Diplomarbeit

ATGL & OLANZAPINE
Interactions with the lipid metabolism

eingereicht von

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zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Pathologie

unter der Anleitung von

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Suman Kumar Das

Ort, Datum …….. (Unterschrift)

Eidesstattliche Erklärung

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Preface

This study is the result of an idea that occurred to me in between a psychology course and my work at the department of pathology. At this time I only knew a few things about lipid metabolism, and I had just heard about olanzapine and its severe clinical side effects, such as weight gain and the development of diabetes to name a few. At the department of pathology I had the opportunity to learn about the newly discovered enzyme ATGL and its role in lipid metabolism, and so it occurred to me that my knowledge of both of these fields could easily be combined into the Hypothesis, which is the focus of this paper.

I spoke with Dr. Gorkiewicz about my idea and I told him what I knew about olanzapine and its side effects, and suggested the possibility that olanzapine interacts with ATGL resulting in the common side effect of obesity in psychiatric patients using this drug. This was the beginning of a long journey. In December 2009, when the concept for the project had just begun to take shape, I just wanted to start research, learn the basics and begin to work on my ideas. The next step was my introduction to Suman Kumar, with whom I have been working on this study until now. He introduced me to the depth of lab work and invisible reagents which in the end supported our ideas and lead to the following results.

From my point of view our results are very satisfying and I sincerely hope that someone will follow our ideas (more and more emerged over time) and will continue to do research in this field, and maybe someday someone will find a way to modify olanzapine in a way that will eliminate its severe side effects and make life a little easier for patients using it. Unfortunately, I will have to leave this work for someone else, as I intend to go into a different field of medicine.

Thanksgiving

First of all I would like to thank Prof. Gerald Hoefler for giving me the opportunity to work in his lipotox team and for supporting my studies in several ways.

I would like to thank Dr. Gregor Gorkiewicz for introducing me into the field of science. Without Dr. Gorkiewicz none of this could have happened. He is the person who guided me for almost two years and who always supported the ideas from which this study eventually emerged. He taught me a lot in these two years and he will continue to be a great influence on my future work in science.

Suman Kumar Das deserves special thanks for introducing me into lab work and for supporting the whole project in an incredible way. Without him, none of this would have been possible. His thoughts and his work have supported me over the past two years and his passion lives in this study.

I would also like to thank everyone on the lipotox team for sharing their ideas about this work and thereby supporting its development. Thanks also to Silvia Schauer, whose organizational support made the lab work possible.

Further special thanks belong to Allison Miller for proofreading, which definitely took some time.

Last but not least, I would like to thank my family and all of my friends for supporting me while I wrote this study.

Thank you very much!

Christopher Pivec

Zusammenfassung

Olanzapin ist eines der meist genutzten Antipsychotika der neuen Generation. Unglücklicherweise besitz es neben seiner hervorragenden Wirksamkeit auch einige ungewünschte Nebenwirkungen, wie das induzieren von Diabetes sowie die Förderung von kardiovaskulären Komplikationen. All diese Nebenwirkungen resultieren in einer reduzierten Lebenserwartung. Die zugrundeliegenden Entstehungsmechanismen der Nebenwirkungen sind im Moment leider noch nicht gänzlich geklärt und bedürfen weiterer Anstrengungen zu deren Entschlüsselung. So ist es das Ziel dieser Studie weiteres Licht auf die Effekte von Olanzapin zu werfen, diese zu entziffern und zu beschreiben. Besonders ein erst kürzlich entdecktes Schlüssel-Enzym des Lipid Stoffwechsels, ATGL (Adipose triglyceride lipase) sollte in die aktuelle Olanzapin-Forschung integriert werden. Zur Erforschung dienten Modelle mit 3T3-L1 Fettzellen, entnommenen gonadalen WAT (white adipose tissue) pads von Wild Typ und verschiedenen Knockout Mäusen. Diese wurden unter basalen und stimulierten Bedingungen untersucht. Um Effekte in Leber und Muskelzellen zu untersuchen wurden HepG2 Zellen und C2C12 Zellen benutzt. Olanzapin reduzierte die Expression und die Enzym-Aktivität von den drei bedeutendsten Enzymen im Fettstoffwechsel, ATGL, HSL und MGL signifikant. Unter höheren Konzentrationen war die Interaktion von CGI-58, einem Aktivator von ATGL mit ATGL durch Olanzapin beeinflusst. Eine erhöhte Aufnahme von gesättigten und ungesättigten Fettsäuren unter Olanzapin Behandlung konnte sowohl in Fett als auch in Muskelzellen beobachtet werden, sowie eine erhöhte Glukose Aufnahme. Durch eine erhöhte Expression von PPAR-gamma, FASN und SCD1 mRNA unter Olanzapin-gabe kann man schlussfolgern, dass Olanzapin direkt oder indirekt die Fettsäure Synthese erhöht. Zugleich ist die ß-oxidation in C2C12 Zellen reduziert. All diese Effekte führen zu einer erhöhten Fettansammlung in Fettdepots. Durch die erniedrigten Cyp7a1, Cyp27a1, ABCG1 und erhöhten SREBP1 Level sind erhöhte Cholesterinansammlungen in HepG2 Zellen zu beobachten. Zusammenfassend kann gesagt werden, dass die durch Olanzapin beobachtete Gewichtszunahme durch ein Zusammenspiel von gesteigerter Fettsäure Aufnahme und Synthese, sowie beeinträchtigter Fettspaltung und Mobilisierung hervorgerufen wird. Des weiteren beeinflusst Olanzapin Stoffwechselwege in Leber und Muskelzellen mit unterschiedlichen Ergebnissen.

Abstract

Olanzapine is one of the most common second-generation antipsychotics. Unfortunately it is known to lead to obesity and its severe consequences, such as diabetes and cardiovascular complications, ultimately resulting in a reduced life expectancy. The underlying molecular mechanisms are mostly unknown, so the aim of this study was to investigate olanzapine's effects on lipid metabolism, including its effects on the newly discovered rate-limiting enzyme in lipolysis, the lipase ATGL. Therefore, differentiated 3T3-L1 adipocytes and gonadal WAT pads from wild type and various knockout mice were treated with various concentrations of olanzapine under basal and stimulated conditions. To investigate olanzapine's effects on the liver, HepG2 cells were used and C2C12 cells were used to investigate olanzapine's effects on myocytes. Olanzapine significantly increased the expression and the activity of the three major enzymes in lipid metabolism, ATGL, HSL and MGL under basal and stimulated conditions. At higher olanzapine concentrations, the interaction of the activator CGI-58 with ATGL was hampered. An increased uptake of saturated and unsaturated FA has been observed in both, myocytes and adipocytes together with an increased glucose uptake. Due to increased expression of PPAR-gamma, FASN and SCD1 mRNA we showed that olanzapine directly or indirectly boosts FA synthesis. In C2C12 cells ß-oxidation is decreased due to olanzapine treatment. All of these effects lead to an increased fat accumulation in adipose depots. Olanzapine treated HepG2 cells showed an increase in cholesterol accumulation and decreased Cyp7a1 and Cyp27a1 levels together with decreased ABCG1 levels and increased SREBP1 levels. In short, olanzapine induces adiposity by increasing FA uptake and de-novo FA synthesis and decreasing lipolysis and affects muscle and liver cells in several ways.

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Glossary and Abbreviations

3T3-L1	Standard fibroblast cell line
5-HT2C	5-hydroxytryptamine serotonin receptor
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ATGL -/-	Adipose triglyceride lipase knock out
ATGL (PNPLA2)	Adipose triglyceride lipase
ATP	Adenosine-5'-triphosphate
C2C12	Mouse myoblast cell line
C57BL/6	Common mouse inbred strain
Ca	California
CD36	Cluster of differentiation 36
cDNA	Complementary deoxyribonucleic acid
CGI-58	α/β hydrolase domain-containing protein 5 (ABHD5)
CPT1a	Carnitine palmitoyltransferase
CYP27A1	Sterol 27-hydroxylase
D2	Dopamine receptor D2
DAG	Diacylglycerol
DG	Diacylglycerol
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylenediaminetetraacetic-acid
EPS	Extrapyramidal side effects
FA	Fatty acid
FABP4	Fatty-acid-binding-protein
FAS	Fatty acid synthase
FASN	Gene for fatty acid synthase
FATP1	Fatty acid transport protein
FBS	Fetal bovine serum or fetal calf serum
G	Gravitational force
GLUT4	Glucose transporter 4
H1	Histamine receptor H1
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HepG2	Human liver carcinoma cell line
HMGCR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HSL	Hormone-sensitive lipase
HSL -/-	Hormone-sensitive lipase knock out
Inh.	Inhibited
KCl	Potassium chloride
kJ	Kilojoule
Ko	Knock out
KOH	Potassium hydroxide
KRPH	Krebs Ringer-Hepes buffer
LBP	Lipid-binding proteins
LDL	Low-density lipoprotein
LDL-r	Low-density Lipoprotein and r for receptor
LPL	Lipoprotein lipase
LXR-a	Liver X receptor alpha
M	Mole
MAG	Monoacylglycerol
MAGL	Monoacylglycerol
MG	Monoacylglycerol
MGL	Monoacylglycerol lipase
MGLL	Monoacylglycerol lipase
MgSO4	Magnesium sulfate
Ml	Millilitre
mM	milli-Mole
mRNA	Messenger ribonucleic acid
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
N	Normality
NaOH	Sodium hydroxide
P/S	Penicillin-Streptomycin
PBS	Phosphate-buffered saline
PPAR-alpha	Peroxisome proliferator-activated receptor alpha
PPAR-gamma	Peroxisome proliferator-activated receptor gamma
qRT-PCR	Real–time-quantitative PCR polymerase chain reaction

