experiment statistical comparison between means and student t test was used. The criteria for statistical significance was $P < 0.05$. Data are presented as means ± SEM.

2.2 Cell culture

2.2.1 Cell line

The 7-F2 cell line derives from the bone marrow of p53 knockout mice. Thompson et al., (1998) isolated and characterized ten clonal cells lines which were grouped into three categories, one of the clones gave rise to the 7-F2 cell line, characterized by indefinite growth *in vitro* and mesenchymal origin.

The 7-F2 cells were kindly donated by Dr. Browen Evans (Department of Child Health, School of Medicine, Cardiff University) and used at passage 10-30.

2.2.2 Cell husbandry

Full culture medium: alpha minimum essential medium containing Earle's salt, sodium pyruvate 1mM and L-glutamine 2 mM, without ribonucleosides and dexoxyribonucleosides, supplemented with 10 % v/v fetal bovine serum (FBS), 100 µg/ml of streptomycin and 100 U/ml of penicillin.

Adipocyte differentiation medium: the above α-MEM medium was supplemented with 50 mM of indomethacin, 50 mg/ml of ascorbic acid and 100 nM dexamethasone to induce adipocyte differentiation (Thompson 1998).

The full culture media as prepared above was kept refrigerated at 4 °C and warmed at 37 °C in a water bath prior to use.

All cell culture ingredients were purchased from Gibco (Invitrogen UK). Indomethacin, ascorbic acid and dexamethasone were purchased from Sigma (UK).

Cells were grown in 25 cm^2 flasks in a humidified incubator at 37 °C, 5% CO_2 and 95% air.

When the cells reached confluence, approximately once every 3-4 days, they were passaged: first, the media was aspirated from the flask by vacuum then the cells were washed with sterile phosphate buffered saline (PBS), 0.5 ml of trypsin was added and incubated at 37 °C for 3-5 minutes. Once the cells were detached from the bottom of the flask, trypsin was inactivated by adding 2 ml of full media, then cells were centrifuged for 3 minutes at 1000 x g, the supernatant was discarded and the cells resuspended in 1.0 ml of full media to be either seeded in new flasks (ratio 1:5) or in a known volume of media in order to be seeded at a known density in 6, 12 or 96 well plates.

To seed the cells at a specific density, cells were counted in a haemocytometer: 8 µl of cell suspension was transferred to both chambers of a haemocytometer and an average count of 10 squares in each of the chambers (x 10^4) provided the number of cells per ml. Once the cell number was determined, an appropriate volume of full media

was added to a known volume of cell suspension to obtain the desired cell seeding density.

2.2.3 Drug exposure

Cells were incubated with clozapine or olanzapine (Kemprotec, UK) at the appropriate concentration while control cells were incubated in full media containing the appropriate volume of DMSO vehicle.

2.2.4 MTS proliferation assay

For this assay, at day -1, cells were seeded in 96-well plates at the density of 3,000 cells per well in a volume of 100 μl of full media. At day 0, full media was replaced with media containing the test-drugs at several different concentrations and then cells were incubated at 37 °C for 72 hours, 5 days or 7 days.

The MTS assay is based on the conversion of the tetrazolium salt MTS (3-(4,5-dimethylthiazol-2yl-)-5-(3-carboximethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) into a coloured and water-soluble formazan.

The dehydrogenase enzymes of the viable cells reduce the yellow MTS solution to a purple colour at 37 °C. Therefore, the amount of formazan produced is directly proportional to the number of living cells providing a good indication of cell viability (Malich et al., 1997).

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The MTS reagent (Promega, UK) was combined with a detection reagent, phenazine methosulfate (PMS) (Promega, UK), in ratio of 20:1, then the mixture was added to the cell culture in a ratio of 1:5 (20 μl).

The plate was then covered in alluminium foil since the reagent is light sensitive, and incubated at 37 °C for 1.5 hours.

The absorbance was measured in a microplate spectrophotometer at 490 nm.

2.2.5 Cell counting with haemocytometer (trypan blue exclusion)

The haemocytometer is a device used to estimate cell number. It consists of a thick glass slide, which has a central H-shaped chamber carrying two grid compartments of known area and volume. Each compartment is divided into 9 squares each covering an area of 1 mm^2 (Fig 2.1). After the cover glass is applied on the top of the chamber, a known volume of cell sample is applied by pipette in the compartments through the edge of the cover glass. The cells lying on the central square, top left and top right, bottom left and bottom right squares are counted (cells lying on the bordering lines are counted if they are on either the top or on the left lines only). The number of cells in 1 ml of the original sample is counted as follow:

$$\frac{\text{Total number of cells in each square}}{\text{Number of squares}} \times 10^4$$

Fig 2.1 A haemocytometer
http://toolboxes.flexiblelearning.net.au/demosites/series4/412/laboratory/study
notes/SNHaemo.htm
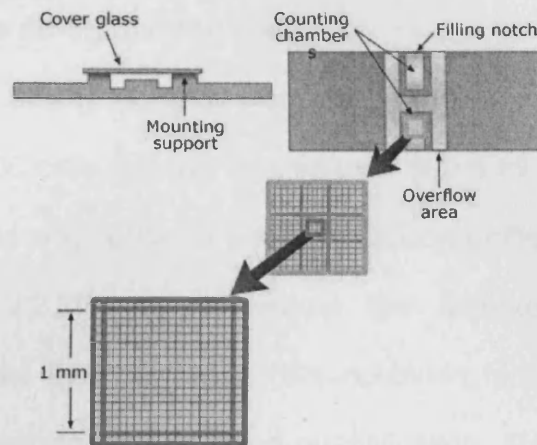
For this assay, at day -1, cells were seeded in 6-well plates at the
density of 30,000 cells per well in a volume of 1 ml of full media. At day
0, the general media was replaced with the media containing the test-
drugs at several different concentrations and then the cells were
incubated at 37 °C for 72 hours.

The cells were then washed with PBS, trypsinized, centrifuged and then
resuspended in 100 μl of full media; to the cell suspension an equal
amount of trypan blue solution 0.4%, 100 μl, was added. The mixture
was then left to incubate for 5 minutes at room temperature and then
placed in haemocytometer for manual cell counting.

Trypan blue is a dye that stains selectively dead cells, therefore, non-
viable cells will appear blue under the light inverted microscope.

2.2.6 Adipocyte differentiation assay

For this assay, at day -1, cells were seeded in 12-well plates at the density of 15,000 cells per well in a volume of 0.5 ml of full media. At day 0, the media was replaced with the adipocyte differentiation media (described in 2.2.2) with or without the test-drugs at different concentration and the cells were then incubated for 2, 5 and 8 days before proceeding with staining and quantification. The media in each well was changed every 3 days.

Oil Red O Staining

Oil Red O stock solution was prepared by dissolving 0.25 g of Oil Red O (Sigma, UK) in 50 ml of isopropanol. The working solution consisted of a mixture 3:2 of the stock solution-dH_2O.

Firstly, the cells were washed with 1.0 ml of PBS then fixed in 0.5 ml formal saline (10% formaldehyde in 0.9% NaCl) for 15 minutes. The formal saline was subsequently removed and cells were washed with distilled water. The cells were then stained with 400 µl of Oil Red O solution for 20 minutes.

Excess stain was washed off with 80 µl of 60% isopropanol followed by further washing with 1.0 ml of PBS. The cells were then stored in fresh PBS prior to qualitative microscopy.

To quantify the adipogenesis (in terms of concentration of Oil Red O), 250 µl of isopropanol was added to each well to dissolve the stain and

the plate shaken briefly; then 150 µl from each well was transferred to a 96-well plate and absorbance was read in a microplate spectrophotometer at 490 nm.

2.2.7 Quantitative Real Time-PCR

7-F2 cells were differentiated for 5 days then total RNA was extracted using Trizol reagent (Invitrogen), 1 µg of RNA was subsequently reverse transcribed into cDNA using random primers (Improm II, RT system, Promega, UK).

The expression of genes of interest was determined relative to housekeeper gene acid ribosomal protein (ARP).

2.2.7.1 RNA extraction and quantification

Cells, seeded in 6 well plates, were lysed with 1 ml of Trizol reagent and homogenized by pipetting up and down. The homogenates were then transferred to 1.5 ml tubes and incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes.

The homogenates were mixed with 0.2 ml of chloroform and the tubes shaken vigorously by hand for 15 seconds then incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4 °C.

Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase (containing RNA).

The aqueous phase was transferred to a fresh tube and RNA precipitated by mixing with 0.5 ml isopropanol. Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g at 4°C. After removing the supernatant, the pellet was washed with 1 ml of 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 4 °C.

At the end of this procedure, the RNA pellet was air-dried and dissolved in 25 µl of RNAse-free water by pipetting up and down and then incubated for 4 minutes a 55 °C.

DNAse treatment

Contaminating DNA was removed using DNA-free kit (Ambion, UK). Samples were mixed with 2.5 µl of 10x DNAse I buffer and 0.5 µl of DNAse 1, agitated gently and then incubated at 37 °C for 30 minutes. To stop the digestion, the samples were treated with 2.5 µl of DNAse inactivation reagent and incubated at room treatment for 2 minutes, then centrifuged at 10,000 x g for 2 minutes and the supernatant, containing RNA, was transferred to a fresh tube and stored a -80 °C.

RNA quantification

RNA was quantified by GeneQuant spectrophotometer (GE Healthcare, UK) by measuring optical density at 260 nm, and quality was checked by the ratio 260/280 nm. The ratio 260/280 nm was ≥1.7 for all the samples, thus indicating pure RNA.

2.2.7.2 Reverse Transcription Reaction (RT)

Reagents employed in this reaction were supplied by Promega, UK.

The following reaction was set up in two DNA/RNA-free tubes:

Reagent	final concentration	volume/reaction
RNA	1 µg	X µl
Random primers (500µg/ µl)	0.5µg/ µl	1µl
Nuclease free water	_	Up to 5µl

Table 2.3a Concentrations and volumes of reagents used for RT

The mix was incubated at 70 °C for 5 minutes then immediately chilled on ice. The following reaction was set up in two DNA/RNA-free tubes:

RT mix	RT-PCR sample	No Enzyme RT-PCR control
5x reaction buffer	4µl	4µl
$MgCl_2$ (25mM)	2.4µl	2.4µl
dNTPs(40mM)	1µl	1µl
RNAsin	0.5µl	0.5µl
Reverse transcriptase	1µl	0µl
Nuclease free water	6.1µl	7.1µl

Table 2.3b Volumes of reagents used for RT

The RNA target + primers was added to the 15 µl above and the mix gently pipetted up and down. The RT reaction was carried at the following thermal profile: 25 °C × 5 minutes / 42 °C × 60 minutes / 70 °C × 15 minutes. The cDNA was then stored at -20 C°.

2.2.7.3 Primers design

The primers have been designed by Dr Borzo Gharibi, Department of Child Health, School of Medicine, Cardiff University.

Primers	Forward	Reverse	Size(bp)
C/EBPβ	CAAGCTGAGCGACGAGTACA	CAGCTGCTCCACCTTCTTCT	157
PPARγ	TTTTCAAGGGTGCCAGTTTC	AATCCTTGGCCCTCTGAGAT	220
LPL	GCGTAGCAHGAAGTCTGACC	CATCAGGAGAAAGGCGACTG	108
ARP	GAGGAATCAGATGAGGATATGGGA	AAGCAGGCTGACTTGGTTGC	72

Table 2.4 Mouse primer sequences. Acid ribosomal protein (ARP) is used as the housekeeper gene.

2.2.7.4 qRT-PCR procedure

Quantitative Real Time-PCR was performed using an MX300p thermal cycler (Stratagene, UK) and Platinum SYBR green qPCR SuperMix UDG (Invitrogen, UK).

The following reaction was set up:

Reagent	Volume/reaction
SYBR green	12.5 μl
cDNA	1 μl
Forward primers	1 μl
Revers primers	1 μl
Nuclease-free water	9.5 μl

Table 2.5 Volumes of regents used for qPCR

The mixture was prepared in a 96-well PCR plate, centrifuged at 1000 x *g* for 5 minutes and then the reaction was performed using the following thermal profile: 50 °C x 2 minutes/ 95 °C x 2 minutes/ 90 °C x 15 seconds + 60 °C x 30 seconds (45 cycles).

All reactions were performed in duplicate and template negative controls were included in the amplification. Results were normalized relative to ARP expression control.

The relative quantitative expression of the genes of interest was analyzed using MxPro software (Stratagene).

2.2.8 Statistical analysis

Statistical analysis was performed using SPSS version 12. Statistical comparison between means was employed using student t test and one- way ANOVA, followed by Dunnett's post-hoc test.

The criteria for statistical significance was P< 0.05. Data are presented as means ± SEM.

2.3 Bibliography

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Thompson D L, Lum K D, Nygaard S C, Kuestner R E, Kelly K A, Gimble J M, Moore E E. The derivation and characterization of stromal cell lines from the bone marrow of p53–/– mice: new insights into osteoblast and adypocite differentiation. J Bone Min Res 1998; 13(2): 195-204.

Chapter 3

Antipsychotic-induced weight gain: a preliminary study to develop an animal model

3. Antipsychotic-induced weight gain: a preliminary study to develop an animal model

3.1 Introduction

Over the past few years several research groups have attempted to develop a rodent model of chronic antipsychotic-induced weight gain and metabolic dysregulation in order to reflect the clinical situation.

Table 3A summaries some of the pertinent results from a number of published studies conducted with the aim of developing such a model, focusing particularly on the effects of clozapine and olanzapine.

Olanzapine has been shown to produce weight gain in female rats and these findings have been replicated in various laboratories (Goudie et al., 2002, Pouzet et al., 2003, Arjona et al., 2004, Fell et al., 2004,

Cooper et al., 2005, Kalinichev et al., 2005-2006, Albaugh et al., 2006). However, experiments involving male rats have encountered difficulties in reproducing the weight gain caused by antipsychotics seen in female models (Pouzet et al., 2003, Albaugh et al., 2006, Minet Ringuet et al., 2006a), and indeed weight loss has actually been reported in one study (Baptista et al., 1993).

Surprisingly, clozapine which has a similar receptor binding profile to olanzapine plus a comparable weight gain inducing propensity and metabolic modifying action in the clinic, has failed to induce weight gain in female rat models (Baptista et al., 1993, Albaugh et al., 2006, Cooper et al., 2008). On the contrary, weight gain has been reported in male rats (Albaugh et al., 2006, Sondhi et al., 2006).

Fewer studies have been performed using the mouse as a model. Kaur and Kulkarni (2002) reported weight gain in female mice treated with clozapine as did Zarate et al., (2004) though this latter finding was observed in male mice. In contrast, Cheng et al., (2005) described a weight loss in clozapine-treated male mice.

Moreover, Cope et al., (2005) and Coccurello et al., (2006-2008) described a weight increase in female mice treated with olanzapine, whilst Albaugh et al., (2006) was unable to show any changes versus olanzapine-treated controls.

It is therefore evident that findings in this field of investigation are quite heterogeneous and at times, contradictory probably due to a number of

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factors that appear to undermine their reproducibility. Hence, not only different species (rat or mouse) but also different strains within the same species have yielded different body weight outcomes.

Furthermore, several research groups have highlighted the importance of gender difference, with female rodents being regarded as more inclined to gain weight than males in response to antipsychotic agents.

In addition, other factors that might potentially account for these discrepancies must be sought within the varieties of methodologies and protocols used by different laboratories. Such factors include disparity of dose, length of treatment and route of administration.

Authors	Species/Strain	Sex	Drugs	Route	Duration	Results
Baptista (1993)	R/Wistar	F/M	CLO 0.5,1,2.5,5,10,20mg/kg	IP	21 days	=BW fem ↓BW male (10,20mg/kg)
Goudie (2002)	R/Wistar	F	OLA 4mg/kg (2Xday)	IP	10 days	↑BW
Pouzet (2003)	R/Wistar	F/M	OLA 5,20mg/kg (2Xday)	OS	21 days	↑BW;FIfem =BW;FI male
Arjona (2004)	R/SD	F	OLA 1.2mg/kg	OS	10 days	↑BW;FI
Fell (2004)	R/HL	F	OLA 0.5,1,4mg/kg	IP	21 days	↑BW;FI; FD
Cooper (2005)	R/H Wistar	F	OLA 1,2,4mg/kg (2Xday)	IP	20 days	↑BW;FI;FD
Kalinichev(2005)	R/Wistar Wistar-SD	F F	OLA 5mg/kg (2Xday) OLA 1,2,10mg/kg (2Xday) ARI 2,4,8mg/kg	OS	14 days 7days	↑BW;FI;FD ↑BW>in Winstar
Kalinichev(2006)	R/SD	F	OLA 2mg/kg (2Xday) ZIP 2,6,10mg/kg (2Xday)	OS	7 days	↑BW ↑BW
Albaugh (2006)	R/SD-Wistar	F/M	OLA increasing dose from 4 to 20mg/kg over time CLO increasing dose from 4 to 8mg/kg over time CLO+OLA 8+4mg/kg	SA	33 days 12 days 13-24th day	↑BW;FI fem;FD;L =BW =BW
Minet-Ringuet (2006a)	R/SD	M	OLA 1mg/kg ZIP 10mg/kg	SA	6 weeks	=BW;FI ↑FD;L
Minet-Ringuet (2006b)	R/SD	M	OLA 0.01,0.1,0.5,2mg/kg	SA	6 weeks	↑BW;FD
Patil (2006)	R/SD	F	OLA 1,2mg/kg	IP	20 days	↑BW;BP
Cooper (2008)	R/Wistar R/H Wistar	F F	CLO 6,12mg/kg (2Xday) CLO 1,4mg/kg (2Xday) CLO 0.25,0.5mg/kg (2Xday)	IP	21 days 20 days 12 days	↓BW ↓BW;=FI,I,L,Glu; ↑FD,Adi ↓BW
Evers (2010)	R/Wistar	F	OLA 5mg/kg (2Xday)	OS	14 days	↑BW;=LA;FI
Kaur (2002)	M/laca	F	CLO 2mg/kg,FLU 10mg/kg CLO+FLU	IP	21 days	CLO↑BW;FI CLO+FLU reverse it
Zarate (2004)	M/AJ M/C57BL/6J	M M	CLO 2,4mg/kg CLO 2,4mg/kg	IP	18 days 18 days	↑BW(4mg/kg) ↑BW(4mg/kg)
Cheng (2005)	M/Balb/c	M	CLO 2,10mg/kg	IP	30 days	↓BW(10mg/kg) =BW (2mg/kg)
Cope (2005)	M/CB57BL/6J	F	OLA 3,6mg/kg QUE 15,30mg/kg RIS 0.125,0.25mg/kg ZIP 7,10.5mg/kg	SA	4 weeks	↑BW;FI;FD
Albaugh (2006)	M/AJ M/C57BL/6J	F F	OLA increasing dose from 4 to 8mg over time	SA	18 days	=BW
Coccurello (2006)	M/CD1	F	OLA 0.75,1.5,3mg/kg OLA 3mg/kg OLA 4,8mg/kg	OS OP	36 days 30 days 28 days	↑BW;FI;FD;I,Tri(3mg/kg) =EE ↑BW;FI
Coccurello (2008)	M/CD1	F	OLA 3,6mg/kg	SA	50 days	OLA 3:↑BW;FI;FD Chol;Glu;Tri;I OLA 6:↑BW;Glu;Tri ↓FI

Table 3A CLO: Clozapine; OLA: Olanzapine; ARI: Aripiprazole; ZIP: Ziprasidone; FLU: fluoxetine; QUE: Quetiapine; IP: intraperitoneal; OS: oral gavage; SA: self-administration; OP: osmotic pump; BW: body weight; FD: fat deposition; FI: food intake; L: leptin; BP: blood pressure; I: insulin; Glu: glucose; Adi: adiponectin; Tri: triglycerides; Chol: cholesterol; LA: locomotor activity.