RPMI 1640	Roswell Park Memorial Institute medium 1640
SCD1	Stearoyl-CoA desaturase 1
SDS	Sodium dodecyl sulfate
SGA	Second generation antipsychotics
SREBP1	Sterol regulatory element-binding protein 1
TAG	Triacylglycerol
TG	Triacylglycerol
Tris-HCl	Tris (hydroxymethyl) aminomethane
USA	United States of America
WAT	White adipose tissue
Wt	Wild type
µCi	µcurie

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1 Introduction

1.1 General Introduction

Approximately one percent of the human population is affected by schizophrenia a psychiatric disease, which is responsible for about 25 % of psychiatric hospitalizations. (Melnik et al. 2010) The general management of Schizophrenia involves behavioral treatment and pharmacological treatment with antipsychotics (Narula et al. 2010), which can be separated into classical antipsychotics such as Haloperidol and second-generation (SGA) "atypical" antipsychotics such as olanzapine, which is the main objective of this study. Second generation antipsychotics are also used in the management of acute manic episodes associated with bipolar I disorders. This results in the fact that more people are influenced by SGAs side effects. (Fini et al. 2010) Antipsychotic drugs are superior to placebo in the treatment of schizophrenia, resulting in improved compliance, effective rehabilitation, relapse prevention and enhanced quality of life. (Voruganti et al. 2000) (Franz et al. 1997). (Davis et al. 1980) (Bridler, Umbricht 2003)

1.2 Important Side effects

Unfortunately one of the main disadvantages of the classical antipsychotics is the association with parkinsonian symptoms, which are described as extrapyramidal side effects (EPS). (Bridler, Umbricht 2003) (Reynolds, Kirk 2010) These EPS where substantially diminished after the introduction of second-generation antipsychotics in the 1990s, however further side effects such as metabolic dysregulation occurred. Metabolic dysregulation results in weight gain and can rapidly lead to obesity, dyslipidemia (Lebovitz 2003) and impaired glucose tolerance, which may extend into type II diabetes leading to cardiovascular diseases. (Reynolds, Kirk 2010) There is also evidence for a reduced life expectancy and an increased risk of cardiovascular death among individuals suffering from severe mental illness. (Osborn et al. 2007)

1.3 A closer look at olanzapine

One of the most frequently subscribed second generation antipsychotics used in the treatment of schizophrenia is olanzapine, which also happens to be one of the most likely SGAs to induce weight gain. (Fini et al. 2010) Olanzapines brand name is Zyprexa (Figure1), and it is mostly administered orally in the form of tablets. (Fini et al. 2010) It is a hydrophobic substance, which contains two basic centers and is poorly soluble in water. (Figure2) (Keltjens R. 2005) (Fini et al. 2010) Because of the importance of olanzapine in the treatment of schizophrenia and its severe side effects on metabolism, the influences of olanzapine on metabolism became a main focus of research.

Figure a (Pivec 2011)

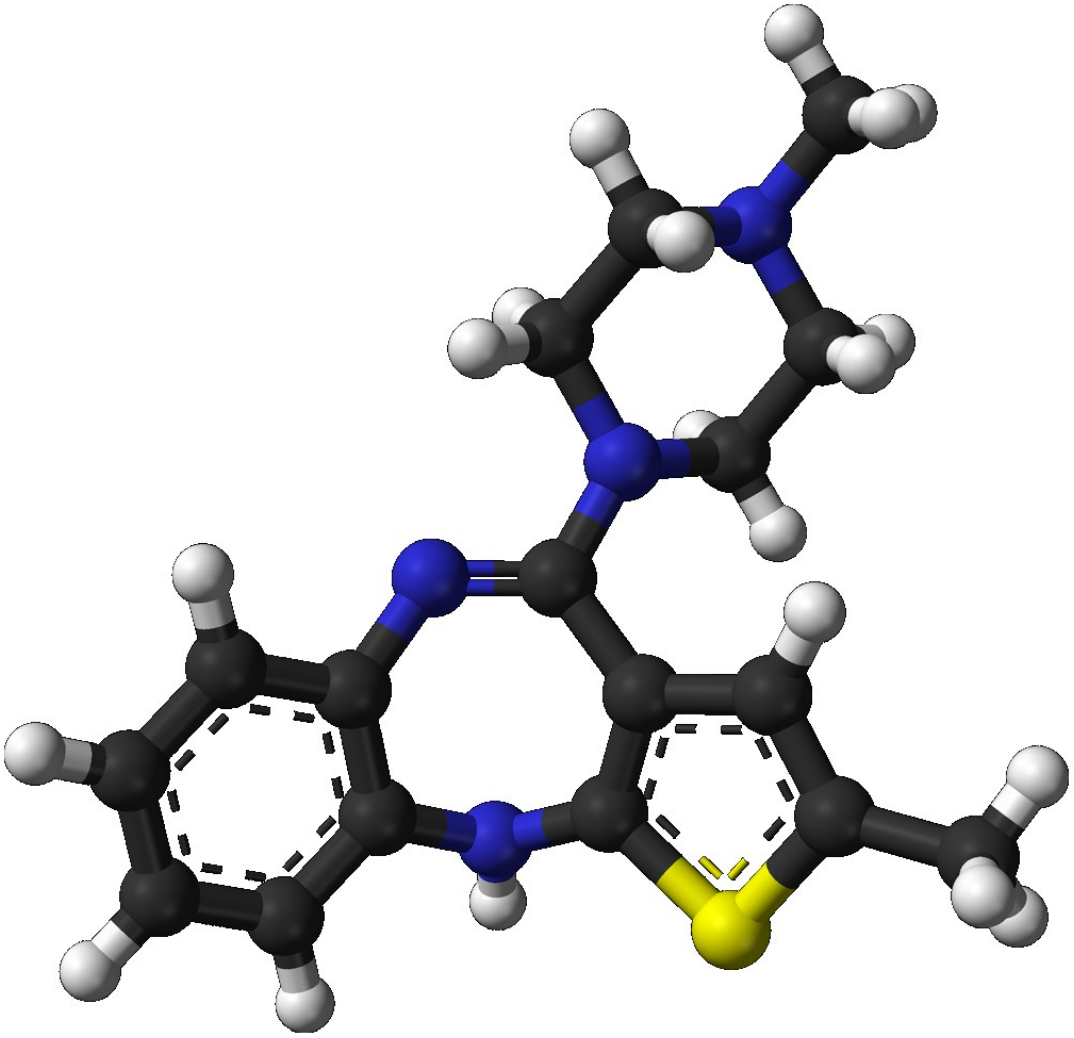


Figure b (Benjah-bmm27 2008)

Weight gain is also the most reported side effect and it is the most frequent cause for discontinuation and noncompliance in psychiatric patients taking Olanzapine. (Cascade et al. 2010) (Park et al. 2010) This can also result in rehospitalization, increased costs to the health system and lower quality of life. It can also lead to less physical activity and can affect self-esteem, which leads to a vicious cycle not only related to weight gain. (Stanton 1995) (Wirshing et al. 1999) (Taylor, McAskill 2000) (Allison, Mackell & McDonnell 2003) (Strassnig, Brar & Ganguli 2003)

1.4 Supposed and confirmed mechanisms of interaction

Although the mechanisms by which treatment with second-generation antipsychotics induces obesity are unclear, there are several possible explanations. For example, there are quite a few parts of the brain, which are suspected to play an important role, such as the hypothalamus, the nucleus accumbens, the amygdale and parts of the limbic brain. (Reynolds, Kirk 2010) (Schwartz et al. 2000) Conclusions from G.P. Reynolds (Reynolds, Kirk 2010) allow us to hypothesize that the extreme weight gain in olanzapine-treated patients is related to its particularly strong antagonism to the 5-HT2C (serotonin) receptor, and some less severe effects are due to H1 (histamine) receptor interaction. The H1 receptor interaction is responsible for the sedating effect of olanzapine, which could also result in weight gain due to less exercise. (Reynolds, Kirk 2010) The D2 antagonism of olanzapine can also influence feeding behavior (Clifton, Rusk & Cooper 1991) by increasing food intake due to blockage of these receptors. (Parada, Hernandez & Hoebel 1988) The inconsequent suppression of food intake, which should occur due to elevated leptin levels, certainly plays a role in the metabolic pathways of olanzapine. There is also a possibility that the signaling mechanisms of leptin are interrupted by pharmacological actions. It is interesting to note that adrenergic receptors show no clear relationship to weight gain and high prolactin levels don't seem to be a major contributor either. (Reynolds, Kirk 2010) Furthermore M. Case et al. showed that there is no consistent correlation between weight changes and changes in appetite. (Case et al. 2010) In addition it must also be noted that the disease process of schizophrenia has a direct effect on metabolic pathology or that risk factors for schizophrenia may be responsible for metabolic disorders. (Reynolds, Kirk 2010)