3.1.1 Pharmacological approach to counteract APS-induced metabolic side effects

Along with the search for the reliable animal model of APS-induced weight gain and/or a drug-perturbed metabolism, a few clinical studies have been performed in order to test the possibility that patients could benefit from the introduction of adjunctive therapy with an anti-obesity medication. However, a lack of understanding of the mechanism underlying APS-induced weight gain complicates the development of a pharmacologic approach to the problem.

One of the drugs approved for the treatment of obesity is orlistat which inhibits enteric lipase to reduce fat absorption. Its absence of any CNS activity might render orlistat a suitable candidate in the above capacity as a treatment for APS-induced weight gain. In this context, there have been two case reports which showed firstly that a patient taking antipsychotic medications displayed a decrease in BMI from 36.3 kg/m^2 to 31.4 kg/m^2 in 12 months of orlistat treatment and another who lost 3 kg after 3 months (Anghelescu et al., 2000). Unfortunately, the use of this drug is restricted by unwanted gastrointestinal side effects, which dictate that orlistat therapy must proceed in combination with a low-fat diet.

Some clinical studies reported that the addition of metformin, the most prescribed oral hypoglycemic agent for the treatment of type 2 diabetes, to olanzapine therapy has been effective not only in improving insulin

sensitivity but also in reducing weight gain (Baptista et al., 2007; Wu et al., 2008). Thus, metformin could be looked upon as a potential agent to be introduced in clinical practice for the management of antipsychotic-induced metabolic side effects.

3.1.2 Aim of the study

The experiments in this study were designed to search for a suitable mouse model since few investigations have been conducted using mice.

The experimental design was based on that of Cope et al., (2005). The same sex and animal strain (female C57BL/6J mice) and a chronic oral self-administration route were used. However, mixing the drug with honey as an oral delivery vehicle was found to be preferable to incorporating the drug in peanut butter pills, as shown in the habituation study previously conducted in the laboratory. This mode of administration proved to be much less invasive for the animals than either intraperitoneal injection or the oral route of administration (gavage). In addition to body weight changes, the rate of fat deposition was also monitored by weighing the intrabdominal, perirenal and periuterine white adipose tissue (post-mortem).

Furthermore, in this pilot study the ability of metformin to attenuate clozapine-induced weight gain was also tested.

3.2 Results

3.2.1 Effect of olanzapine (10 mg/kg) on body weight and fat deposition

There was, as expected, a linear increase in body weight for both treatment groups (Fig 3.1a). Mice treated with olanzapine (10 mg/kg) exhibit some initial weight loss in the first three days of treatment. Moreover, there was not significant weight difference between the two groups for the first 16 days while a significant increase in body weight was seen after 17 days until the end of the treatment (P<0.05) compared with the placebo controls group.

In addition, weight gain was accompanied by a highly significant augmentation of fat deposition (P<0.01) (Fig 3.1b).
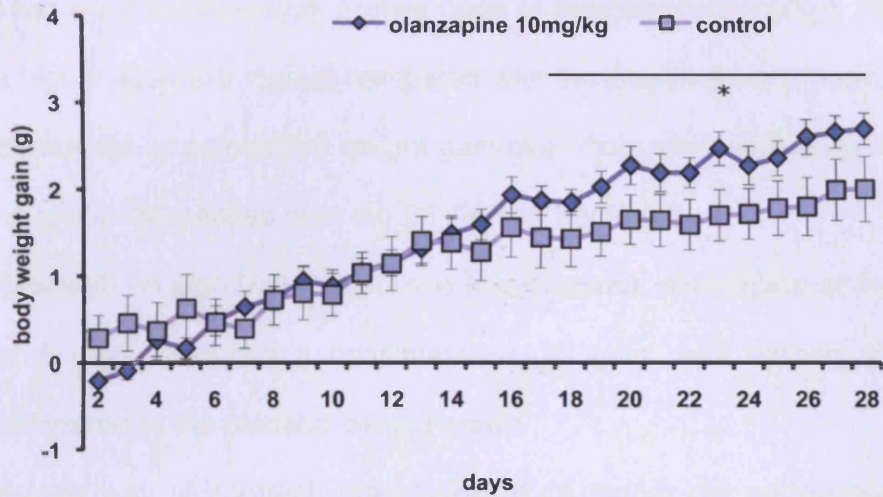
Fig 3.1a Experiment 1. Effect of chronic oral administration of olanzapine (10 mg/kg) on mouse body weight over 28 days. $n=12$; values represent the means +/- SEM; (*$P<$ 0.05).

Fig 3.1b Experiment 1. Effect of chronic oral administration of olanzapine (OLA) (10 mg/kg) on fat deposition (pooled fat pads) in mice. $n=12$; values represent the means +/- SEM; (**$P<0.01$).

3.2.2 Effect of olanzapine (5 mg/kg and 15 mg/kg) on body weight and fat deposition

Mice were administered a lower dose of olanzapine (5 mg/kg), and also a higher dose (15 mg/kg) compared with the previous experiment.

Evaluation of cumulative weight gain over days showed that there were no group differences over the 28 days of treatment.

Although no significant difference was attained, olanzapine at the dose of 5 mg/kg induced a consistent weight gain, with respect to time, compared to the placebo control group.

By the way of contrast, olanzapine at 15 mg/kg did not produce any significant trend towards weight loss over the period of the treatment (Fig 3.2a).

Contrary to the first dose tested of 10 mg/kg, no significant difference was seen in terms of enhanced deposition of white adipose tissue between groups (Fig 3.2b).

Fig 3.2a Experiment 2. Effect of chronic oral administration of olanzapine (5 and 15 mg/kg) on mouse body weight over 28 days. $n=10$; values represent the means ± SEM.
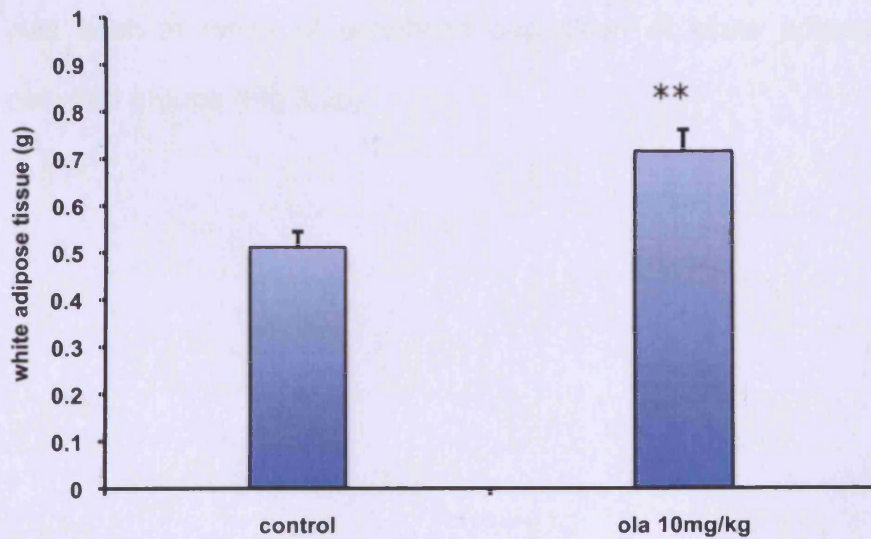

Fig 3.2b Experiment 2. Effect of chronic oral administration of olanzapine (OLA) (5 and 15mg/kg) on fat deposition (pooled fat pads) in mice. $n=10$; values represent the means ± SEM.

3.2.3 Effect of clozapine (4 mg/kg and 8 mg/kg) on body weight and fat deposition

Oral administration of clozapine (4 mg/kg and 8 mg/kg) did not promote any extra weight gain compared with the placebo control group (Fig 3.3a).

Both doses of clozapine increased white adipose tissue deposition compared to the placebo control group instead, although only the lower dose of 4 mg/kg attained significance (P<0.05), (Fig 3.3b).
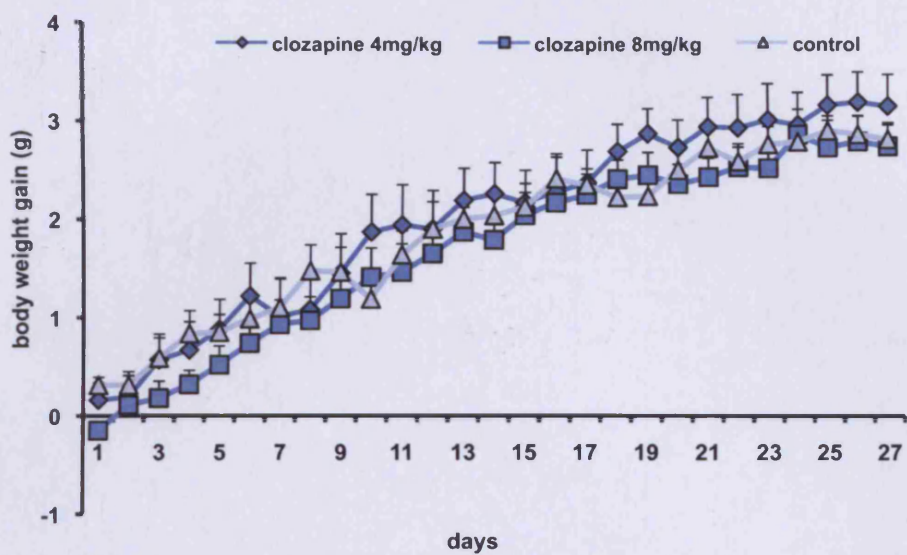
Fig 3.3a Experiment 3. Effect of chronic oral administration of clozapine (4 and 8 mg/kg) on mouse body weight over 28 days. n=10; values represent the means ± SEM.

Fig 3.3b Experiment 3. Effect of chronic oral administration of clozapine (CLO) (4 and 8 mg/kg) on fat deposition (pooled fat pads) in mice. n=10; values represent the means ± SEM; (*$P<0.05$).

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3.2.4 Effect of clozapine (15 mg/kg and 25 mg/kg) on body weight and fat deposition

Evaluation of cumulative weight gain over the 28 day treatment period showed that clozapine (15 mg/kg) significantly increased body weight compared to the placebo control group throughout the period of the treatment (P<0.01).

The higher dose (25 mg/kg), also significantly increased body weight on several intermittent treatment days, 3-7, 11-14 and 22, in comparison to the control group (Fig 3.4a).

Notably, in the first 7 days of treatment, the control group animals lost weight but there is no obviously identifiable causative explanation. However, clozapine treatment at doses of 15 mg/kg and 25 mg/kg did not enhance white fat deposition (Fig 3.4b).
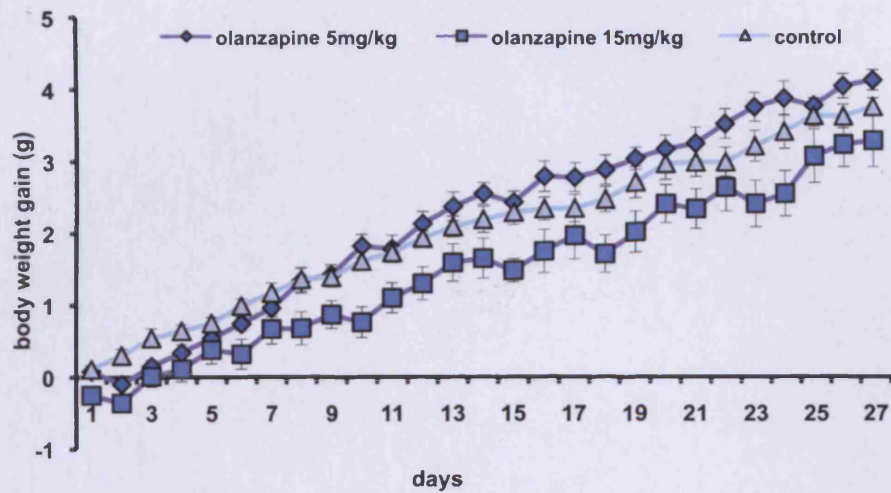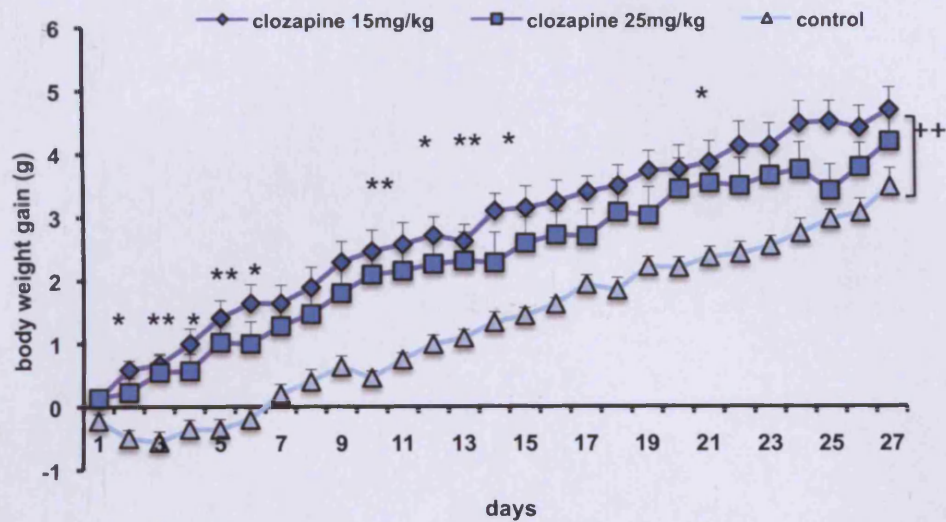
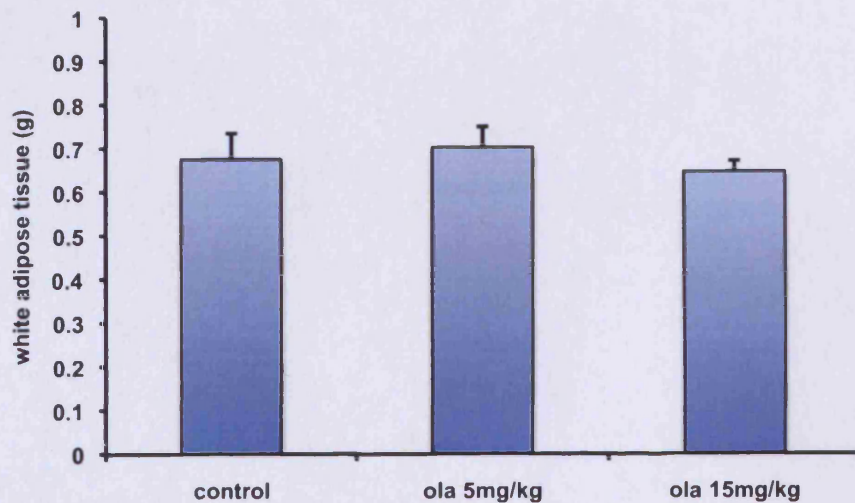**Fig 3.4a** Experiment 4. Effect of chronic oral administration of clozapine (15 and 25 mg/kg) on mouse body weight over 28 days. n=10; value represent the means ± SEM; (clozapine 25mg/kg vs. control: *P<0.05 **P<0.01; clozapine 15mg/kg vs. control: ++P<0.01).

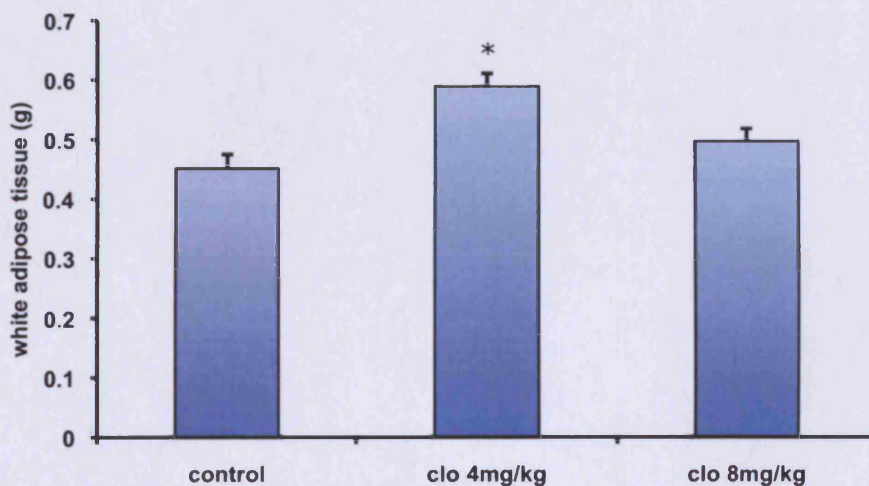**Fig 3.4b** Experiment 4. Effect of chronic oral administration of clozapine (CLO) (15 and 25 mg/kg) on fat deposition (pooled fat pads) in mice. n=10; values represent the means ± SEM.

3.2.5 Effect of clozapine (15 mg/kg), metformin (500 mg/kg) and clozapine (15 mg/kg) + metformin (500 mg/kg) on body weight and fat deposition

In experiment 5, clozapine at the dose of 15 mg/kg, which in experiment 4 caused significant weight gain throughout the treatment, was administered again alone and in combination with metformin 500 mg/kg. The aim of this experiment was to evaluate whether metformin was capable of counteracting the increase in weight induced by clozapine.

Contrary to what was observed in the previous experiment, mice treated with clozapine (15 mg/kg) did not increase their body weight compared to the control group and generally, no difference was noted between any groups compared to control (Fig 3.5a). Notably, the control group weight gain was similar to that observed in experiment 4.

In accord with the results of experiment 4, clozapine (15 mg/kg) did not enhance fat deposition in a significant manner. Likewise, neither the treatment with metformin alone, nor a combination with clozapine, affected fat deposition (Fig 3.5b).

Fig 3.5a Experiment 5. Effect of chronic oral administration of clozapine (15 mg/kg), metformin (500 mg/kg) and clozapine (15 mg/kg) + metformin (500 mg/kg) on mouse body weight over 28 days. $n=9$; values represent the means ± SEM.
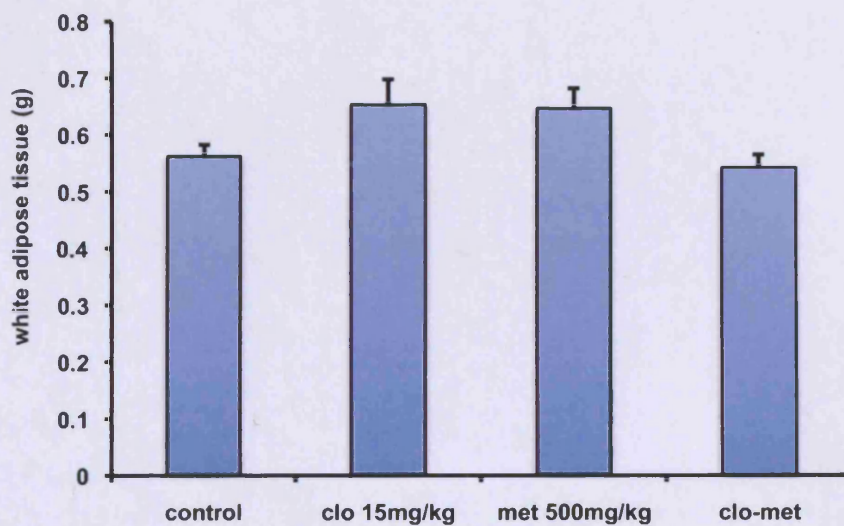
Fig 3.5b Experiment 5. Effect of chronic oral administration of clozapine (CLO) (15 mg/kg), metformin (MET) (500 mg/kg) and clozapine (15mg/kg) + metformin (500 mg/kg) on fat deposition in mice. $n=9$; values represent the means ± SEM.

3.2.6 Effect of clozapine (15 mg/kg and 25 mg/kg), and olanzapine (10 mg/kg and 12.5 mg/kg) on body weight and fat deposition

In previous experiments (1 and 3) it was observed that olanzapine (10 mg/kg) and clozapine (15 mg/kg) induced a significant weight gain in female mice. However, when clozapine (15 mg/kg) had been tested for a second time alone and in combination with metformin, none of the groups showed significant weight differences compared to the control group and clozapine did not enhance body weight gain. Thus, it was considered essential to repeat the experiment administering clozapine and olanzapine at the doses previously capable of inducing significant weight gain. The dose of clozapine 25 mg/kg was also tested again. Furthermore, one additional dose of olanzapine was introduced i.e. 12.5 mg/kg.

Unfortunately, none of the results achieved previously were reproduced in this experiment. All the groups of animals treated with the test compounds at any dose lost weight compared to the control group, and this weight loss achieved statistical significance ($P< 0.05$) on a few intermittent days for olanzapine 10 mg/kg (day 20, 25, 26) and clozapine 25 mg/kg (day 12, 17, 19, 24), (Fig 3.6a).

In the earlier experiment 1, olanzapine (10 mg/kg) increased fat deposition in a highly significant manner, and clozapine (15 and 25mg/kg) slightly increased it but without achieving statistical significance. In this repeated experiment however, neither of the antipsychotic compounds at any doses tested enhanced fat deposition

and olanzapine at 10 mg/kg even yielded a slight reduction (P>0.05) (Fig 3.6b).
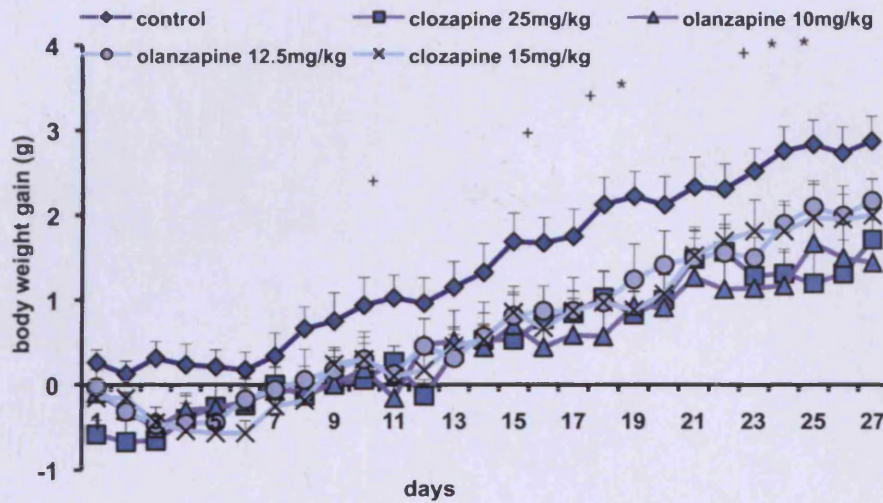
Fig 3.6a Experiment 6. Effect of chronic oral administration of clozapine (15 and 25 mg/kg) and olanzapine (10 and 12.5 mg/kg) on mouse body weight over 28 days. $n=7$; values represent the means ± SEM; (clozapine 25mg/kg vs. control: *$P<0.05$; olanzapine 10mg/kg vs. control: +$P<0.05$).
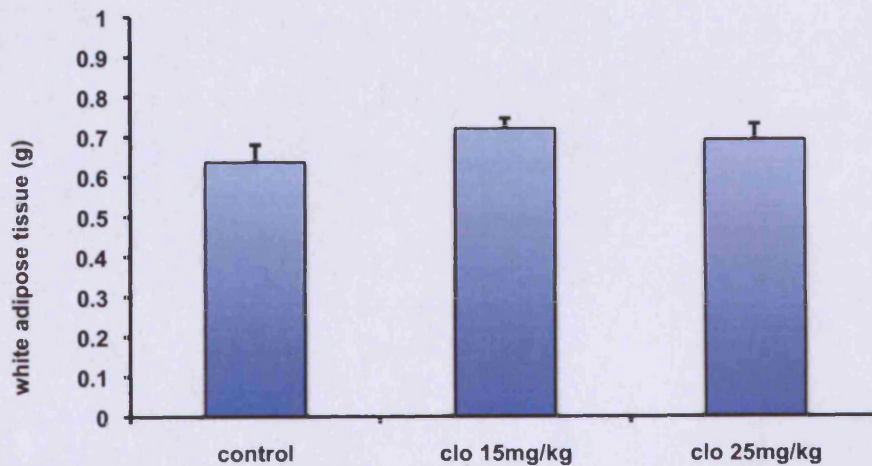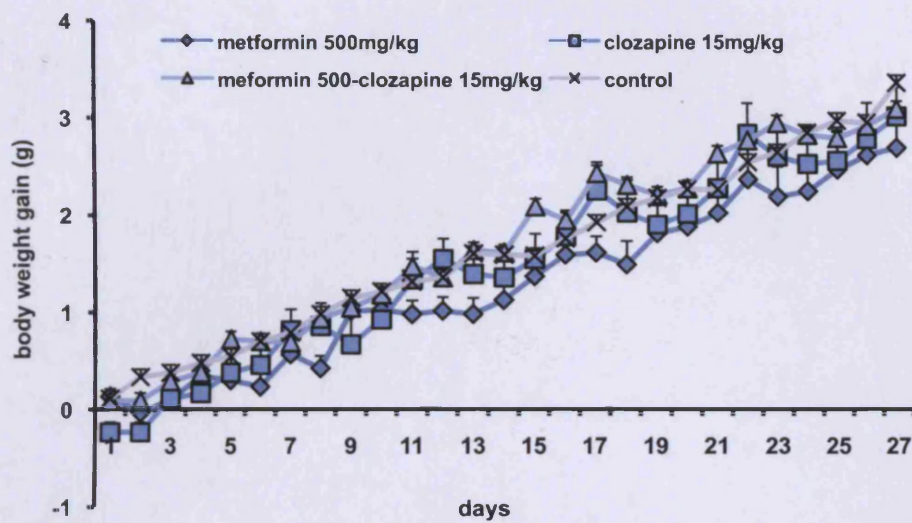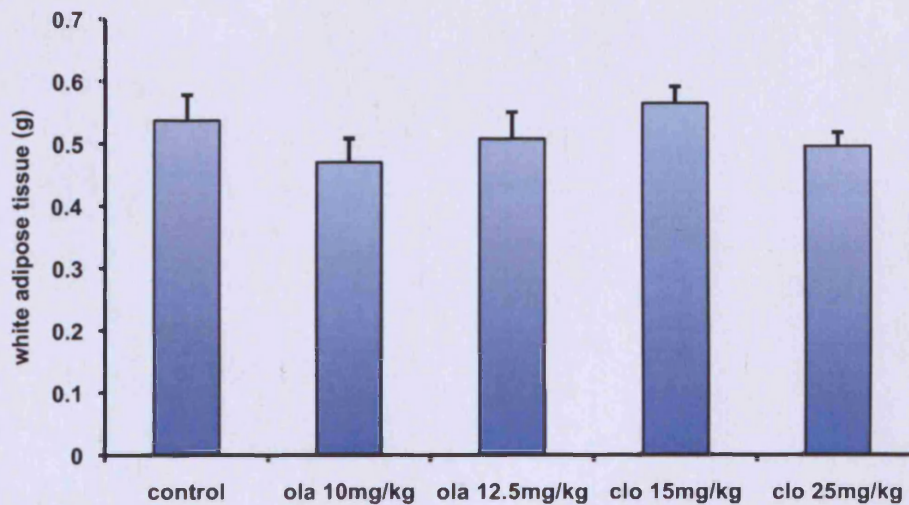

Fig 3.6b Experiment 6. Effect of chronic oral administration clozapine (CLO) (15 and 25 mg/kg) and olanzapine (OLA) (10 and 12.5 mg/kg) on fat deposition in mice. $n=7$; values represent the means ± SEM.

3.3 Discussion

The study shows that female C57BL/6J mice chronically treated with the antipsychotic drugs clozapine and olanzapine *via* the oral route, do not develop a significant repeatable increase in body weight nor reproducibly enhance the overall accumulation of white adipose tissue in the intrabdominal, perirenal and periuterine areas.

Thus, olanzapine (5, 12.5 and 15 mg/kg) did not produce any significant weight gain or excessive fat deposition. While at a dose of 10 mg/kg it had no effect on body weight for the first 16 days of treatment, but subsequently increased body weight significantly, i.e. the difference in weight gain between the two groups being maintained from day 17 to day 28.

However, when the treatment was repeated under the same experimental conditions (using different animals), to validate the previous result, olanzapine failed to produce any significant weight gain throughout the length of the treatment.

Similarly, in the first attempt to reproduce an augmentation of fat deposition consistent with that observed in the clinic, olanzapine (10 mg/kg) significantly enhanced fat accumulation (P<0.01) but this result was not observed when the experiment was repeated.

Furthermore, this pattern was also witnessed in mice chronically treated with clozapine. Hence, clozapine at the doses of 4 and 8 mg/kg did not generate any significant body weight gain compared to controls, while

at a dose of 15 mg/kg it induced weight gain on all 28 days of treatment, and on several intermittent days at the dose of 25 mg/kg.

In an identical fashion to olanzapine described above, when the experiment was repeated, clozapine (15 and 25 mg/kg) seemed to produce weight loss relative to the steady weight gain of the control group when the experiment was repeated.

Consistent with the previous data however, clozapine at both of the higher doses of 15 and 25 mg/kg did not promote accumulation of visceral fat tissue.

On the other hand, clozapine at 4 mg/kg significantly enhances fat deposition without promoting weight gain, although the experiment was not duplicated. This pattern of observation is not novel and has been reported in other studies where clozapine and olanzapine induced fat deposition but no weight gain respectively in female rats (Cooper et al., 2008) and male rats (Minet-Ringuet et al., 2006; Cooper et al., 2007). It might be suggested that the increase in adipose tissue following antipsychotic treatment may be somehow independent of weight gain and this requires further investigation to establish whether these drugs exert a direct effect on lipolysis and lipogenesis.

In the light of these findings, however, the contradictory results on body weight gain and fat deposition are not readily explicable given the fact that weight changes in mice in response to olanzapine and clozapine using the conditions and parameters in the current study do not represent a robustly reproducible animal model.

On the basis of dose alone, rather than other factors (species, strain, sex, route of administration and treatment duration), these results are in disagreement with those of Zarate et al., (2004) who reported that clozapine (4 mg/kg) induced significant weight gain, and those of Cope (2005) and Coccurello et al., (2005-2008) where olanzapine at doses of 3, 4, 6, 8 mg/kg significantly increase body weight. Such findings however, contrast with those of Albaugh et al., (2006) where olanzapine administered in increasing doses from 4 to 8 mg/kg did not affect body weight.

The wide range of doses of antipsychotics chosen in the current study, as well as those used by others in the literature, are intended to reflect doses employed in the clinic. The choice of the right antipsychotic drug and its optimal dose clinically normally requires a few weeks of monitoring and are matched to the individual patient.

To complicate matter further, the dosage of clozapine used in the clinic is in the range 150 and 450 mg/day and this is 15 times higher than that employed for olanzapine (Baldessarini et al., 2001). According to Albaugh et al., (2006) the correspondent dose-transformation to rodent is 40-50 mg/kg/day for clozapine, which would certainly produce a marked sedative effect potentially interfering not only with food intake but also energy expenditure.

Evidence of sedation was noted in the laboratory when clozapine was administered at a dose of 30 mg/kg and consequently treatment was suspended.

Nevertheless, any veiled sedative effect of clozapine and olanzapine at the highest doses administered cannot be excluded in the present study because locomotor activity, food intake and energy expenditure were not measured.

Even though food intake is a simple variable to be measured in rodents, it is more difficult in small sized animals like individual mice due to the relatively small amount consumed and the degree of spillage. In addition, this would necessitate the animals being housed individually for the duration of the habituation plus treatment, and previous behavioral studies have proved that this leads to social isolation and distress, which may interfere with animal feeding behavior (Coccurello et al., 2006).

Ultimately, the range of doses currently used in the clinic and those employed in animal models, raise the question of dosage appropriateness in rodents that reflect those used for psychosis.

Furthermore, the average half-lives of clozapine and olanzapine in humans are respectively 12 and 30 hours (Baldessarini et al., 2001), while they are only 1.5 hours for clozapine and 2.5 hours for olanzapine in rodents (Kapur et al., 2003). Hence, while human drug plasma levels are likely to be steady, in almost all the animal models that have been studied so far, the dosage regimen is no more than twice daily so plasma drug levels inevitably fluctuate compared to the clinical situation. This issue has been discussed by Kapur et al., (2003), who administered either by injection or by continuous infusion through

osmotic mini-pump (MP), with several doses of drugs in both single and multiple dose regimens. Plasma concentrations and D2 receptor occupancy were measured simultaneously. The goal of Kapur's study was to identify the appropriate doses capable of achieving plasma levels and receptor occupancy comparable to clinical occupancy levels and maintain them to a stable level. The rationale behind this study was that, not only such a short half-life of clozapine and olanzapine in rodents results in faster metabolism and elimination of these drugs, but that once or twice daily administration would not effectively mimic the drugs pharmacokinetics in humans.

Kapur et al., identified a range of doses administered several times a day by injection, were able to reach 70-80% D2 receptor occupancy but they did not maintain steady plasma levels. Conversely, the MPs delivery system did maintain stable plasma levels throughout the 24 hours of infusion, accompanied by plasma level more representative of the conditions in the clinic.

The MP approach has several drawbacks however: firstly, the broad range of receptors that clozapine and olanzapine bind do not allow modeling of the dose-receptor occupancy relationship as a function of just D2 receptors so, in this respect, it represents an approximation of receptor binding.

Furthermore, injecting drugs several times a day does not represent a practical reality and although the MP system does lead to steady drug levels, it is invasive and labour intensive.