As mentioned before the development of diabetes is one of the major side effects of olanzapine, which has been proven by a meta-analysis from Newcomer, (Newcomer 2007) who demonstrated that olanzapine is associated with an increased risk of diabetes, while treatment with risperdone or quetiapine is not associated with diabetes. Diabetes can be a long-term consequence but olanzapine can also cause rapid-onset glucose intolerance. The development of diabetes can also emerge without obesity or the existence of any family risk factors. (Jin, Meyer & Jeste 2002)

It doesn't appear to be the case that olanzapine and clozapine, the two drugs with the highest capability for inducing weight gain, are also the ones with the greatest affinity for rapid-onset glucose intolerance, which can produce a potentially fatal ketoacidosis by inducing hyperglycemia. (Reynolds, Kirk 2010) Wirshing et al. reported that patients treated with olanzapine show a significant increase in glucose levels and Triglyceride levels but a decrease in LDL, HDL and total cholesterol. At this point it is unclear if these changes in glucose and lipid levels occur independent of weight gain or not. (Wirshing et al. 2002) Many papers support the hypothesis that olanzapine is one of the most problematic drugs in association with weight gain and impaired glucose tolerance. (Reynolds, Kirk 2010) Furthermore, it is undoubtedly investigated that hypertriglyceridemia shown in olanzapine treated patients increases the risk for cardiovascular disease. (Lebovitz 2003) (Osser, Najarian & Dufresne 1999) (Drexel et al. 1994) (Wirshing et al. 2002)

1.5 Olanzapine's interactions with lipid metabolism

Although we know much about olanzapine's effects on the central nervous system, we only know very little about its cellular effects, such as its impact on lipid metabolism, which seems to play an essential role in causing olanzapine's side effects and opens the door to a novel research focus. From the metabolic point of view, obesity is a pathological accumulation of lipids in the white adipose tissue. So becoming obese is a disturbed homeostasis between triacylglycerol (TAG) synthesis and triacylglycerol degradation whereas a shift from degradation to synthesis results in TAG accumulation, which in turn leads to obesity. Lipases such as ATGL, HSL and MGL are responsible for the breakdown of TAG. HSL was once considered to be the rate-limiting enzyme in TAG degradation, (Duncan et al. 2007) but in 2004, three different groups reported an enzyme, which seemed to be the rate-limiting enzyme in TAG breakdown. (Zimmermann et al. 2009) This enzyme is commonly known as adipose triglyceride lipase (ATGL). The results of many studies indicate that ATGL is the rate-limiting enzyme on TG breakdown, whereas HSL is mainly responsible for DG breakdown. (Haemmerle et al. 2002b) The last lipase in the TG breakdown pathway is monoglyceride lipase (MGL), which hydrolyzes monoglycerides to glycerol and one fatty acid. (Fredrikson, Tornqvist & Belfrage 1986)

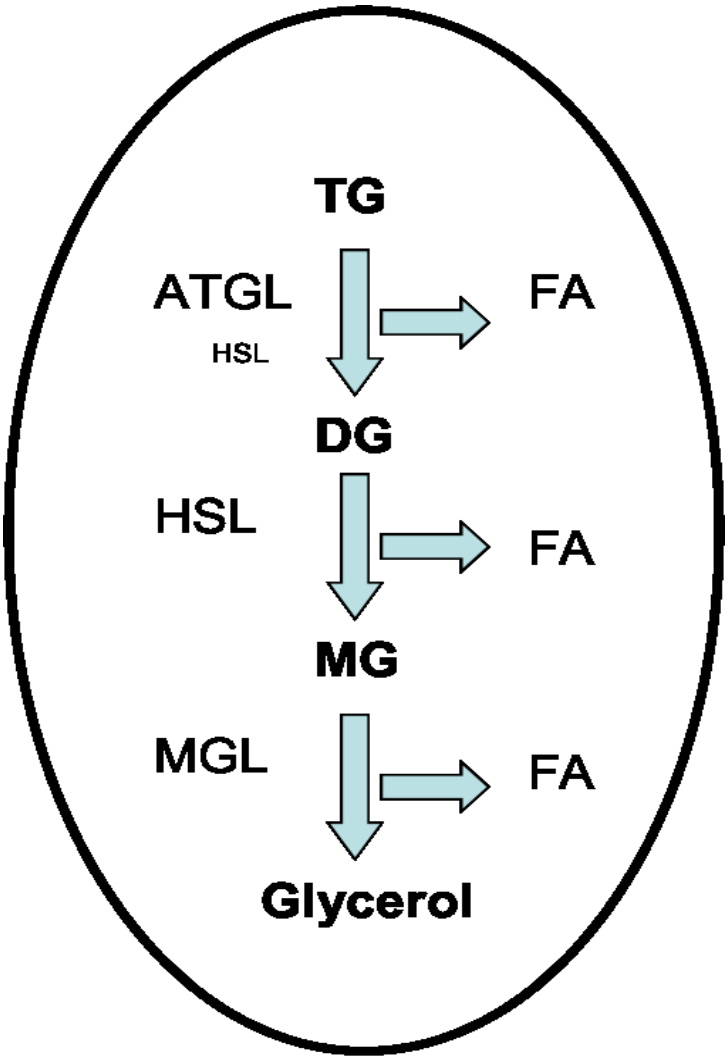


Figure c, Pivec 2010

Vestri et al. reported that olanzapine has the astonishing effect of reducing lipolysis and increasing lipogenesis. These effects lead to the accumulation of intracellular lipids, which enlarge adipocytes and results in obesity. (Vestri et al. 2007) The group also reported that conventional drugs such as haloperidol, did not show these effects. This may be an explanation for the different side effects between the old and the new generation of antipsychotics. Understanding olanzapine's effect on lipolysis is an important focus for investigating SGA's side effects. Minet-Ringuet et al. went deeper into the subject and investigated the effects of SGAs on HSL and FAS, revealing an over expression of FAS and an under expression of HSL, which resulted in stimulated liponeognesis and lower lipolysis. (Minet-Ringuet et al. 2007)

1.6 Aims of the study

Until now the effects of olanzapine on the entire TAG breakdown including ATGL, the rate-limiting enzyme of lipolysis have not been elucidated. Therefore, the subject of this study is the investigation of effects of olanzapine conferred on lipid metabolism on a cellular level using 3T3-L1, C2C12 and HepG2 cell lines. Triacylgycerol and Monoacylglycerol hydrolase assays, LPL assays, glycerol and FFA release assays are used to investigate lipolysis. Fatty Acid uptake assays, 2-Deoxyglucose uptake assays and cellular TG measurements are used to measure lipid accumulation (or lipogenesis) and glucose uptake. Fatty acid oxidation assays are used to measure Beta-oxidation. This study also investigates olanzapine's effects on expression of genes involved in lipid homeostasis by qRT-PCR (ATGL, CGI-58, HSL, MGL, LPL, CD36, PPAR-a, PPAR-g, FASN, FABP4, SCD1, HMGCR, LDL-r, ABCA1, ABCG1, CYP27A1, LXR-A, CPT1a, SREBP1, GLUT4 and FATP1). The results show that olanzapine inhibits lipolysis while increasing FFA uptake, which leads to TAG accumulation in adipocytes. It also increases basal glucose uptake but blunts insulin stimulation in C2C12 myotubes. In addition to the increased FFA uptake it also impedes ß-oxidation.

2 Materials and Methods

2.1 Introduction and explanation of materials and methods in order of appearance in the general part

2.1.1 C57BL/6

C57BL/6 (Figure 3) is probably the most widely used mouse inbred strain. These mice are commonly used in the production of transgenic mice such as the ATGL -/- and HSL -/- used in this study. These mice generally breed well, don't seem to have a high susceptibility to tumors and are long-lived. (Sarna, Dyck & Whishaw 2000)

Other characteristics:

1) high susceptibility to diet-induced obesity, type 2 diabetes, and atherosclerosis;
2) a high incidence of microphthalmia and other associated eye abnormalities;
3) resistance to audiogenic seizures;
4) low bone density;
5) hereditary hydrocephalus (early reports indicate 1 - 4 %);
6) hairloss associated with overgrooming,
7) a preference for alcohol and morphine;
8) late-onset hearing loss; and
9) increased incidence of hydrocephalus and malocclusion

(The Jackson Laboratory)

Figure d (The Jackson Laboratory)

2.1.2 3T3 and 3T3-L1

The original 3T3 cells where developed by George Todaro and Howard Green in New York at the department of pathology. They became the standard fibroblast cell line and where originally obtained from Swiss mouse embryo tissue. (TODARO, GREEN 1963)
The 3T3-L1 cell line is a modified version of the 3T3 cells and is used in the research of adipose tissue because of their ability to differentiate into adipocyte-like cells.