Overall, the approach used by Kapur et al., although simplistic, it is nonetheless a further step towards the search for reliable criteria to match animal models to clinical treatments.

As in previous reports reviewed here, different methodologies account for different end results. In this context the age of the animals has a major influence. In the present study young animals (4-5 weeks old) were chosen in order to maximize any possibility of detecting distinguishable increase in body weight, which would have been less discernable in adult animals.

Additionally, 4 weeks was selected as appropriate treatment duration in order to increase the likelihood of an effect since the average length of previous studies conducted by others was 3 weeks.

Furthermore, whilst the influence that animal gender exerts is questionable since clinical observations report that weight gain is observed in male patients as it is in females (Meltzer et al., 2002; Ascher-Svanum et al., 2005), differences in species and strain (hence metabolism), might account for discrepancies in results. In this study C57BL/6J inbred mice were employed due to a lower degree of intrinsic variability distinctive of inbred strains compared to outbred strains. Nonetheless, outbred strains have also been employed by other authors but to a lesser extent. Additionally, inbred balb/c and AKR mice were utilized in a series of preliminary experiments but they were

averse to ingestion of the honey vehicle and as a consequence, the treatment was forced to be discontinued.

Taken together, several groups have reported models of APS-induced weight gain in rodents, but the findings of this investigation suggests that at least in mice, caution should be exercised in interpreting their reproducibility. An added perspective to this concept may be derived from the finding that the atypical antipsychotic ziprasidone, although it has little or no effect on human body weight (Allison et al., 1999) it does significantly promote weight gain in an animal model (Kalinichev et al., 2006).

3.4 Conclusions

The main goals of this study were firstly, to produce a reliable and consistent mice model of clozapine and olanzapine-induced weight gain, and secondly, to test the ability of some agents to attenuate this weight increase. The drugs to be chosen for this purpose were either based on their application as anti-obesity agents or for their pharmacological action(s) on specific receptors to which olanzapine and clozapine bind.

Since the reproducibility of a mouse model of atypical antipsychotic-induced weight gain was not established by the study, it was not possible to perform any detailed experiments to probe agents as anti-weight gain treatment against antipsychotics.

Despite all the above-mentioned limitations, the search for a suitable animal model of weight gain should still be pursued, as well as further examination of metabolic dysfunction caused by these drugs and possible underlying mechanisms, which to date, are poorly understood.

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Chapter 4

The effect of clozapine and olanzapine on adipogenesis: an *in vitro* cell study

4. The effect of clozapine and olanzapine on adipogenesis: an *in-vitro* cell study

4.1 Introduction

The mechanism by which atypical antipsychotics, olanzapine and clozapine in particular, increase weight and cause metabolic disturbances, is yet to be elucidated. However, it is believed that the weight gain could, at least in part, be mediated by the action of these drugs on the hypothalamus: in the arcuate nucleus region of the hypothalamus there are two groups of neurons responsible for the feeding regulatory process, namely agouti-related neuropeptide Y (AgRP/NPY) and pro-opiomelanocortin/cocaine- and amphetamine-related transcript (POMC/CART). The former stimulates appetite while the latter suppress it (Schwartz et al., 2000).

Several hormones regulate food intake, leptin and insulin play a major role in this control regulation, and their receptors have been found in AgRP/NPY and POMC/CART neurons. Leptin is secreted by adipocytes and its levels increase proportionally to body fat storage, thus, a decrease in fat storage results in lower circulating leptin and an increase in appetite (Meier and Gressner, 2004). Elevated plasma levels of leptin have also been found to be associated with antipsychotic treatment, however, patients under antipsychotic medications often do not experience a decrease in food appetite, suggesting a possible disruption of the mechanisms regulating the response to leptin in the hypothalamus (Jin et al., 2008).

As well as hormones, neurotransmitters such as serotonin, noradrenaline and histamine, participate in the regulation of eating behaviour and metabolism, their receptors being found in the hypothalamus. It has been observed that $5HT_{1A}$ agonists increase food intake while $5HT_{2c}$ agonists decrease it (Clifton et al., 2000). The noradrenergic system also plays a role since the administration of α_1 - adrenoceptor agonists decreases food intake while the stimulation of α_2 -adrenoceptor increases it (Ramos et al., 2005).

The role of histamine receptors in hypothalamic regulation of energy balance and food intake has also been studied (Yoshimatzu, 2006) and H1 antagonists have been shown to potently increase food consumption. Moreover, H1 receptor knockout mice appear to develop obesity (Deng et al., 2010). Olanzapine and clozapine have also been

shown to activate H1 receptor-mediated AMP kinase (AMPK) in the hypothalamus, which results in an increase in food intake (Kim et al., 2007).

4.1.1 Adipocyte differentiation

While several research groups have focused on the central, neurotransmitter-mediated effect of antipsychotic drugs to uncover the mechanisms by which these drugs cause weight gain and metabolic abnormalities, little has been investigated on their direct biological effect on adipogenesis in cultured pre-adipocytes and mature adipocytes.

The generation and increase of adipose tissue in the body is a consequence of an enlargement of mature adipocytes due to lipid accumulation (hypertrophy), and an augmentation of adipocyte numbers due to enhanced adipocyte differentiation from precursor cells (hyperplasia). In humans, the ability to generate new fat cells persists even at an adult stage (Gregoire et al., 1998), therefore, the goal of this study was to investigate whether the increase in body weight caused by clozapine and olanzapine is due to new formation of mature adipocyte from precursor cells.

Adipocytes derive from mesenchymal stem cells (MSCs). These progenitor cells have the ability to undergo differentiation into osteblasts, chondrocytes, adipocytes and myocytes.

Over the past decades, the establishment of several in-vitro models of adipogenesis have aided the advancement of knowledge of the cellular and molecular events involved in the differentiation of fibroblastic-like

preadipocytes into mature adipocytes. This process occurs in several stages: first, the influence of hormones and growth factors (in a process that is yet poorly understood) drives the uncommitted cells to the specific adipogenic lineage. Pre-adipocytes then undergo a phase of growth arrest followed by mitotic clonal expansion, characterized by several more rounds of cell division, under the influence of adipogenic inducers, before terminal differentiation and formation of mature adipocytes (Gregoire et al., 1998; Lefterova and Lazar 2009). During this process cells change from a fibroblastic (stellate) to a spherical shape followed by accumulation of lipid droplets.
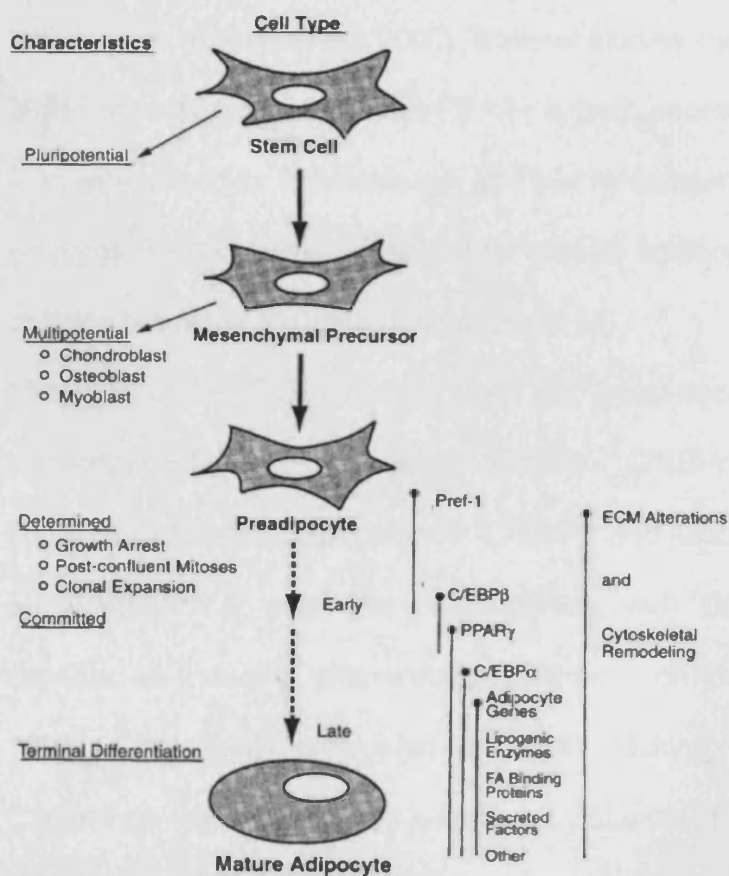

Fig 4A Adipocyte differentiation process. (Adapted from Gregoire et al., 1998).

Among the transcription factors involved in the differentiation and maturation process, peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) appear to play a key role (Cowherd et al., 1999).

PPARγ is a member of the nuclear hormone receptor family. It is expressed in two isoforms PPARγ1 and PPARγ2 with the latter being most abundant in fat tissue (Tontonoz et al., 1995). The expression of PPARγ has been linked to morphological changes in fat cells, lipid accumulation and insulin sensitivity, which emphasizes its critical role in the process (Rosen et al., 2000). Several studies (Lefterova and Lazar 2009) have demonstrated that PPARγ is both necessary and sufficient to initiate adipocyte differentiation and that its deficiency is lethal in mice embryos. PPARγ is also essential for mature adipocyte survival and its deletion leads to cell death (Imai et al., 2004).

Members of the C/EBP family have also been recognized as crucial transcription factors. In particular C/EBPα, C/EBPβ and C/EBPδ are known to promote adipogenesis. C/EBPβ and C/EBPδ appear to be early markers of adipocyte differentiation, with C/EBPβ even being capable of inducing differentiation without hormonal inducers (Yeh 1995). Conversely, embryonic fibroblasts lacking both C/EBPβ and C/EBPδ completely lose their adipogenic potential (Tanaka et al., 1997). C/EBPβ and C/EBPδ are thought to promote adipogenesis through the induction of C/EBPα and PPARγ expression (Yeh et al., 1995).

C/EBPα, when activated, in turn induces the expression of several genes involved in terminal differentiation and lipid metabolism such as aP2, LPL, FAS and GLUT4 (Gregoire et al., 1998; Cowherd et al., 1999).

In this study cultured mouse fibroblast-like cells 7-F2 are employed as a model to study adipogenesis. 7-F2 is a bone marrow-derived stromal cell line characterized by indefinite growth *in-vitro* and mesenchymal origin. These cells express osteoblastic markers but have the ability to differentiate into adipocytes, as demonstrated by mRNA over-expression of adipocyte specific markers i.e. PPARγ, adiponectin, aP2, adipsin, and confirmed by Oil Red O staining (Thompson et al., 1998; Muruganandan et al., 2010). Moreover, the conversion of 7-F2 cells into adipocytes is accompanied by a loss of the osteoblastic phenotype (Thompson et al., 1998).

4.1.2 Aim of the study

To determine whether clozapine and olanzapine have a direct influence on the formation of fat cells we investigated their effect in a fibroblast-like cell line, 7-F2. In addition, we studied the effect of clozapine and olanzapine on the mRNA expression of several target genes involved in adipocyte differentiation and metabolism: PPARγ, C/EBPβ and lipoprotein lipase (LPL).

4.2 Results

4.2.1 Effect of clozapine and olanzapine (1-100 μM) on the proliferation and viability of undifferentiated 7-F2 cells

The MTS assay and cell counting with haemocytometer were used to monitor the growth pattern of the cells under the influence of the test drugs, clozapine and olanzapine. A range of antipsychotic concentrations spanning 2 log units was first tested.

MTS assay

7-F2 cells were seeded into 96 well-plates (3,000 cells/well), then clozapine or olanzapine were added after 24 hours and cells allowed to proliferate for an additional 72 hours. The assay was carried out as described in paragraph 2.2.4. The optical density reading represents the concentration of formazan and its is proportional to the number of living cells.

Clozapine did not appear to affect cell proliferation at concentrations between 1 and 75 μM but it significantly (P< 0.05) reduced cells numbers at the highest concentration of 100 μM compared to control (Fig 4.1).

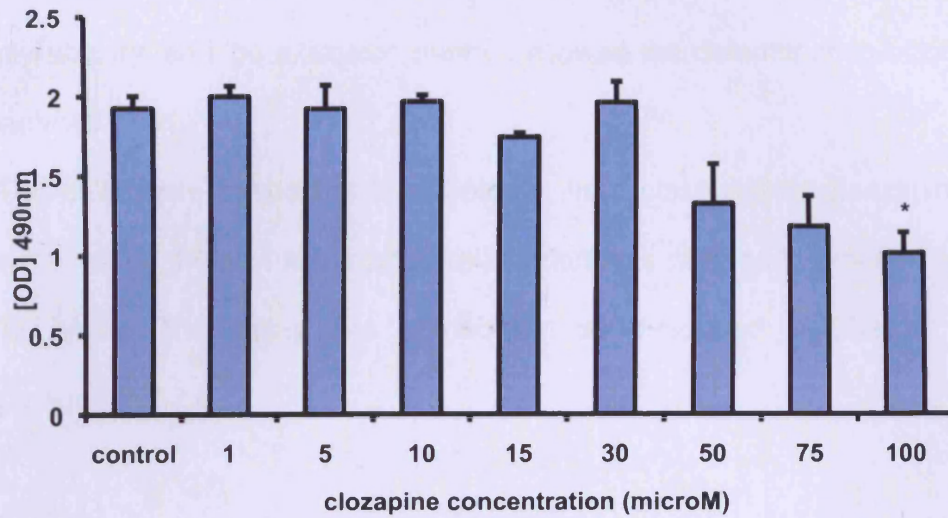Olanzapine had no effect on proliferation (P> 0.05) at any of the concentration studied (Fig 4.2).

Fig 4.1 The effect of clozapine (1-100 µM) on 7-F2 cell proliferation determined by MTS assay. The values represent the means ± SEM;* P< 0.05. *n*=3 from 3 independent experiments .
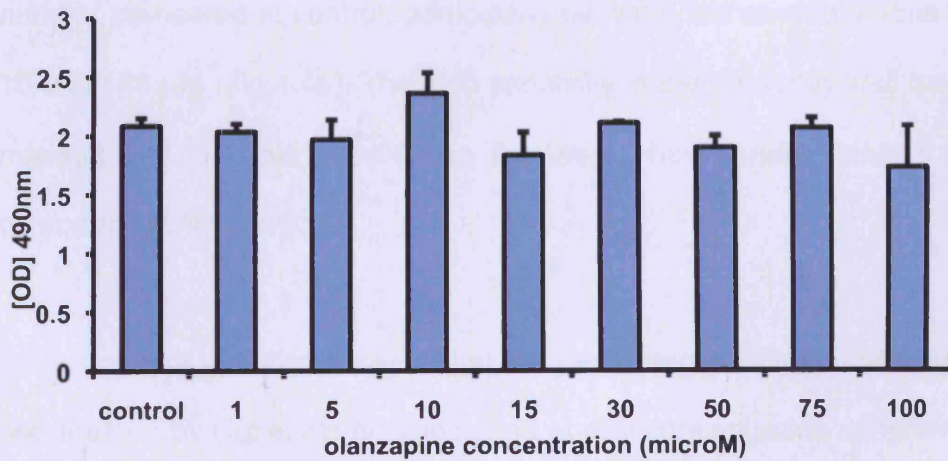

Fig 4.2 The effect of olanzapine (1-100 µM) on 7-F2 cell proliferation determined by MTS assay. The values represent the means ± SEM; *n*=3 from 3 independent experiments.

Cell counting with haemocytometer

The rate of proliferation was also assessed by manual cell counting, and the trypan blue exclusion method allowed the determination of cell survival.

The cells were seeded in 6 well-plates, then clozapine or olanzapine were added after 24 hours and proliferation was allowed to proceed for 72 hours. The assay was carried out as described in Chapter 2 paragraph 2.2.5.

Clozapine significantly decreased ($P<0.05$) 7-F2 cell numbers at concentrations between 15 and 100 μM (Fig 4.3a).

Although statistical analysis did not detect any significant decrease in cell number, suggesting that olanzapine does not affect the rate of cell growth, the graphic data did show a trend towards a decrease in cell number compared to control, particularly between the concentrations of 10 and 100 μM (Fig 4.4a). The high variability in the data may well have masked any possible significance that might have been detected in comparison with controls.

Statistical analysis also showed that the percentage of viable cells was not affected by clozapine or olanzapine at any concentration compared with controls (Fig 4.3b; 4.4b) which overall suggests that both drugs may slow down the rate of cell proliferation without causing cell death.

Fig 4.3a The effect of clozapine (1-100 μM) on 7-F2 cell proliferation determined by manual counting after 72 hr of incubation. The values represent the means ± SEM; * P< 0.05. *n*=3 from 3 independent experiments.

Fig 4.3b The effect of clozapine (1-100 μM) on % 7-F2 cell viability derived from the ratio (viable cells/total cell number) x 100 as determined by trypan blue exclusion. The values represent the means ± SEM. *n*=3 from 3 independent experiments.
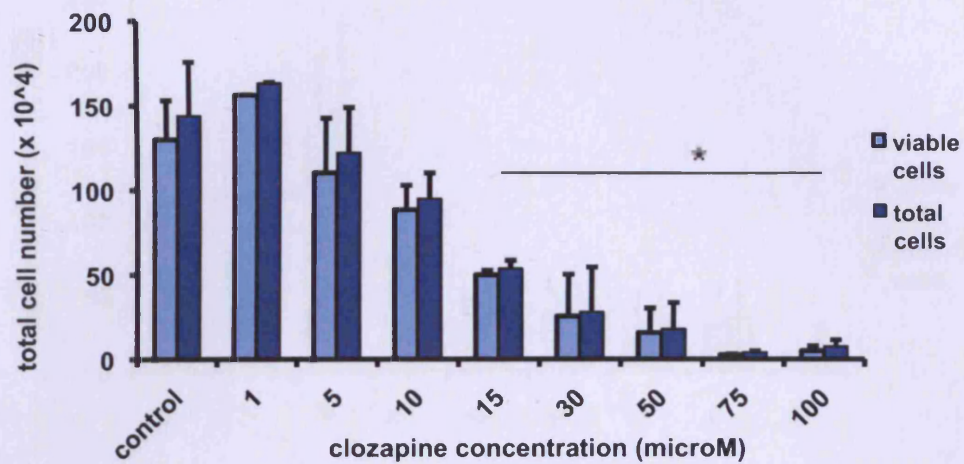
Fig 4.4a The effect of olanzapine (1-100 μM) on 7-F2 cell proliferation determined by manual counting after 72 hr of incubation. The values represent the means ± SEM. *n*=3 from 3 independent experiments.
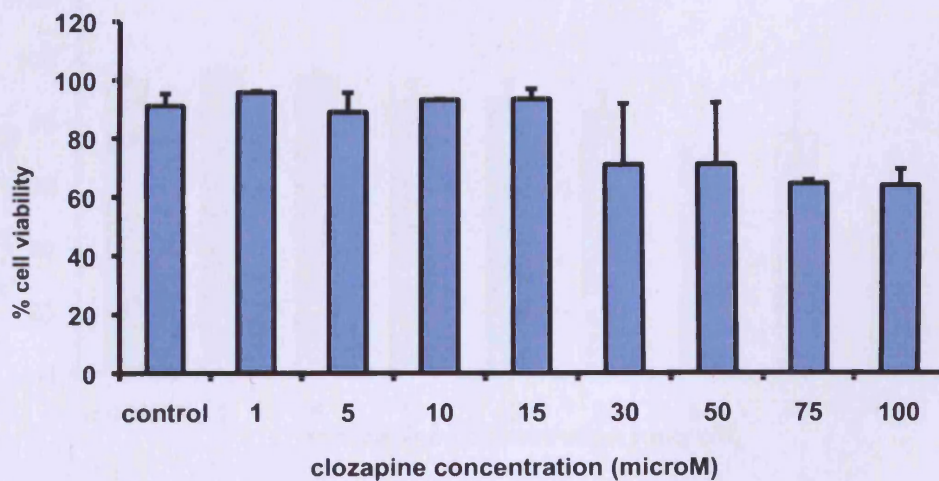

Fig 4.4b The effect of clozapine (1-100 μM) on % 7-F2 cell viability derived from the ratio (viable cells/total cell number) x 100 as determined by trypan blue exclusion. The values represent mean ± SEM. *n*=3 from 3 independent experiments.
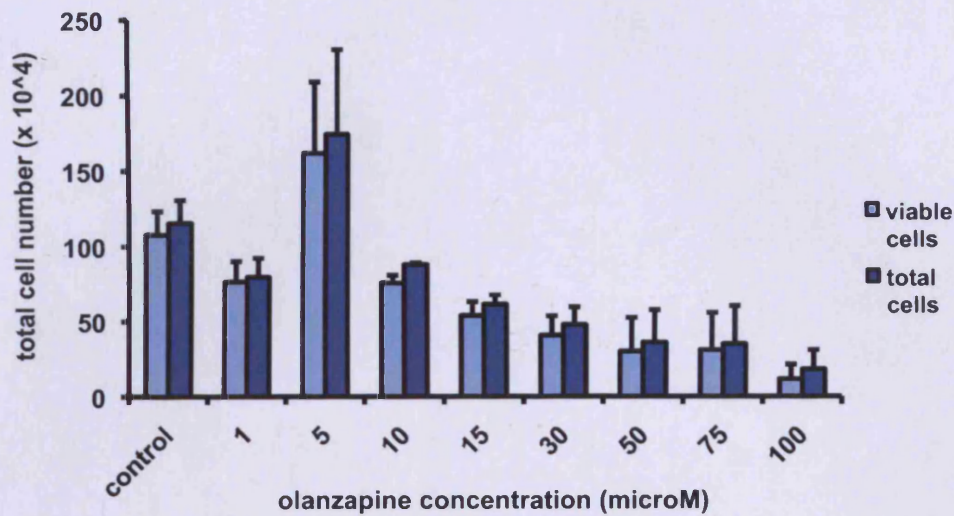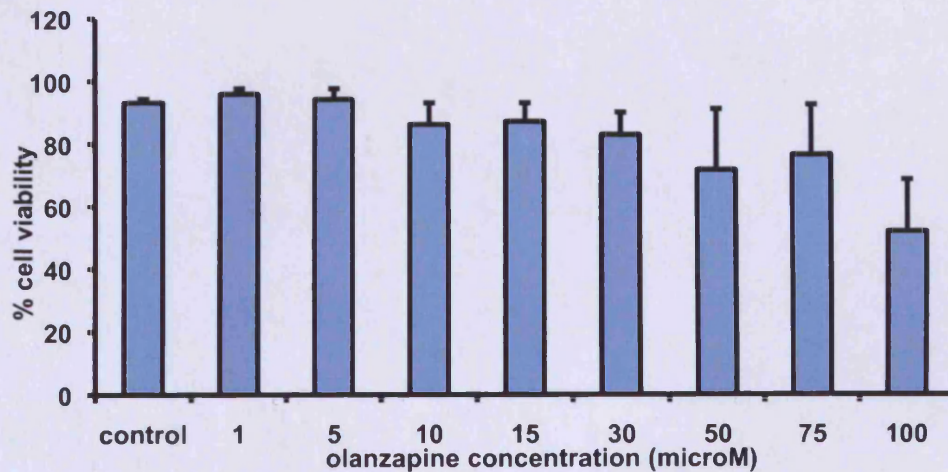
4.2.2 Effect of clozapine and olanzapine (1-10 µM) on the proliferation and viability of undifferentiated 7-F2 cells

MTS assay

The effect of clozapine and olanzapine on cell growth was evaluated with respect to time. Hence, the cells were incubated for 3, 5 and 7 days and the rate of proliferation was measured.

The effect of clozapine was both time and dose dependent beyond 3 days. Thus, after 3 days of incubation, clozapine at all concentrations had no significant effect ($P > 0.05$) on cell proliferation compared to control.

In contrast, at day 5, clozapine at 3, 5 and 10µM significantly decreased cell proliferation, and after 7 days the rate of proliferation decreased at all concentrations compared to control (Fig 4.5).

The effect of olanzapine was also time and dose dependent. Thus, like clozapine, olanzapine did not affect cell growth at day 3 compared to control. However, after 5 days of incubation, only the highest concentration (10µM) inhibited cell proliferation but at day 7, olanzapine significantly reduced ($P < 0.05$) cell proliferation at all doses compared to control (Fig 4.6).

Fig 4.5 The effect of clozapine (1-10 μM) on 7-F2 cell proliferation determined by MTS assay. The values represent the means ± SEM between corresponding durations (days) of incubation; (* P< 0.05) *n*=10 from 2 independent experiments.
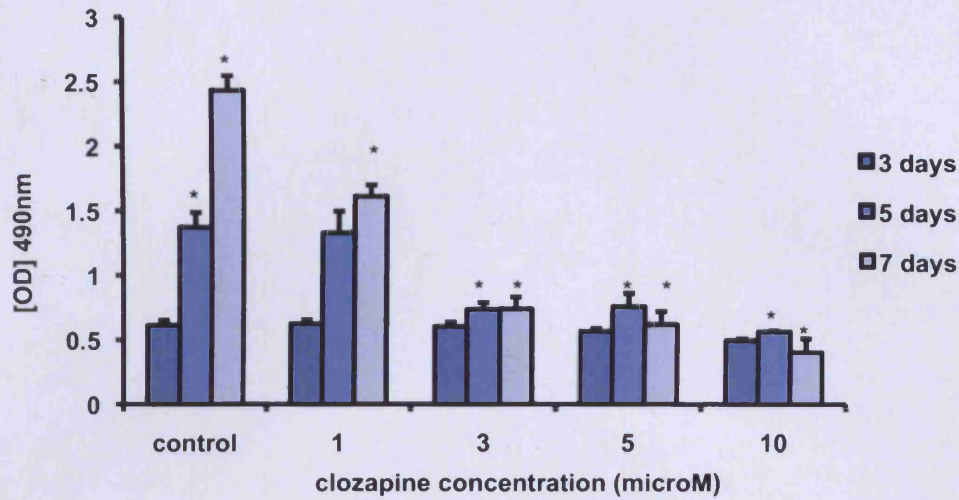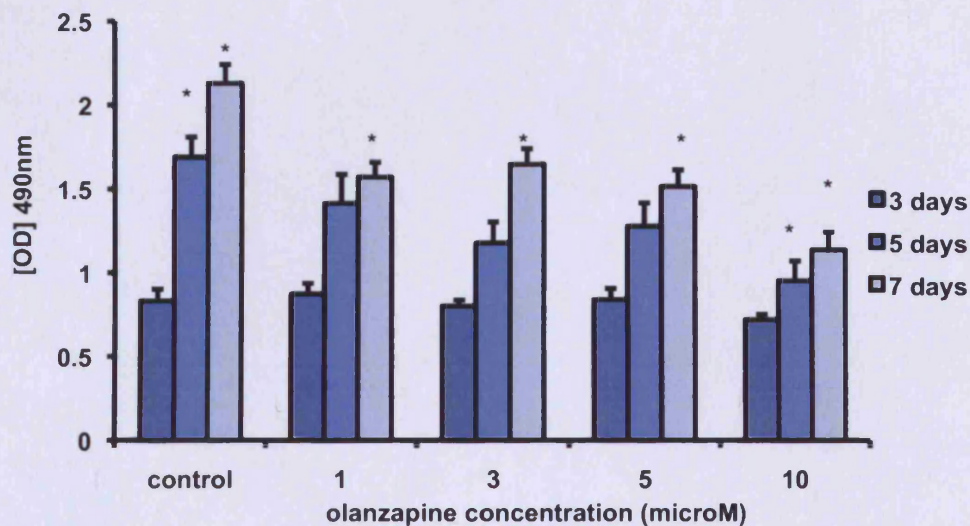

Fig 4.6 The effect of olanzapine (1-10 μM) on 7-F2 cell proliferation determined by MTS assay. The values represent the means ± SEM between corresponding durations (days) of incubation; (*P< 0.05) *n*=10 from 2 independent experiments.

4.2.3 Effect of clozapine and olanzapine (1-10 µM) on cell differentiation:

Adipogenesis assay

7-F2 cells can differentiate into mature adipocytes if cultured in the presence of the necessary mix of adipogenic inducers.

During the process of adipogenic differentiation 7-F2 cells adopt a rounded morphology and develop accumulations of lipid droplets in the cytoplasm which are visible by phase contrast light microscopy. These lipid droplets within the cytoplasm of 7-F2 cells were stained with the lipophilic dye, Oil Red O, before qualitative analysis by light microscopy. To achieve a quantitative measure of lipid droplet accumulation in 7-F2 cells lipid-bound Oil Red O was extracted with alcohol and assayed by UV-Vis spectrophotometry where the absorbance at 490 nm is assumed to be directly proportional to the lipid concentration in the cells (Kuri-Harcuch, 1978), (Fig 4B).
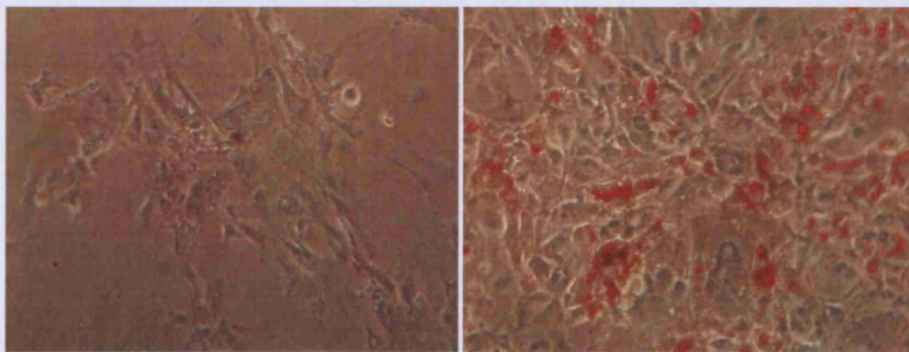
Fig 4B Undifferentiated 7-F2 cells (left), and 7-F2 cells after 5 days of adipogenic differentiation (right).

Previous methodology adopted by the laboratory was slightly modified due to initial difficulties in extracting the Oil Red O.

Despite the fact that the volume of Oil Red O used was the same in both cells treated with normal (full) media and cells treated with the adipogenic inducer media, the inability to remove it from the wells resulted in no difference (P> 0.05) detected between undifferentiated and differentiated cells in the colorimetric Oil Red O assay (Fig 4.7a).
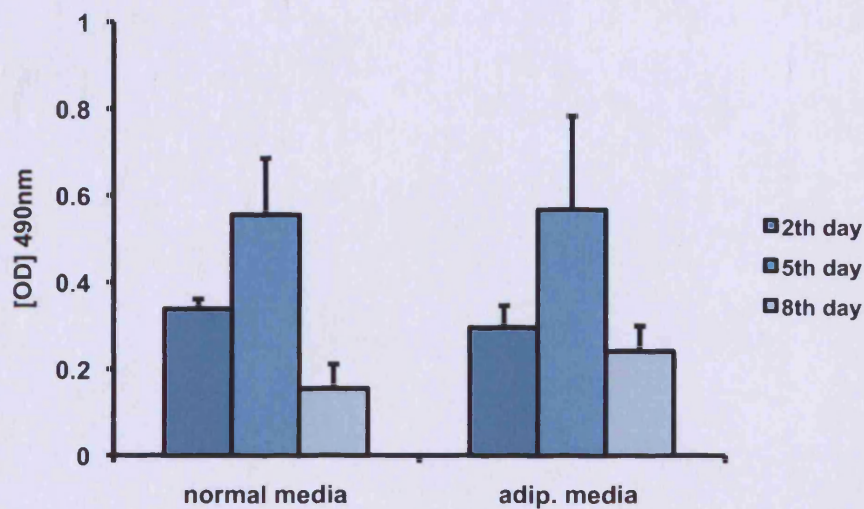
Fig 4.7a Induction of adipogenic differentiation in 7-F2 cells. The initial method developed for the Oil Red O assay did not distinguish between undifferentiated cells (treated with normal growth media) and differentiated cells (treated with normal media+adipogenic mix inducers).

Fig 4.7b shows that by modifying the protocol (as described in paragraph 2.2.6), the colorimetric assay was able to show how the differentiation process was not initiated in cells cultured in normal (full) media (in absence of adipogenic inducers). Two days after the differentiation started, no difference (P> 0.05) was seen between the cells treated with normal media and the cells treated with adipogenic media. After 5 days however, there was a 2.5 fold difference (P< 0.05), and a > 2 fold increase at day 8 (P< 0.05).

Fig 4.7b Induction of adipogenic differentiation in 7-F2 cells with adipogenic mix. The modified Oil Red O method afforded a distinction between undifferentiated and differentiated 7-F2 cells. The values represent the means ± SEM between corresponding durations (days) of incubation (*P< 0.05).

7-F2 cells were first seeded in 12 well-plates (day -1), then, 24 hours later either clozapine or olanzapine were added (day 0), and

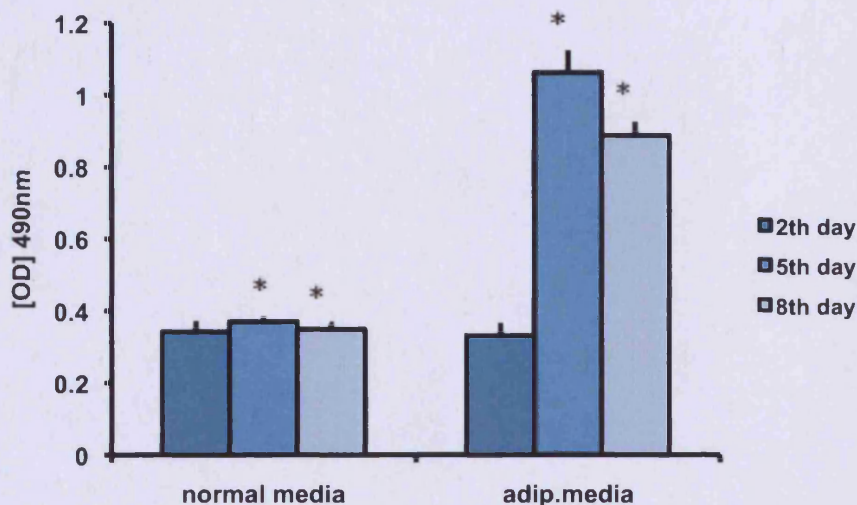differentiated over a period of 8 days. The rate of differentiation was evaluated at specific time-points at 2nd, 5th and 8th day.

Fig 4.8 and Fig 4.9 show that the differentiation process reached its peak at day 5, and declined by day 8. Moreover, both drugs decreased adipogenesis in a time and concentration dependent manner.

At day 2 both clozapine and olanzapine did not affect cell differentiation at any concentration compared to control (Fig 4.8). This could be explained by the previous observation that at day 2 Oil Red O uptake was comparable in control cells (treated with adipogenic media) (Fig 4.7b).

However, after 5 days of culture in adipogenic media, clozapine at 5 and 10 μM reduced adipocyte formation from precursor compared to control. After 8 days of culture, adipogenesis was significantly reduced at the 3, 5 and 10 μM concentrations compared to control ($P<0.05$) (Fig 4.8).