2.1.3 DMEM (Dulbecco's Modified Eagle's Medium)

Harry Eagle and Renato Dulbecco developed several media for cell culture, such as the EMEM (Eagle's Minimum Essential Medium) and the DMEM (Dulbecco's Modified Eagle's Medium), which was used in this study. (EAGLE 1955a, EAGLE 1955b) The DMEM contains approximately two times as much glucose and four times as many amino acids and vitamins as the original one. It also contains iron and phenol red. (Pombinho et al. 2004)

2.1.4 FBS (Fetal bovine serum or fetal calf serum)

Fetal bovine serum is produced out of a bovine fetus' blood and is commonly used as a serum supplement for cell culture of eukaryotic cells. It promotes the growth of various tissue cultures and functions as a source of growth hormones and growth factors. (biology-online 2005)

2.1.5 C2C12

The C2C12 cell line is a mouse myoblast cell line firstly obtained by Yaffe and Saxel due to experiments with C3H mice. These C2C12 cells are able to differentiate into myoblasts and osteoblasts and are used to study various pathways within these cells. (Yaffe, Saxel 1977)

2.1.6 HEPG2

HEPG2 is a human liver carcinoma cell line, which was isolated from a 15 year old Caucasian Americans liver biopsy in 1979 by Alden et al. (Aden et al. 1979) HEPG2 cells still have the possibility to produce most of the plasmaproteins such as albumin, apolipoproteins, fibrinogen and alpha-fetoprotein. (Knowles, Howe & Aden 1980) These cells also respond to human growth hormone to stimulate their growth. We used particularly the ones involved in weight gain and other side effects of olanzapine.

2.1.7 RPMI 1640 medium

RPMI 1640 is a cell culture medium originally developed by Moore et. al. at the Roswell Park Memorial Institute, which is why it is called RPMI. It is a bicarbonate buffering system based on the RPMI-1630, and contains glucose, salts, amino acids and vitamins. This medium is widely used as a supporting medium for growing many types of cultured cells. (Lindl 2002)

2.1.8 Triacylgycerol

Triacylglycerol is the most important storage fat in the human body. It is an ester derived from glycerol and three fatty acids. In the human body mostly palmitic and stearic acids were used as FA for building up TAG's. Burning TAG's provides the most energy per unit (about 39kj/mol). (Horn et al. 2005)

2.1.9 Monoacylglycerol

A Monoacylglycerol has the same basics as a TAG but it has only one fatty acid. (Horn et al. 2005)

2.1.10 Phosphate-buffered saline (PBS)

PBS is one of the most commonly used biological buffers, which is isotonic and non-toxic and in the right field of osmolarity for cells. As in this study's case PBS is often used for washing procedures. It contains sodium phosphate, sodium chloride, potassium chloride (in some mixtures) and potassium phosphate. (Morris et al. 2001)

2.1.11 Ethylenediaminetetraacetic-acid (EDTA)

EDTA is a commonly used chelating agent. It is also used as an anticoagulant for blood samples, it binds metal ions in chelation therapy and finds use in many other fields. (Hart 2000)

2.1.12 Non-labelled triolein and 9,10-3H(N)- triolein

Triolein is a chemical compound composed of a triglyceride and an unsaturated fat formed from an oleic acid. (Stuer-Lauridsen 2005) Its labeled form 9,10-3H(N)- triolein is for example used to monitor digestion. (PerkinElmer 2011)

2.1.13 Froskolin

Forskolin is produced by the Indian Coleus plant (Coleus forskohlii) and can be reproduced for research by certain methods. Forskolin is also known under the name Coleonol. In research forskolin can be used to raise cAMP (cyclic AMP) levels to study cell physiology. It raises cAMP levels by activating the enzyme adenylyl cyclase, which directly raises the cAMP levels. (Hänsel, Sticher 2009)

2.1.14 cDNA

Complementary DNA (cDNA) is produced with the help of the enzyme reverse transcriptase and the enzyme DNA polymerase. It is basically a DNA copy of mRNA and doesn't include introns. cDNA can be used to clone eukaryotic genes into prokaryotic cells, for example. (Alberts et al. 2005)

2.1.15 CGI-58

CGI-58 is a 38-kDa protein and it is also known as α/β hydrolase domain-containing protein 5 (ABHD5). It plays a key role in the breakdown of TAG not only in adipocytes, but also in almost every other human cell. It facilitates lipolysis as a coactivator of ATGL. The interaction between CGI-58 and ATGL has not been fully investigated, as it is unclear if it is a direct interaction between these two proteins. (Yamaguchi 2010)

2.1.16 PPAR-α and PPAR-γ

PPAR's full name is peroxisome proliferator-activated receptor and they belong to a group of nuclear receptor proteins regulating the expression of genes by acting as transcription factors. They are so called ligand-activated transcription factors. PPAR-γ plays a key role in adipose tissue differentiation and it also regulates lipid storage in white adipose tissue and energy dissipation in brown. PPAR-γ also improves insulin sensitivity and thus increases glucose uptake into cells. PPAR-α's major role is the regulation of energy homeostasis in the liver by, for example, activating fatty acid catabolism, stimulating gluconeogenesis and ketone body synthesis and controlling the gathering of lipoproteins. PPAR-α also stimulates cholesterol catabolism and reduces adiposity. (Michalik et al. 2006)

2.1.17 LPL

LPL's full name is lipoprotein lipase and its key role is hydrolyzing lipids in lipoproteins. Because of the lipophilic nature of TAG's, they have to be transported in lipoproteins such as chylomicrons and very low-density lipoproteins (VLDL). To shift the TAG's into the cells they have to be broken down by enzymes to pass the cell membrane. The LPL is located in endothelial cells lining the capillaries, and so its purpose is to break down the TAG's into glycerol and free fatty acid to enable their transport into cells. (Horn et al. 2005)

2.1.18 MGL

MGL, also called MAG lipase, MAGL or MGLL is believed to fulfill the rate-limiting role in the breakdown of monoacylglycerol to glycerol and fatty acids. Therefore it is responsible for the last step in TAG breakdown. (Zechner et al. 2005)(Fredrikson, Tornqvist & Belfrage 1986)

2.1.19 HSL

Hormone-sensitive lipase also known as HSL was supposed to be the rate-limiting enzyme for TAG breakdown. (Duncan et al. 2007) In 2004, three different groups reported an enzyme, which seems to be the actual rate-limiting enzyme in TAG breakdown. (Zimmermann et al. 2009) This enzyme is commonly known as adipose triglyceride lipase (ATGL). Nevertheless HSL's role seems to be the breakdown of TG to DG and DG to MG where its main purpose seems to be the second step. The breakdown of TG to DG seems to be predominantly preformed by ATGL. (Zechner et al. 2005)

2.1.20 ATGL (PNPLA2)

Three different groups found the novel lipase adipose triglyceride lipase also known as ATGL or PNPLA2 in 2004. (Zimmermann et al. 2009) It seems to be the rate-limiting enzyme in TAG breakdown, and its main purpose is to break down TG to DG. Many results indicate that ATGL is the rate-limiting enzyme on TG breakdown, whereas HSL is mainly responsible for DG breakdown. (Haemmerle et al. 2002b)

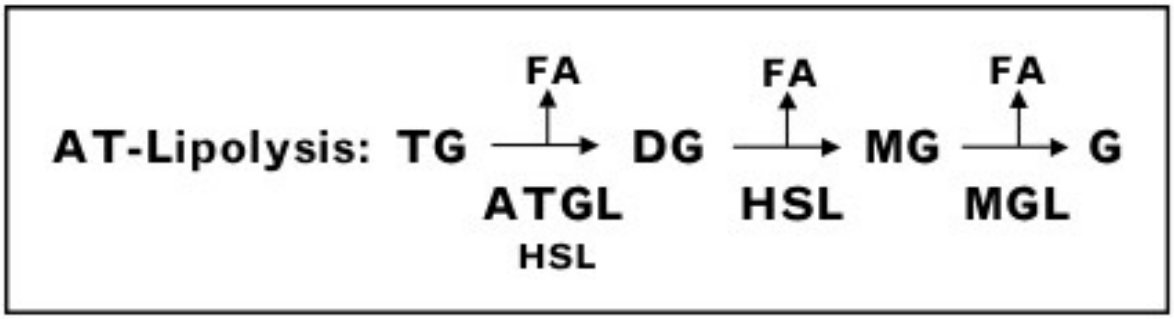


Figure e adapted by (Zechner et al. 2005)

2.1.21 FASN and FAS

The multifunctional enzyme fatty acid synthase (FAS) is encoded by a gene named FASN. The main purpose of FAS is to produce fatty acids, mostly palmitic acid, which is the prototype of nearly every other fatty acid. (Wakil 1989) (Horn et al. 2005)

2.1.22 FABP4

FABP stands for fatty-acid-binding-protein and they are carrier proteins for fatty acids as the name implies. They also carry other lipophilic substances such as eicosanoids, retinoids and others. They belong to the superfamily of lipid-binding proteins (LBP). At the moment 9 different FABP's are known acting in the liver, intestines, muscle and heart, adipocytes, epidermis, ileum, brain, myelin and in testis. FABP4 predominantly acts in adipocytes. (Chmurzynska 2006)

2.1.23 CD36

CD36 stands for cluster of differentiation 36. They are integral membrane proteins found on a variety of cells and fulfill a variety of purposes such as interacting as receptors or ligands on white blood cells. (Horn et al. 2005) CD36 in our case is important due to its ability to bind long-chain fatty acids. (Baillie, Coburn & Abumrad 1996)

2.1.24 SCD1

SCD1 stands for stearoyl-CoA desaturase, and catalyzes the rate-limiting step in monounsaturated fatty acid synthesis. Furthermore it plays an important role in energy metabolism. SCD1 is one of four isoforms in mice, which is predominant in the liver. (Biddinger et al. 2006)