Olanzapine significantly reduced ($P<0.05$) cell differentiation (3, 5 and 10 μM) after 5 days of treatment with adipogenic inducers, while after 8 days, only the highest concentration (10 μM) decreased adipogenesis in a significant manner ($P<0.05$) (Fig 4.9).
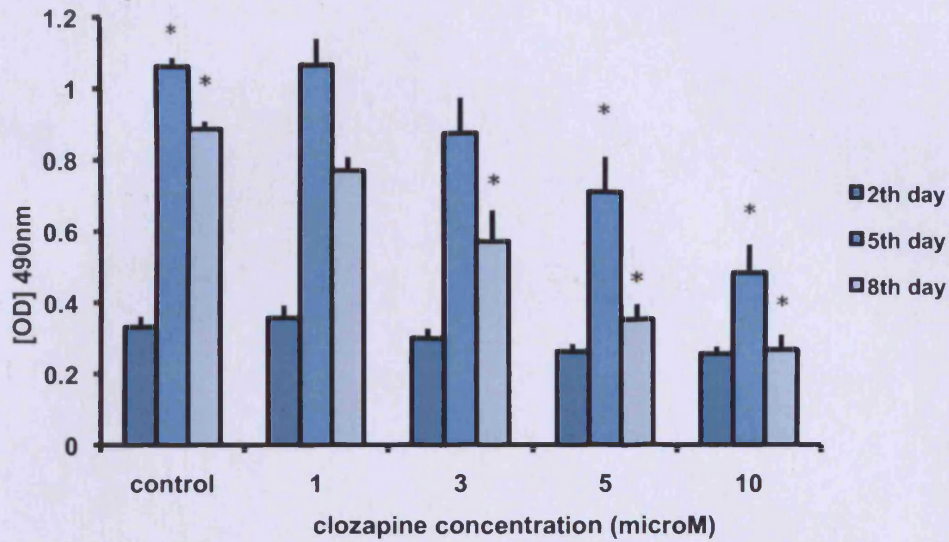
Fig 4.8 Quantification of adipogenesis using Oil Red O staining in clozapine-treated 7-F2 cells. The values represent the means ± SEM.*P< 0.05 denotes statistical significance between clozapine-treated cells and corresponding control cells at a given day of culture n=8 from 4 independent experiments.
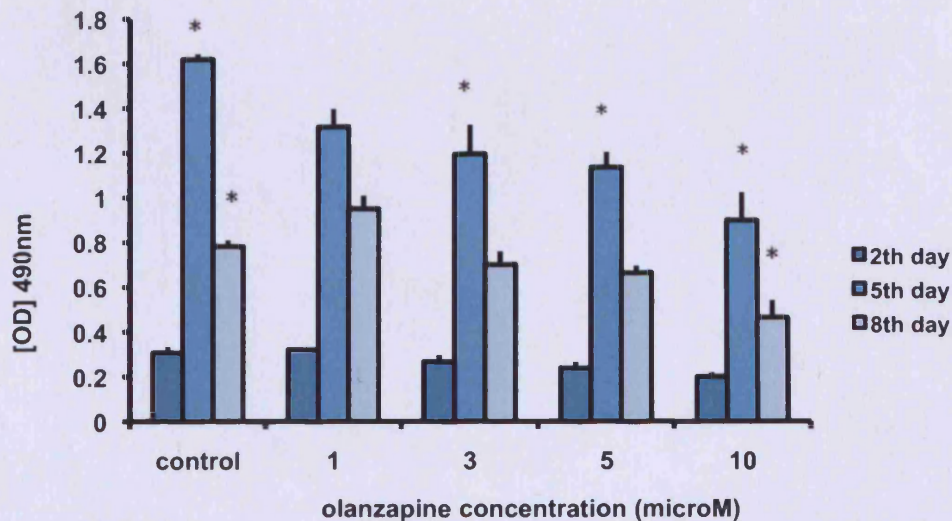
Fig 4.9 Quantification of adipogenesis using Oil Red O staining in olanzapine-treated 7-F2 cells. The values represent the means ± SEM.*P< 0.05 denotes statistical significance between olanzapine-treated cells and corresponding control cells at a given day of culture n=8 from 4 independent experiments.

Day 2　　　　**Day 5**　　　　**Day 8**
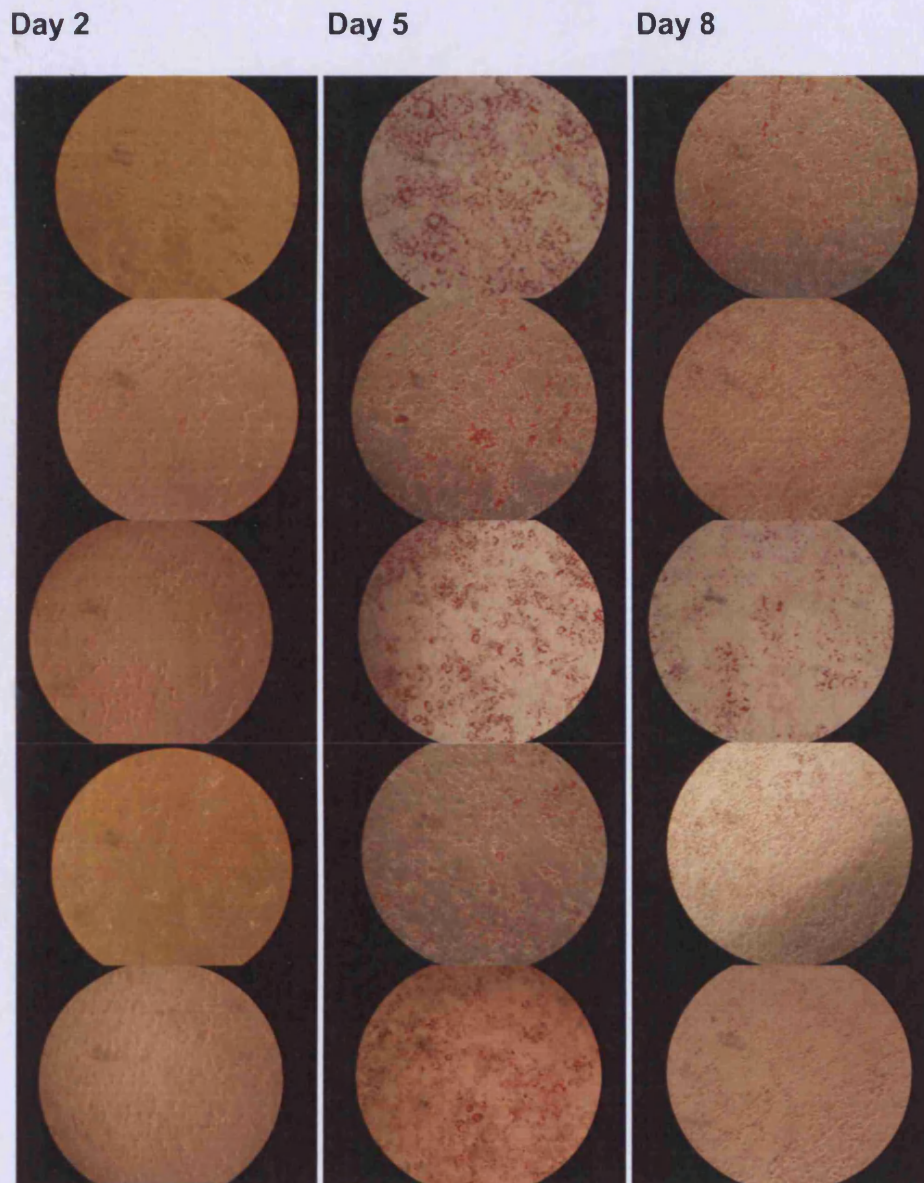
Fig 4C Effect of clozapine on the time-dependent differentiation of 7-F2 cells into adipocytes when cultured in the presence of adipogenic inducers. Lipid droplets are stained using Oil Red O (top (first row)= vehicle control cells; second row= clozapine 1μM; third row= clozapine 3μM; forth row= clozapine 5μM; fifth row= clozapine 10μM).

Day 2   **Day 5**   **Day 8**

Fig 4D Effect of olanzapine on the time-dependent differentiation of 7-F2 cells into adipocytes when cultured in the presence of adipogenic inducers. Lipid droplets are stained using Oil Red O (top (first row)= vehicle control cells; second row= olanzapine 1μM; third row= olanzapine 3μM; forth row= olanzapine 5μM; fifth row= olanzapine 10μM).

4.2.4 Effect of clozapine and olanzapine (1-10 µM) on cell proliferation and viability during the differentiation of 7-F2 cells

Cell counting with haemocytometer

Cells were seeded in 6 well-plates, then, 24 hours later, clozapine or olanzapine (dissolved in adipogenic inducers media) were added for a period of 8 days.

Over the 8 day period the 7-F2 cells differentiate into adipocytes during which time changes in cell number and cell viability were evaluated.

Neither clozapine nor olanzapine affected cell growth at any of the incubation periods up to 8 days or any at concentration up to 10 µM compared to control although, as previously observed, this result was characterized by high variability (Fig 4.10a; 4.11a).

Moreover, both clozapine and olanzapine did not affect cell viability at any day and at any concentration (Fig 4.10b and 4.11b).

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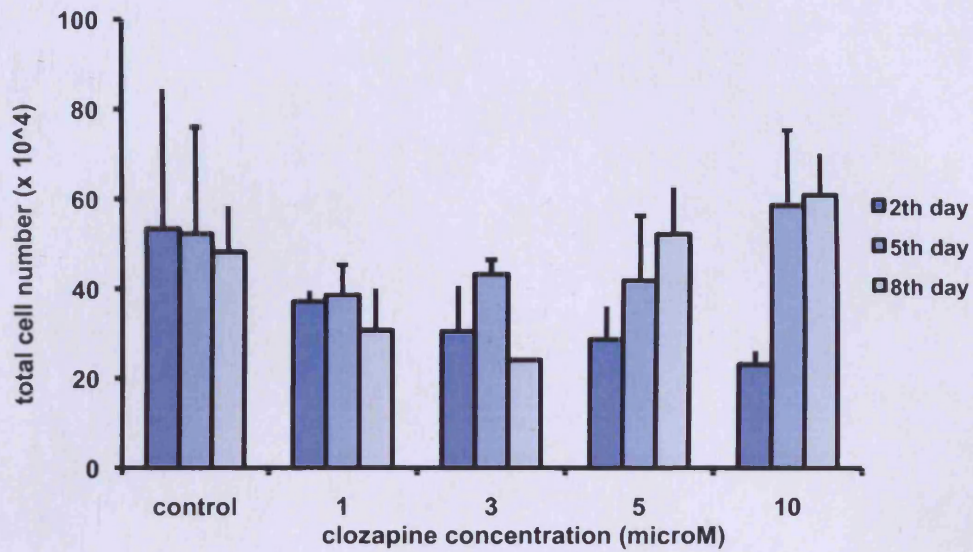
Fig 4.10a The effect of clozapine determined by manual cell counting. The values represent the means ± SEM between corresponding durations (days) of incubation. $n=3$ from 3 independent experiments.
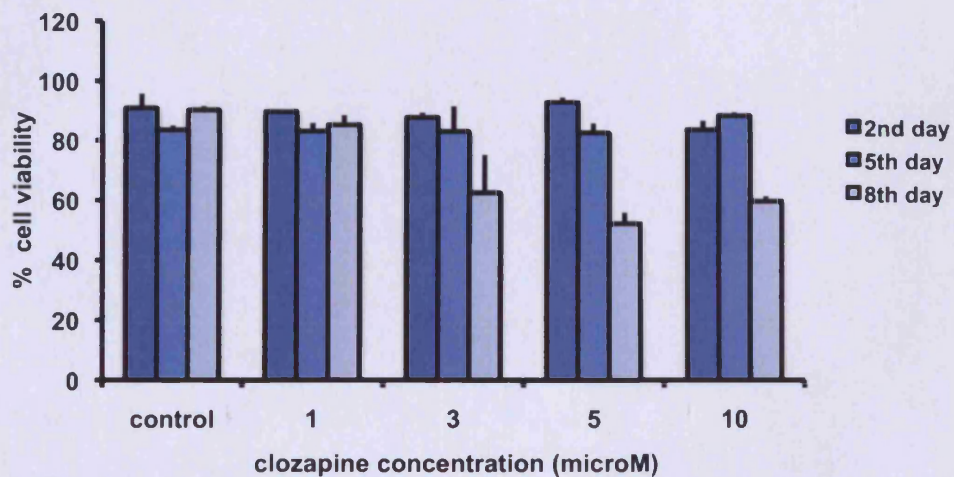
Fig 4.10b The effect of clozapine on % cell viability derived from the ratio (viable cells number/total cell number) x 100. The values represent mean ± SEM between corresponding durations (days) of incubation. $n=3$ from 3 independent experiments.
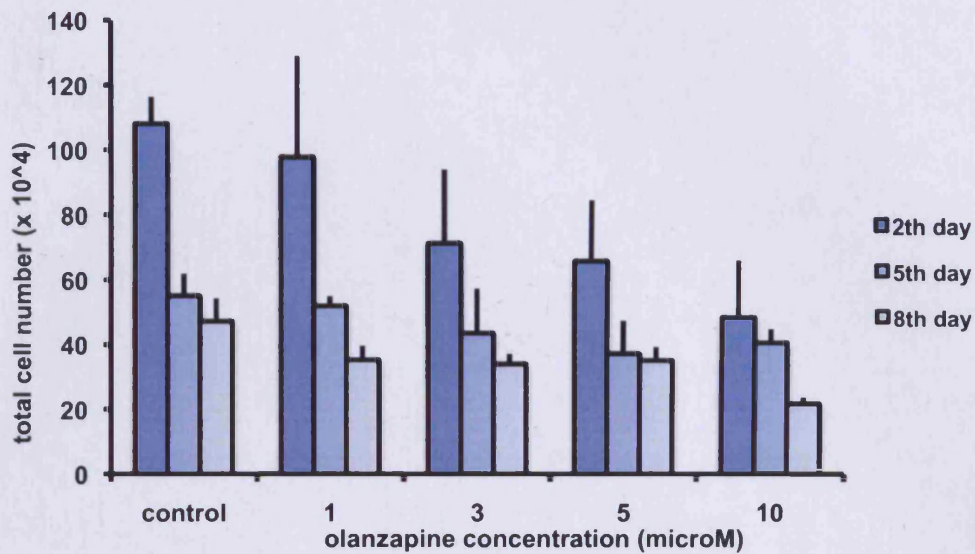
Fig 4.11a The effect of olanzapine determined by manual cell counting. The values represent the means ± SEM between corresponding durations (days) of incubation. *n*=3 from 3 independent experiments.
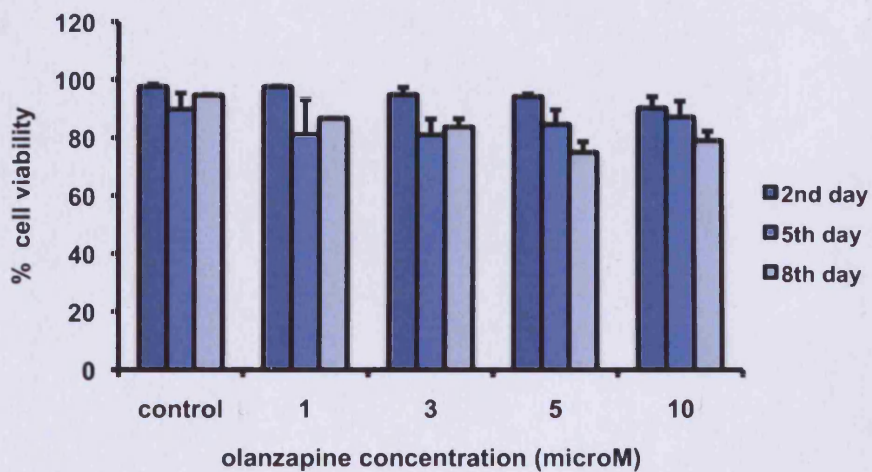
Fig 4.11b The effect of olanzapine on % cell viability derived from the ratio (viable cells number/total cell number) x 100. The values represent mean ± SEM between corresponding durations (days) of incubation. *n*=3 from 3 independent experiments.

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4.2.5 Effect of clozapine and olanzapine on gene expression of PPARγ, C/EBPβ and LPL in 7-F2 cells

7F2 cells were cultured for five days in the presence of adipogenic-inducer media, then total RNA was extracted, reverse transcribed in cDNA and analyzed using quantitative-Real Time PCR to evaluate the expression of three genes of interest: PPARγ, C/EBPβ and LPL. These genes were chosen since they are key markers of adipocyte differentiation.

Clozapine (5 μM) significantly increased C/EBPβ expression by 4 fold (P< 0.05). The expression of PPARγ was increased 3 fold although this increase was not significant (P> 0.05). Similarly, there was a slight 0.7 fold increase in relative LPL mRNA expression although this was not significant (P> 0.05) (Fig 4.12).