2.1.25 HMGCR

HMGCR stands for HMG-CoA reductase, or (3-hydroxy-3-methyl-glutaryl-CoA reductase), and it is the enzyme responsible for the rate limiting step in cholesterol biosynthesis. The HMGCR is located in the membrane of the endoplasmic reticulum, and reduces, with help of NADPH/H+, ß -HMG-CoA to mevalonate. (Horn et al. 2005)

2.1.26 LDL-r

LDL stands for Low-Density Lipoprotein and the r for receptor. The receptor is located at the surface of almost every cell in the body and functions as a mosaic receptor for mediating endocytosis of cholesterol-rich LDL. It functions due to the apoprotein B100 on LDL particles and recognizes them this way. (Horn et al. 2005)

2.1.27 ABCA1

ABCA1 belongs to the protein superfamily of ATP-binding cassette transporters (ABC-transporters). They use ATP's energy to translocate solutes across cell membranes. They transport many different substrates, such as lipids, sterols and drugs. (Jones, George 2004) ABCA1's specific function is to regulate cellular cholesterol and phospholipid homeostasis by working as a cholesterol efflux pump. (Schmitz, Langmann 2001)

2.1.28 ABCG1

ABCG1 also belongs to the protein superfamily of ATP-binding cassette transporters (ABC-transporters) mention before. They also use the energy of ATP to transport solutes across cellular membranes. (Jones, George 2004) ABCG1's specific function is to mediate the transport of cholesterol from macrophages and some other cells to HDL. There are several splice variants of ABCG1 with slightly different functions. (Engel et al. 2006)

2.1.29 LXR-alpha

LXR-alpha stands for Liver X receptor alpha and is a nuclear receptor protein regulating macrophage function involved in controlling lipid homeostasis and inflammation. Together with PPARs they control the regulation of lipid uptake and efflux, lipoprotein metabolism and lipogenesis. (Ricote, Valledor & Glass 2004)

2.1.30 CPT1-A

CPT stands for carnitine palmitoyltransferase and we can differentiate between CPT1, located in outer mitochondrial membranes and CPT2, located in inner mitochondrial membranes. CPT1 has three tissue specific isoforms, CPT1-A in liver, CPT1-B in muscle and CPT1-C in brain. CPTs mediate the long chain fatty acid transport by binding to carnitine. If CPT1-A is lacking, current attacks of fasting hypoketotic hypoglycemia can occur. (Bonnefont et al. 2004)

2.1.31 SREBP1

SREBP1 stands for sterol regulatory element-binding protein 1, which belongs to the basic-helix-leucine zipper family of transcription factors. The SREBF 1 & 2 genes encode SREBPs in mammals. SREBP1s purpose is to regulate the synthesis of sterol biosynthesis by upregulating its enzymes. (Wang et al. 1994)

2.1.32 GLUT4

GLUT4 belongs to a family of glucose transporters expressed in various cell types. GLUT4 is specific for adipose tissue and striated muscle and controls insulin-regulated glucose transport. It is different from GLUT 1 and 3, which are insulin-independent. (Horn et al. 2005)

2.1.33 FATP1

FATP stands for fatty acid transport protein. FATP1 belongs to this group of transport proteins and is responsible for the active transport of fatty acids into various tissues. (Horn et al. 2005)

2.1.34 CYP27A1

CYP27A1 is commonly known as sterol 27-hydroxylase and is a gene that encodes a cytochrome P450 oxidase and plays an important role in bile acid synthesis. It is specifically located in various tissues and is found in the mitochondria of various cells. Its specific function is to degrade cholesterol to bile acid, therefore when inhibited accumulation of cholesterol can be observed. (Chiang 2009)

2.2 General part

2.2.1 Harvesting of tissue and animals

All animal studies were performed in accordance with the guidelines and provisions of the Commission for Animals Experiments of the Austrian Ministry of Science and recommendations of the local ethics committee.

Female C57BL/6J mice were kept on a normal 12 hours light, 12 hours dark cycle feeding on a standard laboratory food diet (4.5 % w/w fat). Using targeted homologous recombination HSL -/- and ATGL -/- mice were generated. (Haemmerle et al. 2002a)(Haemmerle et al. 2006) The further processed tissue was harvested from 8 to 10 week old mice, which were anesthetized with isoflurane and killed by cervical dislocation. Tissues were immediately excised and frozen in liquid nitrogen or handled as described later on.

2.2.2 Cell lines (3T3-L1, C2C12 & HEPG2)

The 3T3-L1 preadipocytes were cultured in DMEM (high glucose) with 10% FBS. Differentiation into adipocytes was done as described earlier (Green, Kehinde 1975). C2C12 myoblasts were cultured in DMEM (high glucose) with 10% FBS and 1% penicillin/streptomycin. The Differentiation into myocytes was done as described earlier (Miller 1990). HEPG2 hepatocytes were cultured in DMEM (high glucose) medium with 10% FBS and 1% P/S.

2.2.3 Primary Hepatocyte Isolation

Following a two-step collagenase perfusion technique as described earlier, primary murine hepatocytes were isolated (Schreiber et al. 2009). Briefly, the liver was perfused with magnesium-free Hank's buffer supplemented with calcium using a perfusion rate of 80-100 ml/min for about 10 to 15 minutes. Afterwards the liver was perfused with a collagenase solution (0.5g/L) using a rate of 50 to 70 mL/min for about 5 to 10 minutes. Subsequently, the capsule of the liver was opened and rinsed with RPMI 1640 medium. The fibrous connective tissue was dismissed and the cell suspension was plated to treat the hepatocytes with more than 80% viability with various olanzapine concentrations for about 30 hours.

2.2.4 Triacylgycerol and Monoacylglycerol hydrolase assay

Gonadal WAT from wild type mice and differentiated 3T3-L1 adipocytes were washed with phosphate-buffered saline (PBS) containing 1mM ethylendiametetraacetic-acid (EDTA). Thereafter were homogeniced in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1mM dithiothreitol, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin, ph 7.0) by using a Magna Lyser instrument (Roche diagnostics GmbH, Mannheim, Germany). The lysate was centrifuged at 20,000 G for one hour at 4°C and the lipid-free infranatant (cytosolic fraction) was sampled. Using a conventional ultrasound sonicator the substrate was further processed for determining the TG hydrolase activity with non-labeled triolein and [9,10-3H(N)- triolein] (NEN Life Science Products, Boston, MA, USA) as a radioactive tracer. Then it was emulsified with phosphatidylcholine/phosphatidylinositol (Sigma).

The cytosolic fractions were incubated either with or without HSL-inhibitor 76-0079 (NNC 0067-0000-0079, Novo Nordisk, Denmark) or with or without recombinant CGI-58 protein and incubated at 37°C for 60 minutes while being constantly shaken. Incubation was done with various concentrations of olanzapine. Subsequently, the reaction was stopped using 3.25 ml methanol/chloroform/heptane (10:9:7), 1 ml of 0.1 M potassium carbonate and 0.1 M boric acid (pH 10.5). After centrifugation at 800 G for 20 minutes the upper phase was sampled and radioactivity was measured in 1 ml of the phase by using a liquid scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, Fullerton, CA, USA).

Monoacylglycerol hydrolase (MGH) activity was measured by incubating the cell lysate with FFA free BSA and 100 µl of 1mM 2-oleoglycerol (2-OG; Sigma Aldrich, St. Louis, USA) containing substrate at a 1:1 ratio in 100mM potassium phosphate buffer. The mixture was incubated at 37° for 20 minutes with various concentrations of olanzapine (0µM, 50µM and 400µM) and subsequently stopped by adding 100 µl chloroform. After centrifugation at 10,000 G for 10 minutes the free glycerol concentration was measured in the upper phase by using a commercial kit (Sigma Aldrich, St. Lois, USA)

2.2.5 LPL assay

The substrate for the LPL activity assay was prepared by mixing non radioactive Triolen with 3H-Triolein (glycerol-tri-[9,10-3H(N)-oleate], 1µCi/µL). The solvent was evaporated with nitrogen and the remaining sample was emulsified in Triton-X and Tris buffer (pH 8.6) using a commercial sonicator. Purified LPL (Sigma) was incubated with a substrate made of FFA free BSA, Triolein and heat inactivated human serum and with various concentrations of olanzapine at 37°C for 1h. Thereafter, the reaction was stopped by adding 3.25 ml methanol/chloroform/heptane (10:9:7), 1 ml of 0.1 M potassium carbonate and 0.1 M boric acid (pH 10.5). After centrifugation at 800 G for 20 minutes lipase activity was measured in I ml of the upper phase by using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, Fullerton, CA, USA).

2.2.6 Glycerol and FFA release assay

To perform the glycerol and FFA release assays, 3T3-L1 or mouse gonadal WAT was used. Cells and tissues were washed with PBS and incubated in the presence or absence of a specific HSL inhibitor (76-0097) and stimulated with or without 10 μM Froskolin in Dulbeco's modified Eagle's medium (DMEM) (PAA, Pasching, Austria) containing 2 % fatty acid-free bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) under various olanzapine concentrations at 37° C for 2 h. Commercial kits (Wako Chemicals, Neuss, Germany and Sigma-Aldrich respectively) were used to analyse aliquots of medium to determine FFA and glycerol release.

2.2.7 Fatty Acid uptake assay

C2C12 myotubes were washed in Krebs Ringer HEPES (KRH) buffer supplemented with 0.1% BSA twice. C2C12 cells were then incubated with the addition of different concentrations of olanzapine in 2.5 mM 5:1 palmitate or oleate-BSA complex containing 10 μCi/ml [1-14C] palmitate or oleate for 30 min at 37 ^{o}C in modified Krebs Ringer HEPES. The cells were washed in ice cold KRH/0.5% BSA to stop the uptake process. The cellular FFA uptake was determined by liquid scintillation counting (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, Fullerton, CA, USA). Protein concentrations for normalisation were determined by lyzing the cells with 0.3 N NaOH and 0.1 % an then using the RcDc kit according to the manufacturers instructions (BIO-RAD).

2.2.8 2-Deoxyglucose uptake assay

The 2-deoxyglucose uptake assay was performed as described previously (Capell et al. 2010). In brief, C2C12 myotubes or *Musculus gastrocnemius* from C57BL6 mice were incubated with or without 100 nM insulin for 1 h in KRPH buffer. Various concentrations of olanzapine (0µM, 50µM and 400µM) and 2-deoxy-[3H] glucose (0.1 mM, 0.5 µCi/ml) were added and incubated for 4 min. The reaction was stopped by adding 10 µM cytochalasin B (Sigma) in ice cold PBS, and the cells were then washed with ice cold PBS again. Cells were lysed in 0.3 N NaOH and 0.1 % SDS and glucose uptake was measured by scintillation counting. Protein concentration was determined by using the DC Kit as recommended (Bio Rad).

2.2.9 Cellular TG measurement and cholesterol content

Whole cellular lipids from 3T3-L1 adipocytes were extracted with 2 ml hexane/isopropanol (3:2, v:v) at 4°C for 1h. The solvent was dried with liquid N_2 and lipids were then dissolved in 1% Triton-X100. The TG content was assessed by using a TG assay kit (Diagnostic Systems, Holzheim, Germany) according to the manufacturer's protocol. Cells were then lysed in 0.3 N NaOH and 0.1 % SDS and protein concentration was determined by using the Dc kit (Bio Rad). Cholesterol content in primary hepatocytes was determined with a colorometric assay according to the manufactureres protocol (Human diagnostics).

2.2.10 Fatty acid oxidation assay

Cell fractions enriched in intact mitochondria were isolated from differentiated C2C12 myotubes as previously described (Rubi et al. 2002). Briefly, the cells were harvested and then resuspended in buffer A (100 mM KCl, 5 mM MgSO4, 5 mM EDTA, 1 mM ATP, and 50 mM Tris-HCl, pH 7.4). Subsequently the cells were homogenized and centrifuged at 2,000 g at 4°C for 3 minutes. The resulting supernatant was collected and centrifuged again at 16,000 g for 15 minutes. Finally the pellet was resuspended in buffer B (220 mM sucrose, 70 mM mannitol, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4).

For palmitate and oleate oxidation assays, 150 µg of mitochondria and 50 µl of a 2.5 mM 5:1 palmitate or oleate-BSA complex containing 10 µCi/ml [1-14C] palmitate or oleate was incubated for 1 h at 37 ^{o}C in KRH buffer supplemented with 5 mM ATP, 1 mM NAD+, 0.5 mM l-carnitine, 0.1 mM CoA, 0.5 mM malate, and 25 µM cytochrome C in 12?24?96? well plates. The wells were covered with a piece of Whatman paper (Whatman, Florham Park, NJ) soaked in 3M KOH. 100 µl of 70% perchloric acid (Sigma) was injected into the wells and CO_2 was collected at room temperature. The filter paper was then dried and ^{14}C-radioactivity was determined.

2.2.11 qRT-PCR

Total RNA from cells was isolated using Trizol reagent (Invitrogen) according to manufacturer's protocol. cDNA was prepared from 1 µg of total RNA using a high capacity reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) and qRT-PCRs were performed using primers for CGI-58, PPAR-a, LPL, CD36, MGL, HSL, ATGL, FASN, FABP4, SCD1, HMGCR, LDL-r, ABCA1, CYP27A1, ABCG1, LXR-A, CPT1a, PPAR-a, SERBP1, GLUT4 and FATP1 and using Taqman Universal PCR master mix (Applied Biosystems) in triplicates. The relative expressions were calculated by the ddCt method using β-actin as the housekeeping gene.

3 Results

3.1 Olanzapine decreases lipolysis

The first step in triglyceride metabolism is the breakdown of TAG to DAG, regulated by ATGL, which represents the rate-limiting step in triglyceride breakdown. Olanzapine is known to impair lipolysis (Vestri et al. 2007) (Minet-Ringuet et al. 2007) (Albaugh et al. 2010). Using gonadal fat pads from C57BL6 *Wt* mice, $ATGL^{-/-}$ mice, and $HSL^{-/-}$ mice, we investigated olanzapine's effects on the enzyme activity of ATGL and HSL. H^3-labeled triolein was used as an artificial substrate to analyse the lipase activity. This analyses revealed that treatment of gonadal fat pads from C57BL6 *Wt* mice, $ATGL^{-/-}$ mice, and $HSL^{-/-}$ mice with various concentrations of olanzapine caused a dose-dependent decrease of total lipase activity (Figure 11). Treatment with 400 µM olanzapine decreased lipase activity in Wt WAT by 68% (+/- standard deviation). Olanzapine also inhibited lipase activity of HSL (Minet-Ringuet et al. 2007). In $ATGL^{-/-}$ mice the remaining TG hydrolase activity was reduced by 27% (+/- standard deviation) when treated with 50 µM and by 66% (+/- standard deviation) when treated with 400 µM olanzapine (Figure 11a). The hydrolase activity of $HSL^{-/-}$ mice was reduced by 27% (+/- standard deviation) when treated with 50 µM and by 60% (+/- standard deviation) when treated with 400 µM olanzapine (Figure 11a). We also treated the lysate of gonadal WAT pads from $ATGL^{-/-}$ mice with olanzapine to dissect the effects on HSL activity. The TG hydrolase activity from gonadal WAT pads of $ATGL^{-/-}$ mice was reduced by 29% (+/- standard deviation) when treated with 50 µM and 56% (+/- standard deviation) when treated with 400 µM olanzapine. Interestingly, at high levels of olanzapine (400 µM), CGI-58, a coactivator of ATGL (Yamaguchi 2010) failed to increase the lipolytic activity in WAT of Wt and $Hsl^{-/-}$ mice (Figure 11b).

We next investigated lipolysis under basal and stimulated conditions by using forskolin or ethanol as a control and evaluated the effects of various concentrations of olanzapine on WAT lipolysis by measuring the FFA and glycerol release. By treatment with 10 µM forskolin both FFA and glycerol release were increased in WAT pads of all three genotypes (Figure 7&9). When treated with various concentrations of olanzapine in stimulated and basal conditions FFA and glycerol release from WAT of *Wt, ATGL*$^{-/-}$ and *HSL*$^{-/-}$ mice was decreased (Figure 7&9). These results confirm that olanzapine decreases lipolysis by blocking lipase activity of ATGL and HSL (Figure 7&9). Subsequently, we assessed the effects of olanzapine on LPL and MGL lipase activity. While olanzapine significantly decreased the activity of MGL by 31% when treated with 400 µM (+/- standard deviation) (Figure 13a) the activity of LPL when treated with olanzapine showed no significant alteration (Figure 14b). As a second model we used differentiated 3T3-L1 adipocytes. To investigate cytotoxicity of olanzapine on 3T3-L1 cells we used the MTT (Berridge, Tan 1993). Cells were incubated, with various concentrations of olanzapine and cytotoxicity was assessed after 50 hrs (Figure 6). Even with a dose of 400 µM olanzapine we couldn't identify any significant toxicity of olanzapine on 3T3-L1 cells. Similar to the experiments with murine WAT, we investigated the decrease of lipolysis in 3T3-L1 cells with and without recombinant CGI-58 (Figure 8&10). Lipolysis in HSL inhibited (HSL inhibitor 76-0097) 3T3-L1 cells was reduced by 28% (+/- standard deviation) when treated with 50 µM and by 85% (+/- standard deviation) when treated with 400 µM olanzapine. By using stable HSL knockdown 3T3-L1 cells lipolysis was reduced by 22% (+/- standard deviation) when treated with 50 µM and by 85% (+/- standard deviation) when treated with 400 µM olanzapine (Figure 12). When treated with 400 µM olanzapine, CGI-58 failed to increase lipolysis in 3T3-L1 cells.

Basal glycerol release from 3T3-L1 adipocytes was reduced by 42% when treated with 50 µM olanzapine and by using the HSL inhibitor or shRNA to knock-down HSL glycerol release was reduced by 47% (+/- standard deviation) and 41% (+/- standard deviation) and by 78% total, 88% using the HSL inhibitor and 91% using shRNA to knock-down HSL (+/- standard deviation), respectively when treated with 400 µM olanzapine. Basal FFA release was reduced by 21% total, 37% using the HSL inhibitor and 33% using shRNA to knock-down HSL (+/- standard deviation) when treated with 50 µM and by 41% total, 84% using the HSL inhibitor and 74% using shRNA to knock-down HSL (+/- standard deviation) when treated with 400 µM olanzapine. Forskolin stimulated glycerol release from 3T3-L1 adipocytes was reduced by 33% total, 19% using the HSL inhibitor and 28% using shRNA to knock-down HSL (+/- standard deviation) when treated with 50 µM and by 69% total, 48% using the HSL inhibitor and 65% using shRNA to knock-down HSL (+/- standard deviation) when treated with 400 µM olanzapine, forskolin stimulated FFA release was reduced by 20% total, 40% using the HSL inhibitor and 38% using shRNA to knock-down HSL (+/- standard deviation) when treated with 50 µM and by 53% total, 91% using the HSL inhibitor and 87% using shRNA to knock-down HSL (+/- standard deviation) when treated with 400 µM olanzapine (Figure 8&10).