A five day exposure of 7-F2 cells to olanzapine (3 μM) had little effect (P> 0.05) on the expression of mRNA encoding for PPARγ, C/EBPβ and LPL (Fig 4.13). Specifically, the relative expression of PPARγ and C/EBPβ increased 0.7 and 3 fold respectively, however, the variability between experimental replicates precluded the achievement of statistical significance. Notably, the mean relative expression of LPL was 30 % lower in 7-F2 cells treated with olanzapine; however this was not significant (P> 0.05).
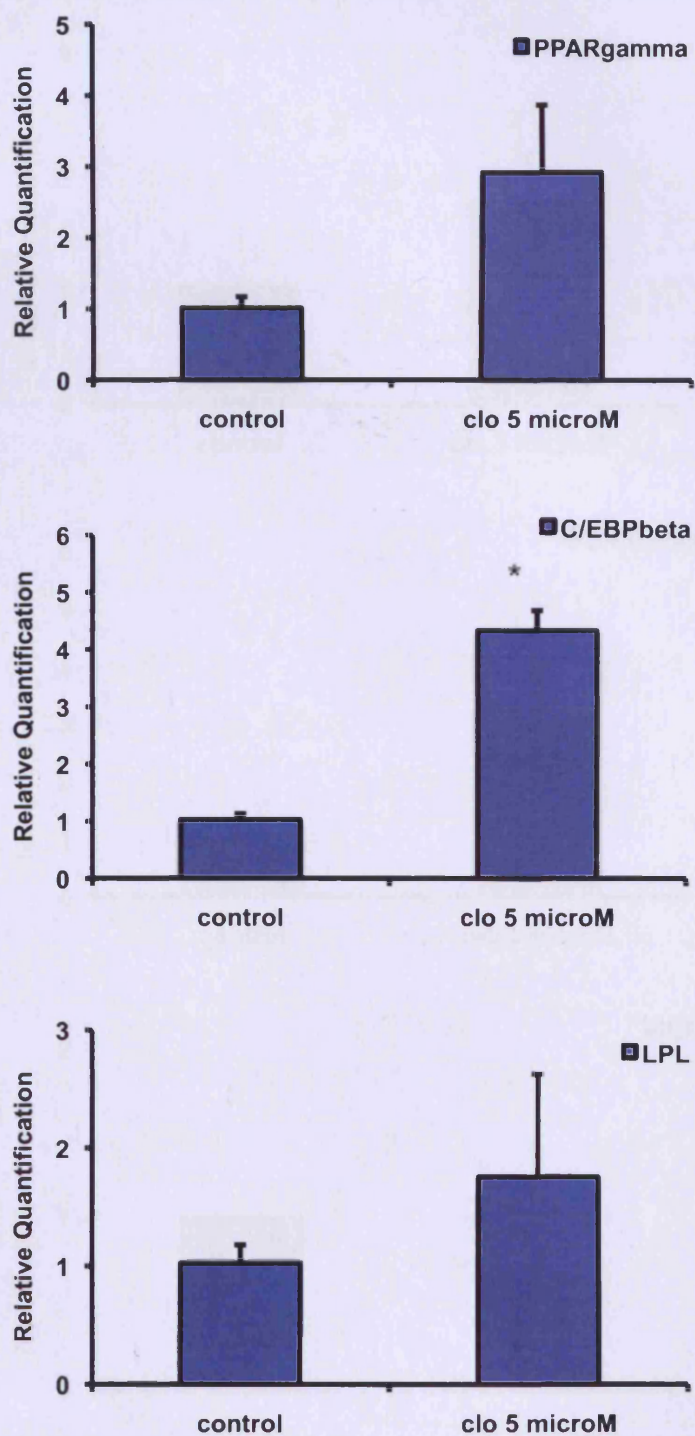
Fig 4.12 Effect of clozapine (CLO) (5 μM) on the relative mRNA expression of PPARγ, C/EBPβ and LPL in 7-F2 cells. The genes of interest are shown relative to that of housekeeper gene ARP as determined by qRT-PCR The values represent the mean ± SEM *P< 0.05. *n*=4 from 2 independent experiments.
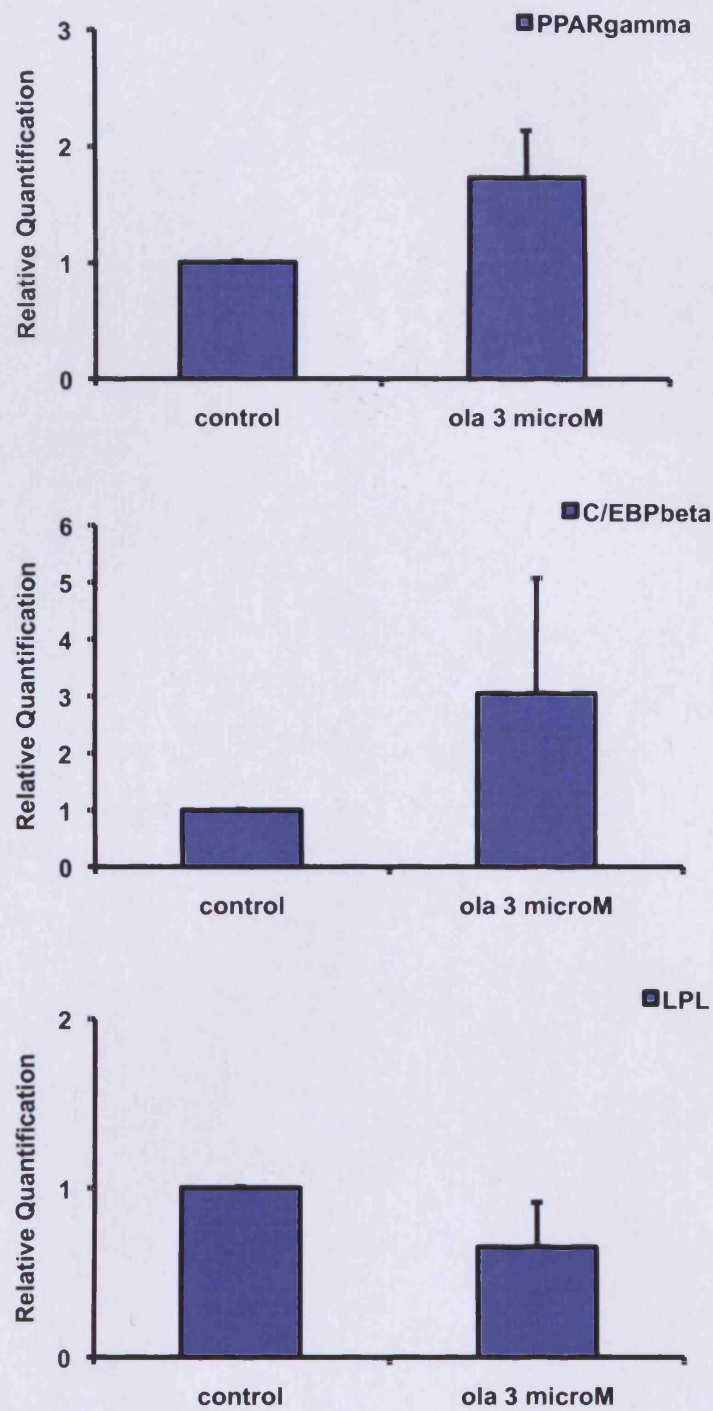
Fig 4.13 Effect of olanzapine (OLA) (3 μM) on the relative mRNA expression of PPARγ, C/EBPβ and LPL in 7-F2 cells. The genes of interest are shown relative to that of housekeeper gene ARP as determined by qRT-PCR The values represent the mean ± SEM. n=4 from 2 independent experiment.

4.3 Discussion

To complement the *in-vivo* study described in chapter 3, a series of *in-vitro* experiments were designed to investigate the effect of APS drugs upon a cell line that may represent a suitable model for adipogenesis *in-vivo*.

7-F2 is a clonal cell line that originates from the bone marrow of p53-/- mice and it is characterized by indefinite growth in vitro and mesenchymal origin. However, despite expressing phenotypic osteoblastic markers, appropriate treatment with adipogenic inducers causes the loss of these markers and 7-F2 cells can convert to mature adipocytes (Thompson et al., 1998).

This preliminary study showed that both clozapine and olanzapine at concentrations up to 10 μM did not promote the differentiation of fibroblast-like cells 7-F2 into adipocytes compared to controls. Moreover, quantitative RT-PCR showed that mRNA expression of PPARγ, C/EBPβ and lipoprotein lipase (LPL) was not affected by olanzapine treatment while clozapine up-regulated gene expression of C/EBPβ but not that of PPARγ and LPL.

In this study clozapine and olanzapine were tested for the first time on 7-F2 cells, therefore the initial aim was to evaluate their effect on the pattern of cell growth using an MTS assay and a manual cell counting technique (trypan blue exclusion). A wide range of concentrations was

initially chosen and incubated with cells for 72 hours (1-100 μM): MTS assay showed that clozapine (100 μM) significantly reduced cell viability while olanzapine did not significantly affect viability at any concentration (Fig 4.1; 4.2). Conversely, manual cell counting revealed that clozapine significantly decreased cell number at concentrations between 15 and 100 μM while olanzapine at all concentrations (except 5 μM) reduced cell number but without achieving statistical significance (Fig 4.3a; 4.4a). Nonetheless, neither of the drugs seemed to affect cell viability by trypan blue (Fig 4.3b; 4.4b), suggesting that both clozapine and olanzapine may slow the rate of proliferation without affecting cell survival.

Subsequently, an MTS was employed to measure 7-F2 cells mitochondrial activity over an extended period of time in the presence of clozapine or olanzapine (1-10 μM). This was used as an indicator of cellular proliferation in the presence of APS drugs. Both drugs, as previously observed by manual cell counting, did not affect the rate of proliferation after 3 days (72 hours), however, after 5 days, both drugs at several concentrations reduced proliferation and, after 7 days of treatment cell proliferation decreased at all concentration tested (1-10 μM) (Fig 4.5; 4.6), overall suggesting that prolonged treatment with clozapine and olanzapine, even at low doses, negatively affected cell survival.

This study also aimed to provide an insight into a possible direct effect of these drugs in enhancing adipocyte differentiation: Fig 4C and 4D show the differentiation process at different stages up until the appearance of mature adipocytes with the characteristic lipid droplets stained in red by the lipophilic Oil Red O dye. The pattern of the differentiation appeared to be the same for control and drug-treated cells. Oil Red O staining of 2-day cultures revealed the virtual absence of lipid droplets which indicate that this is an early stage of the differentiation process. After 5 days culture, numerous lipid droplets were seen as an indicator of a mature adipocyte phenotype. A decrease in lipid droplets was however noted after 8 days of induction.

Contrary to what was hypothesized initially, that clozapine and olanzapine might directly promote the differentiation of fibroblast-like cells into adipocytes, the adipogenesis assay appeared to indicate that these drugs actually prevent their formation (Fig 4.8; 4.9). However, the qRT-PCR data, although limited in sample size, did not fully support these results i.e. olanzapine (3 µM) had no significant effect on the mRNA expression of PPARγ, C/EBPβ and LPL compared to controls (Fig 4.13). Similarly, the treatment with clozapine (5 µM) had no significant effect on the mRNA expression of PPARγ and LPL, but up-regulated C/EBPβ expression by 4 fold (Fig 4.12). The drug treatment conditions used in these qRT-PCR studies equaled the lowest respective drug concentration that produced a significant increase in

lipid accumulation as determined by quantitative Oil-Red O assay (Fig 4.8, Fig 4.9) and the duration of culture (i.e. 5 days) that produced the peak of lipid droplet accumulation in control cell cultures treated with adipogenic factors.

The low number of experimental replicates employed in the qRT-PCR and the high variability between the samples limited the statistical power of these experiments, however, further studies may show the subtle trend in gene expression to be significant.

It is not clear however, whether reduced adipocyte differentiation reflects a direct effect on adipocyte formation or if it is a consequence of the effect of clozapine and olanzapine on the inhibition of cell proliferation and survival. Thus, a cell counting was run parallel to the adipogenesis assays to evaluate cell proliferation and viability during the differentiation process. Both clozapine and olanzapine did not affect cell number or survival (Fig 4.10a-b; 4.11a-b).

Few studies to date, have investigated the potential influence of these drugs on recruitment and differentiation of adipocyte precursor cells and findings are conflicting: clozapine enhanced the differentiation of human primary pre-adipocytes (Hemmrich et al., 2006) and that of 3T3-L1 pre-adipocytes (Yang et al., 2009). Similarly, olanzapine was also found to promote adipocyte formation from 3T3-L1 pre-adipocytes (Yang et al., 2007). By contrast, and in accordance with the study presented here,

Hauner et al., (2003), did not find clozapine to promote adipocyte differentiation in primary pre-adipocytes.

However, the idea that clozapine and olanzapine may directly perturb lipid metabolism has been the object of a number of investigations.

It was shown that clozapine and olanzapine (but the latter to a less extent) have a direct effect on cell metabolism through their action on peripheral tissues, and up-regulation of cholesterol and fatty acid biosynthesis (mediated by sterol regulatory element binding proteins SREBPs), was reported in glioma cells and hepatocytes (Ferno et al., 2005- 2006-2009; Reader et al., 2006). Moreover, an increase in lipid accumulation accompanied by a decrease in lipolytic activity in adipocytes has also been described (Minet-Ringuet et al., 2006; Vestri et al., 2007).

Although the results reported in this study suggest that clozapine and olanzapine do not promote *in-vitro* new cell recruitment and formation of fat cells, this might indirectly support some of the findings reported by others which suggest that these drugs may directly disturb lipogenesis and lipolysis in adipocytes (Minet-Ringuet et al., 2006; Vestri et al., 2007). Therefore, body weight gain and adiposity might be, at least in part, caused by a direct peripheral effect of clozapine and olanzapine on cell metabolism, possibly promoting accumulation of lipids and thereby causing adipocyte enlargement (hypertrophy) rather than tissue remodelling and increase in *de- novo* adipocyte formation.

An additional consideration to take into account is that bone marrow-derived mesenchymal cells might not be the most appropriate cell model for studying adipogenesis. A recent study comparing the human phenotype of MSCs derived from adipose tissue (AMSCs) and bone marrow (BMSCs) showed that both share a similar immunophenotypic profile and, based on morphological changes (Oil Red O staining), both display positive adipocyte differentiation capabilities, but with AMSCs enhancing differentiation 7 fold more than BMSCs. However, no difference was seen between AMSCs and BMSCs in terms of mRNA expression of marker genes involved in adipogenesis and lipid metabolism (Pachon-Pena et al., 2010).

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Chapter 5

The effect of clozapine on adipogenesis: an *ex-vivo* study

5. The effect of clozapine on adipogenesis: an *ex-vivo* study

5.1 Introduction

In the previous chapter the effect of clozapine and olanzapine in influencing adipogenesis in cultured bone marrow-derived fibroblast-like cells was described. In this chapter we aimed to investigate clozapine *ex-vivo* effect on bone marrow cells obtained from the C57BL/6J mouse strain that was utilised in body weight gain experiments.

Since the generation of marrow fat is considered to be identical to adipogenesis in other tissues (Rosen et al., 2009), it is believed that the process follows the same highly regulated pathway. Under genetic, hormonal and dietary influence, bone marrow mesenchymal stem cells (MSCs) undergo a program of differentiation events under the overall control of transcription factors such as peroxisome proliferator activated

receptor gamma (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs). PPARγ and C/EBPs assume the role of master regulators by initiating a cascade of key factors involved in the various stages of the adipogenic differentiation.

Moreover, bone marrow MSCs have the potential to differentiate not only into adipocytes but also in osteoblasts, chondrocytes and myoblasts depending upon a complex interaction of extracellular mediators activating lineage-specific transcription factors which in turn drive the differentiation program towards a specific lineage (Takada et al., 2007) (Fig 5A).
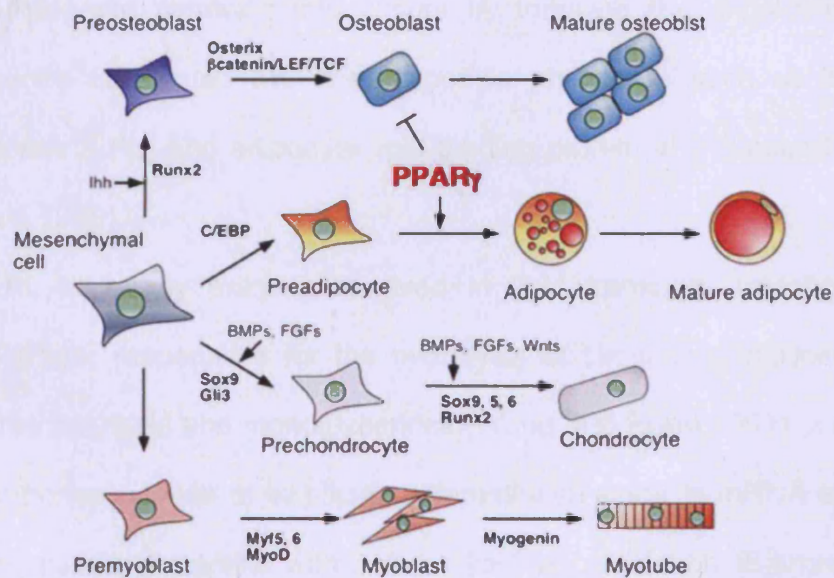

Fig 5A Differentiation potential of bone marrow MSCs. (From Takada 2007).

Interestingly, a certain degree of plasticity has been demonstrated between osteoblasts and adipocytes with a reciprocal relationship that enables the interconversion between these two lineages (Fig 5B).

For example, when cultured under appropriated *in-vitro* conditions in-vitro single MSCs were shown to be capable of differentiating into adipocytes, then later dedifferentiating and adopting an osteoblast phenotype. Similarly, mature osteoblasts are capable of differentiating to adipocytes, as well as the reverse whereby mature adipocytes dedifferentiate and become osteoblasts (Kassem et al., 2008).

When PPARγ is expressed in osteoblasts it is able to suppress the osteoblast phenotype and redirect the differentiation towards the adipogenic pathway: this occurs by inducing the expression of the genes associated with the adipocyte phenotype such as lipoprotein lipase (LPL) and adipocyte lipid-binding protein aP2 (Lecka-Czernik et al., 1999).

LPL is a key enzyme involved in lipid transport, metabolism and storage, responsible for the hydrolysis of circulating triglycerides into free fatty acid and monoglycerides (Wang and Eckel 2009). It is also an important marker of adipocyte differentiation since its mRNA expression increases in parallel with cellular lipid accumulation (Bjorntorp et al., 1978).

aP2 is part of a family of intracellular proteins capable of binding lipophilic ligands such as fatty acids (Benlohr et al., 1997). Although it is expressed in several tissues, is most abundant in adipose tissue

(MacDougald and Lane 1995), and it is known to act as carrier to facilitate intracellular-lipid traffic forming a complex with long-chain fatty acids (Coe et al., 1999). It also represents an important marker of terminal adipocyte differentiation (Tontonoz et al., 1994).