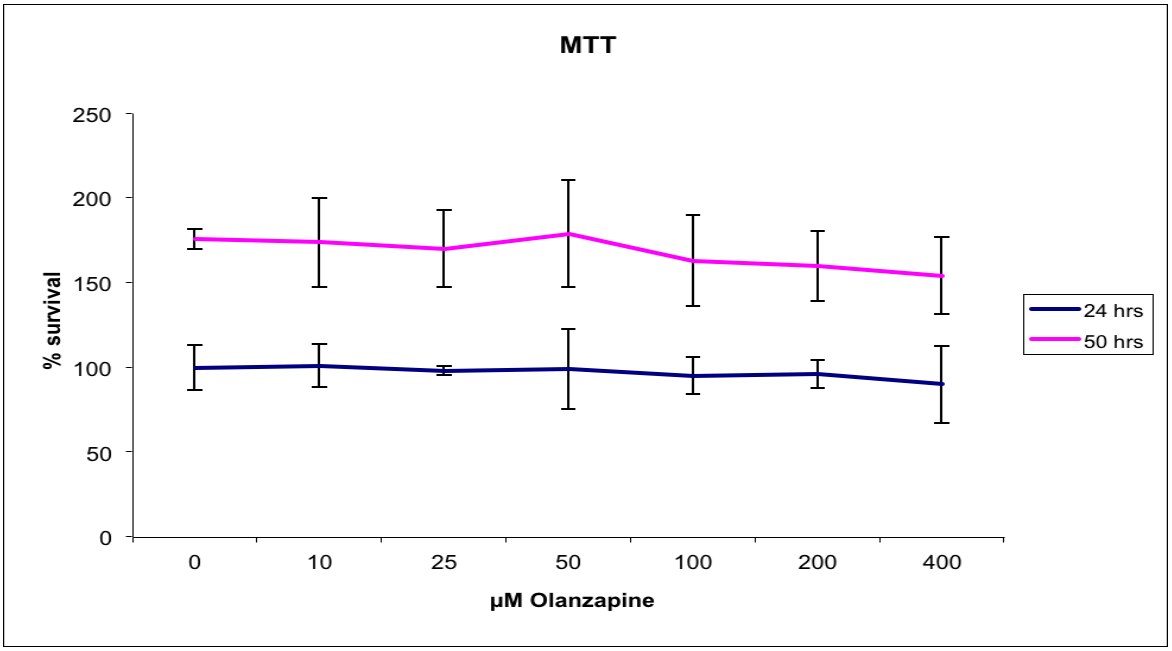
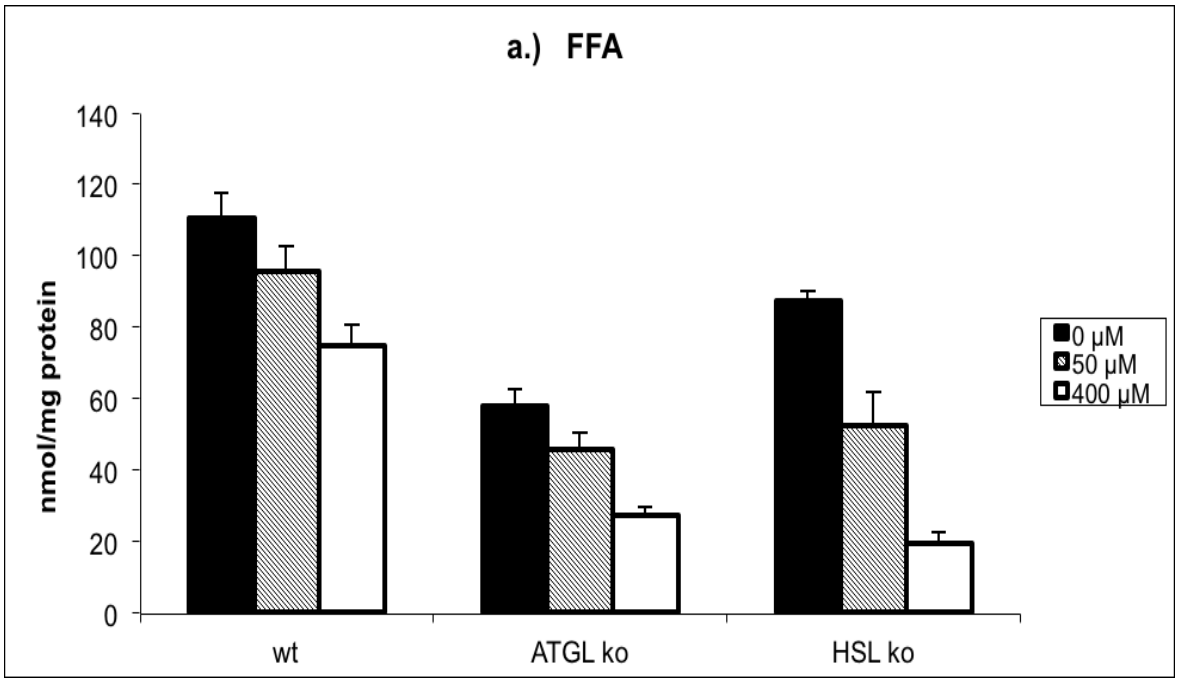


Figure 6 3T3-L1 toxicity assay. Cytotoxicity of various Olanzapine concentrations on 3T3-L1 adipocytes was assessed by using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT test). Olanzapine concentrations up to 400 µM showed no cytotoxic effects conferred on 3T3-L1 adipocytes.

3.1.1 Effect of olanzapine on basal and stimulated lipolysis



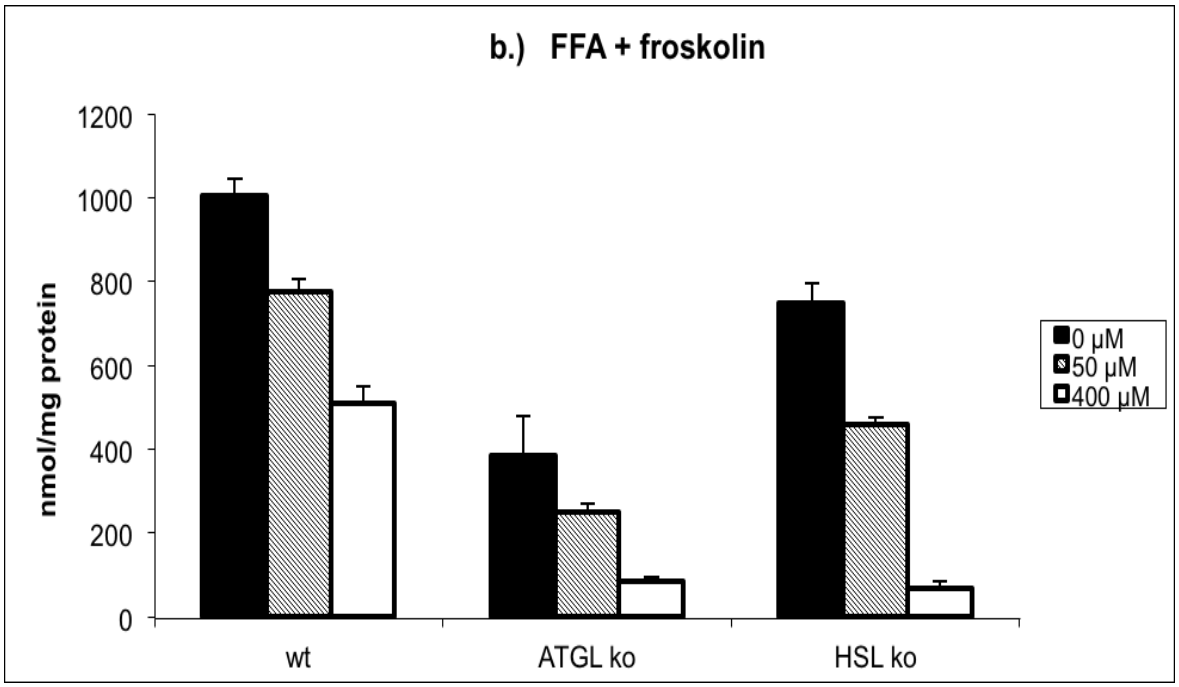
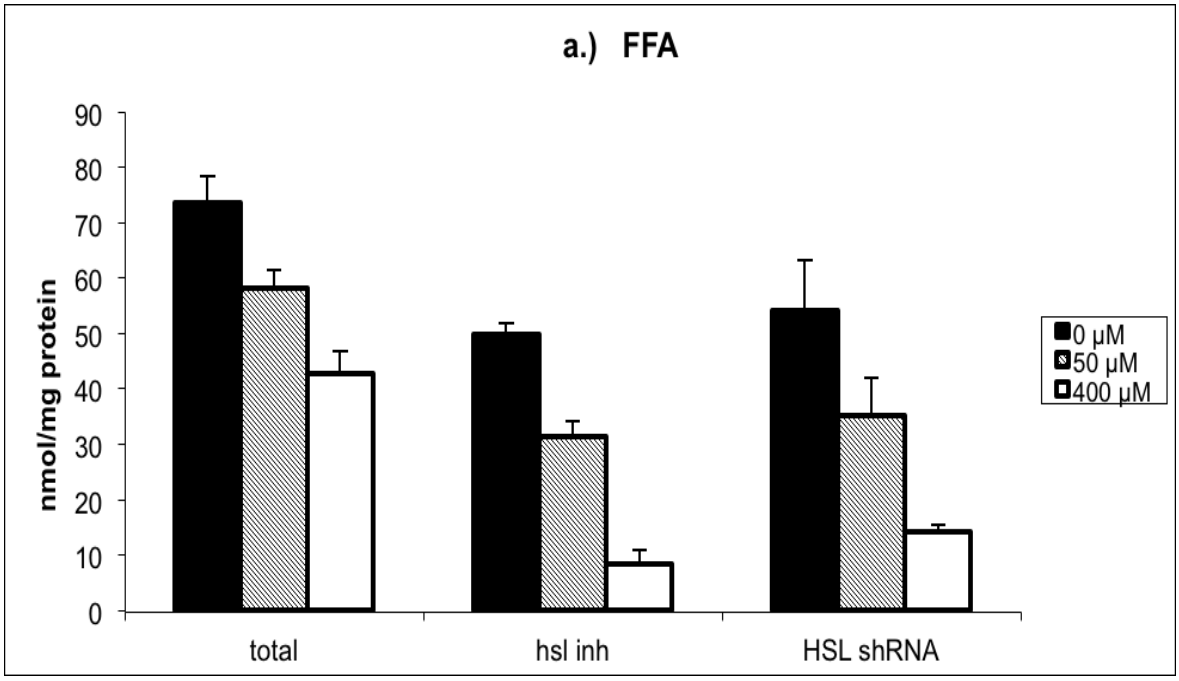