5.1.1 Aim of the study

It was observed earlier in this thesis (chapter 3) that clozapine at the doses of 15 and 25 mg/kg induced a significant weight gain in female mice compared to the controls (Fig 3.4a). The dose of 15 mg/kg in particular promoted body weight gain through the duration of drug treatment.

Following this observation, the animals employed in the experiment were killed under terminal gaseous anaesthesia and the bone marrow from femur bones was collected and subjected to RNA extraction and qRT-PCR to evaluate the expression of four genes of interest. These genes were namely PPARγ, aP2 and LPL, because of their role in adipocyte differentiation and lipid metabolism, and osteomodulin (OMD), a protein belonging to the leucine rich repeat protein family of proteoglycans; the over-expression of which is a reliable marker of increased osteoblast differentiation and mineralisation (Rehn 2008).

The aim of this study was to establish using qRT-PCR, whether clozapine, administered to C57BL/6J mice at a dose of 15 mg/kg could direct the differentiation of BMSCs towards the adipogenic pathway. Furthermore, we sought to investigate the effect of clozapine (15 mg/kg)

on the expression of an osteoblastic marker gene (OMD), which may reveal whether the interconversion between adipocytes and osteoblasts can occur not only *in vitro* but also *in vivo* (Fig 5B).
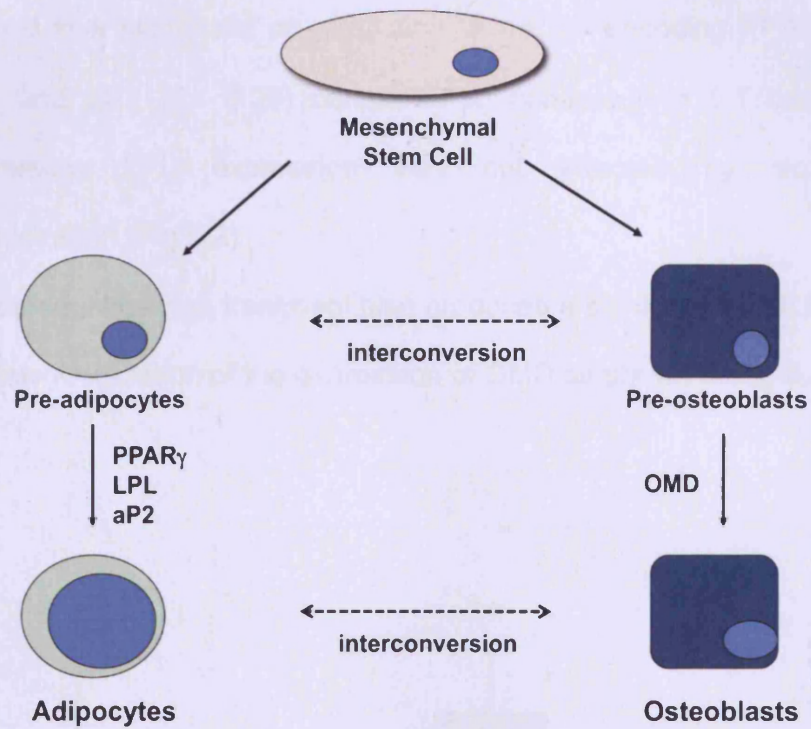

Fig 5B Differentiation and interconversion of bone marrow mesenchymal stem cells (BMSC) into adipocytes and osteoblasts.

This data may provide some evidence that clozapine (and possibly olanzapine) have a weight increasing effect mediated at the cellular level in addition to other possible central mechanisms.

5.2 Results

5.2.1 Effect of clozapine (15 mg/kg) on gene expression of PPARγ, LPL, aP2 and OMD in the bone marrow of C57BL/6J mice

Clozapine orally administered for 28 days at a dose of 15 mg/kg resulted in a significant up-regulation of mRNA encoding PPARγ (P< 0.05) and aP2 (P< 0.05) compared to controls (Fig 5.1 and 5.2). Conversely, LPL expression was not affected by clozapine administration (Fig 5.2).

In addition, clozapine treatment also produced a significant (P<0.01) 0.3 fold down-regulation of the expression of OMD as shown in Fig 5.2.

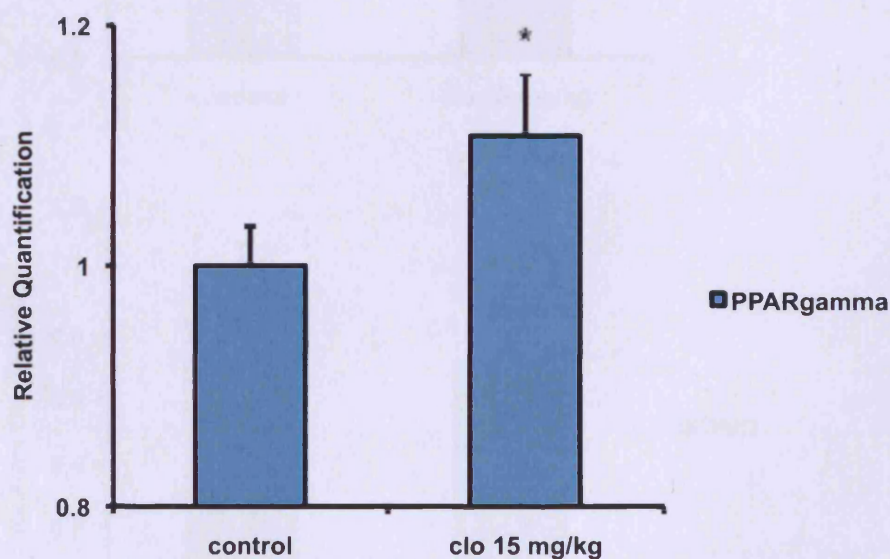**Fig 5.1** Effect of clozapine on mRNA expression of PPARγ in bone marrow as determined by qRT-PCR. The values represent the mean ± SEM *P< 0.05; *n*=10(10 animals/group).
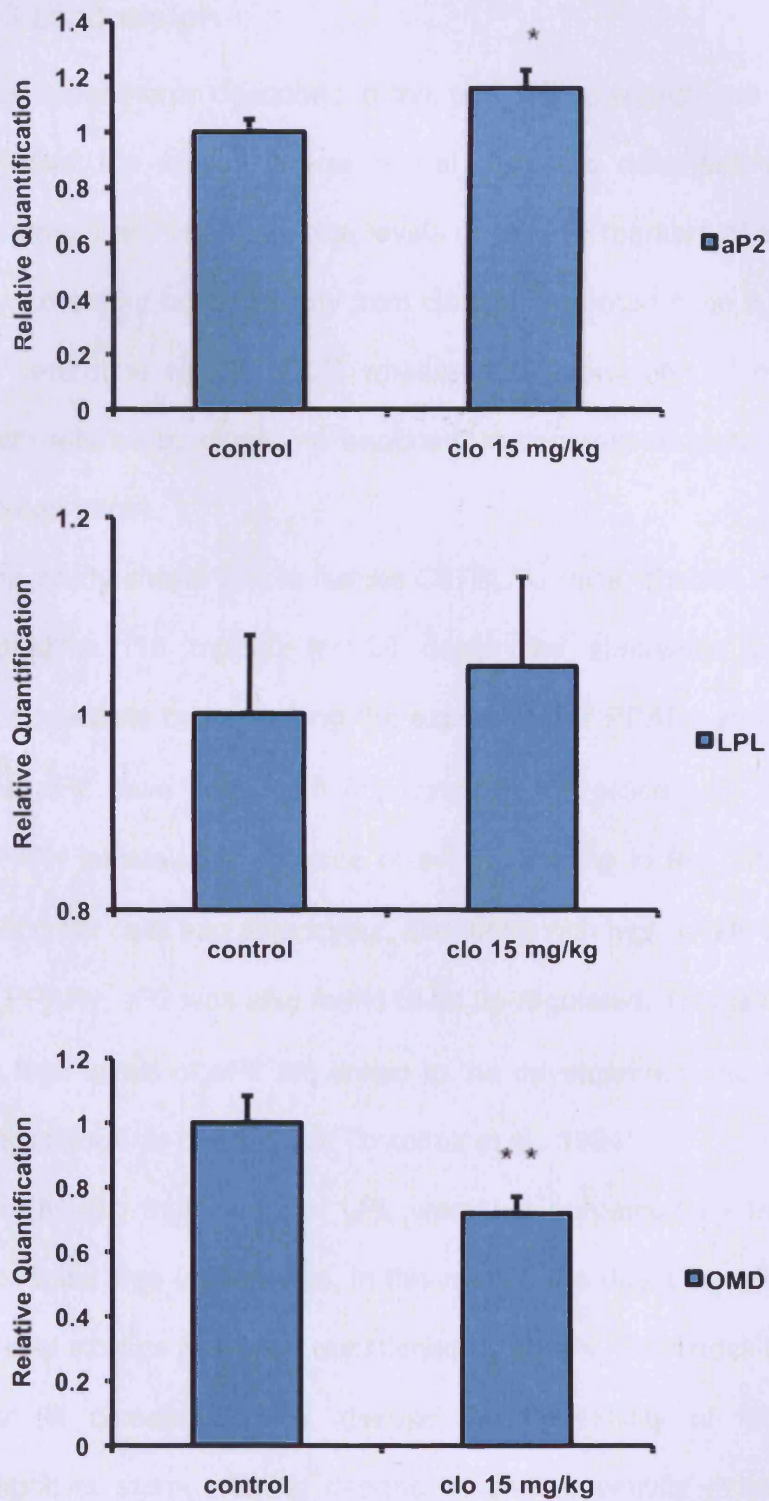
Fig 5.2 Effect of clozapine on mRNA expression of aP2, LPL and OMD in bone marrow as determined by qRT-PCR. The values represent the mean ± SEM. *P< 0.05; ** P<0.01; n=10 (10 animals/group).

5.3 Discussion

The experiments described in this chapter were designed to determine whether the *in-vivo* mouse model that was described in chapter 3 displayed any change in the levels of genetic markers of adipogenesis. By extracting bone marrow from clozapine-treated mice it was possible to determine by qRT-PCR whether the expression of marker genes were altered by clozapine treatment in comparison to control (vehicle-treated mice).

This study shows that in female C57BL/6J mice, chronic treatment with clozapine (15 mg/kg) for 28 days may stimulated bone marrow adipogenesis by increasing the expression of PPARγ and aP2. PPARγ and aP2 have been both implicated in the process of adipogenesis: PPARγ initiates the cascade of events leading to the differentiation of precursor cells into adipocytes, and along with high levels of expression of PPARγ, aP2 was also found to be up-regulated. This is not surprising as high levels of aP2 are linked to the development and maturation of the adipogenic phenotype (Tontonoz et al., 1994).

The finding that levels of LPL were not enhanced by treatment with clozapine was unexpected. In this matter, the direct contribution of LPL to lipid storage has been questioned by others. Weinstock *et al.* showed that in crossbred mice, despite the availability of fatty acids for adipocyte storage being dependent on the activity of LPL, when its expression is suppressed, there is little effect on the adipose tissue mass. This appeared to be due to a compensatory increase in *de-novo*

endogenous synthesis of fatty acids (Weinstock et al., 1997). However, it was later shown that reducing LPL expression through siRNA knockdown, caused a reduction in intracellular lipid levels of 80% indicating that LPL is necessary for lipid uptake and storage (Gonzales and Orlando, 2007).

In this study we observed a highly significant reduction of the levels of OMD mRNA. This may indicate a decrease in the differentiation of bone marrow cells into osteoblasts, thus suggesting that the observed *in-vitro* reciprocal relationship between osteoblasts and adipocytes (Kassem et al., 2008) may also occur *in-vivo* in clozapine-treated mice.

Despite the cellular heterogeneity of the bone marrow samples employed here was a limitation of the study, an up-regulation of PPARγ and aP2 mRNA levels, and a down-regulation of OMD mRNA levels seem to indicate that there are higher levels of bone marrow adiposity in animals chronically treated with clozapine compared to non-treated controls. As is the case with all qPCR quantification of mRNA levels it is desirable to follow-up these studies with experiments looking at relative protein levels.

Despite the function of marrow fat being largely unknown thus far, the generation of adipocytes from bone marrow MSCs (BMSCs) follows the same pathway of adipocyte formation from adipose MSCs (AMSCs) (Rosen et al., 2009; Pachon-Pena et al., 2010). In a recent study it was shown that histochemical assays suggested that AMSCs display a

greater lipid accumulation potential than BMSCs. However, during adipogenesis, mRNA levels for PPARγ, C/EBPα, adiponectin, leptin, LPL and aP2 were over-expressed in both AMSCs and BMSCs without any significant difference between them (Pachon-Pena et al., 2010).

It must be borne in mind that this is a preliminary investigation and further studies employing primary adipocyte culture should be undertaken. Moreover, other parameters such as adipocyte size, lipogenic and lipolytic activity should be investigated. Such an approach would provide additional evidence as to whether clozapine or olanzapine can directly affect adipogenesis and lipogenesis in an *in-vivo* model with relevance to the clinical situation.

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Chapter 6

General discussion

6.1 General discussion and conclusions

The development and introduction to clinical practice of second-generation antipsychotic drugs ("atypical") represented a major breakthrough in the treatment of psychosis (Sheen, 1999). These agents improved the life of millions of patients with schizophrenia by substantially diminishing the disabling extrapyramidal side effects that had negatively impacted the lives of patients previously treated with the older antipsychotics.

In the past decade however, it has been recognized that atypical antipsychotic therapy may be associated with disturbing metabolic derangements (Allison et al., 1999; Newcomer, 2004; Lambert et al., 2005). Body weight gain, adiposity, glucose dysregulation, insulin resistance and dyslipidemia, which are strongly associated with the onset of obesity, type 2 diabetes and cardiovascular diseases, are raising great concern among clinicians. The biological mechanisms

underlying atypical antipsychotic-induced side effects remain largely unknown and therefore the development of preclinical animal models is essential for translational research.

In this study clozapine and olanzapine were investigated because of their well-established propensity to induce metabolic alterations compared to other atypical drugs such as risperidone and ziprasidone (Allison et al., 1999). Body weight gain and adiposity were chosen as parameters of interest because they represent the most perceptible metabolic side effects of these medications and as such, they are the source of major distress for patients, often leading to inadequate adherence to treatment (i.e. poor patient compliance), discontinuation and a consequent relapse of psychotic symptoms (Fontaine et al., 2001).

The primary aim of the study was to mimic the degree of weight gain and adiposity seen in the clinic using female C57BL/6J mice. However, when significant weight gain in clozapine or olanzapine-treated animal groups was observed, these findings were not replicated in subsequent experiments. Thus, the development of a consistent and robust model was obstructed by variability and inconsistency of the experimental results. As shown in a number of previous studies, human weight gain and fat deposition has proved to be extremely challenging to model in rodents. In this context some studies have presented evidence of weight increase, some of weight loss and others have reported no

difference (see Chapter 3, table 3A). Moreover, numerous rodent models appear to support the belief that weight gain is a more prominent feature in females rather than in males, although this does not truly reflect the clinical situation (Meltzer et al., 2002; Ascher-Svanum et al., 2005).

Doses and frequency of drug administration in these studies also represent another issue that might undermine the reliability of the final experimental outcomes. Clozapine and olanzapine have short half-lives in rodents (Kapur et al., 2003) which in turn give rise to faster drug elimination from the organism impeding sustained steady plasma levels to reduce the likelihood of observing weight increase (Kapur et al., 2003).

Overall, the mixed success accomplished thus far in modeling weight gain and adiposity in rodents directly question the predictive validity of these models so much that it is suggested that rodents, and rats in particular, do not represent an optimal species to mimic the atypical antipsychotic-induced body weight gain and fat deposition seen in the clinic. Nevertheless, the search for a suitable animal model that reflects the range of metabolic alterations seen in patients treated with atypical antipsychotic medications is still a necessary step towards uncovering the mechanisms responsible for these major side effects, and ultimately, to drive the search towards a possible adjunctive treatment strategy to counteract them.

The current *in-vitro* studies (chapter 4) indicate that clozapine and olanzapine do not promote adipogenesis based on the morphological assays in 7-F2 cells. This might lead to hypothesis that both drugs do not exert their metabolic effects through a direct action on adipocytes but rather that they increase body weight via central pathways. However, the *ex-vivo* investigation of bone marrow extracts from clozapine-treated mice showed that clozapine up-regulates mRNA expression of PPARγ and aP2 which are well recognized markers of adipocyte differentiation and lipid formation. The limited nature of these observations using qRT-PCR only necessitate further investigation on lipolytic/lipogenic drug activity employing additional correlative methodologies.

As previously suggested, bone marrow-derived mesenchymal stem cells (BMSCs) may not be the optimal cell model to study adipogenesis and adipocyte metabolism (see Chapter 4), therefore, testing the adipogenic potential of these agents in a more established cell model of pre-adipocites such as 3T3-L1 cells and/or primary adipose tissue-derived mesenchymal stem cells (AMSCs) may provide a better understanding of whether these drugs can, at least in part, directly modify stem cell differentiation and affect adipocyte formation.

Additional studies are also needed to corroborate any possible direct effects at the mRNA transcriptional level of target genes driving the first stages of adipocyte formation. These include PPARγ and C/EBPs, and

genes involved in terminal differentiation and maturation such as aP2, LPL, FAS, GLUT4, and leptin (Cowherd et al., 1999).

Finally, previous reports have demonstrated that both clozapine and olanzapine are capable of activating lipogenesis and, in parallel, inhibiting lipolysis (Minet-Ringuet et al., 2006; Vestri et al., 2007). It would therefore be valuable to further investigate and establish in cultured adipocyte cell line and primary adipocytes, whether lipid accumulation and reduced lipolysis might conceivably be the preferential pathways by which clozapine and olanzapine directly alter adipocyte biology peripherally.

6.2 Bibliography

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