Figure 7 (a, b) Basal (a) and froskolin stimulated (b) Free fatty acid (FFA) release from gonadal white adipose tissue (WAT) of Wt (C57BL6), Atgl-/- and Hsl-/- mice measured after treatment with various concentration of olanzapine. Olanzapine decreases the FFA release from WAT in an dose-dependent manner under stimulated and non-stimulated conditions.



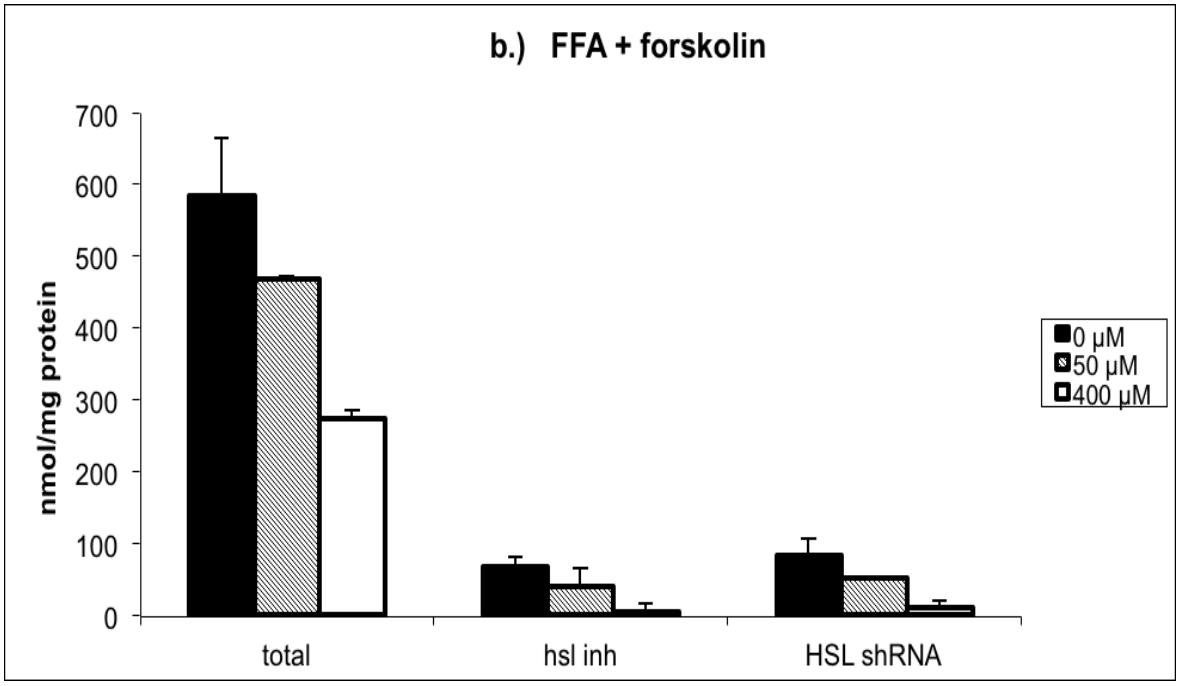


Figure 8 (a, b) 3T3-L1 cells were differentiated to adipocytes and basal (a) as well as froskolin stimulated (b) FFA release was measured after treatment of various concentrations of olanzapine or DMSO as control. HSL was stably downregulated in 3T3-L1 adipocytes or HSL inhibitor (76-0097) was used to examine the effect of olanzapine on ATGL mediated lipolysis. Olanzapine decreases basal and forskolin stimulated FFA release in a dose dependent manner in 3T3-L1 adipocytes, 3T3-L1 adipocytes treated with a HSL inhibitor and even if HSL was knocked-down by a shRNA approach.

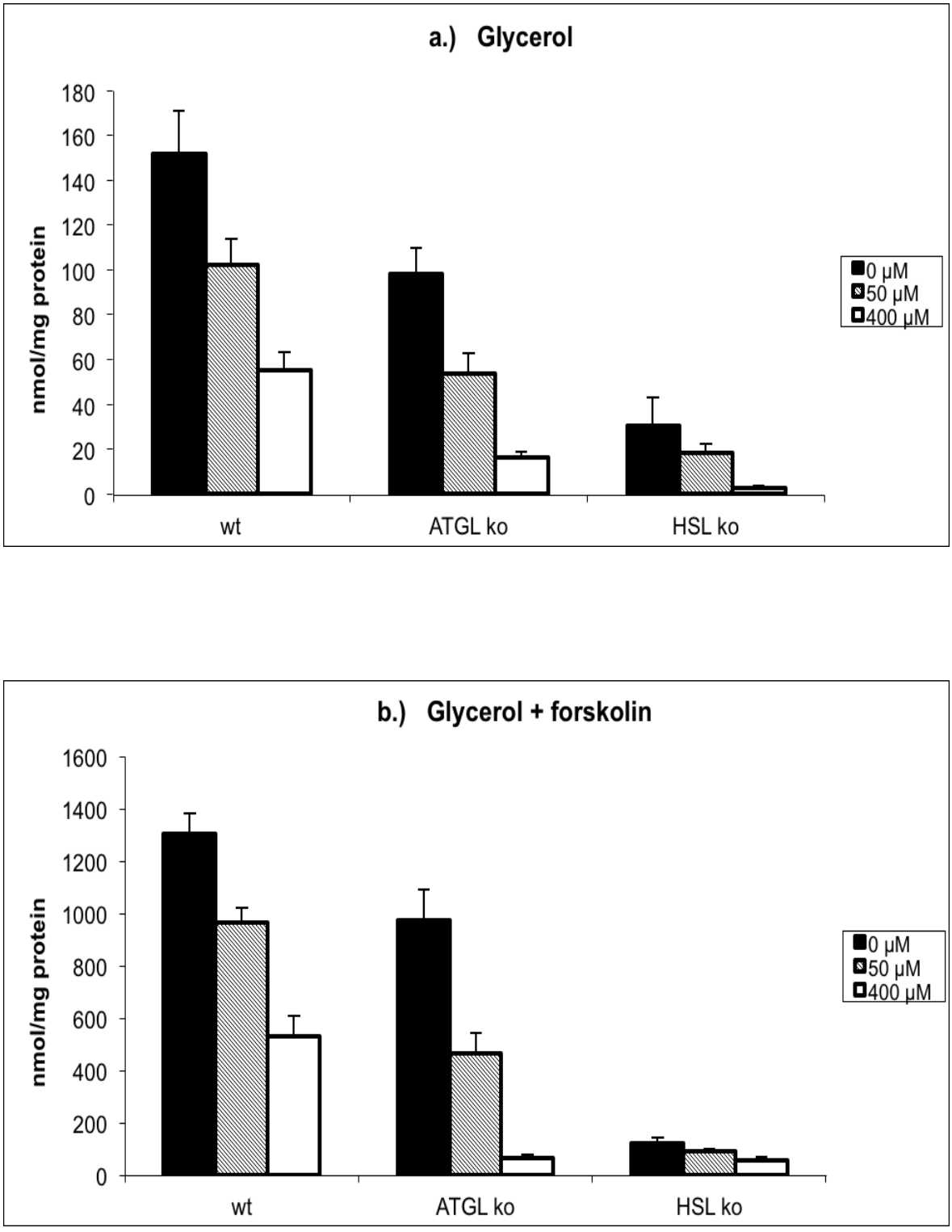
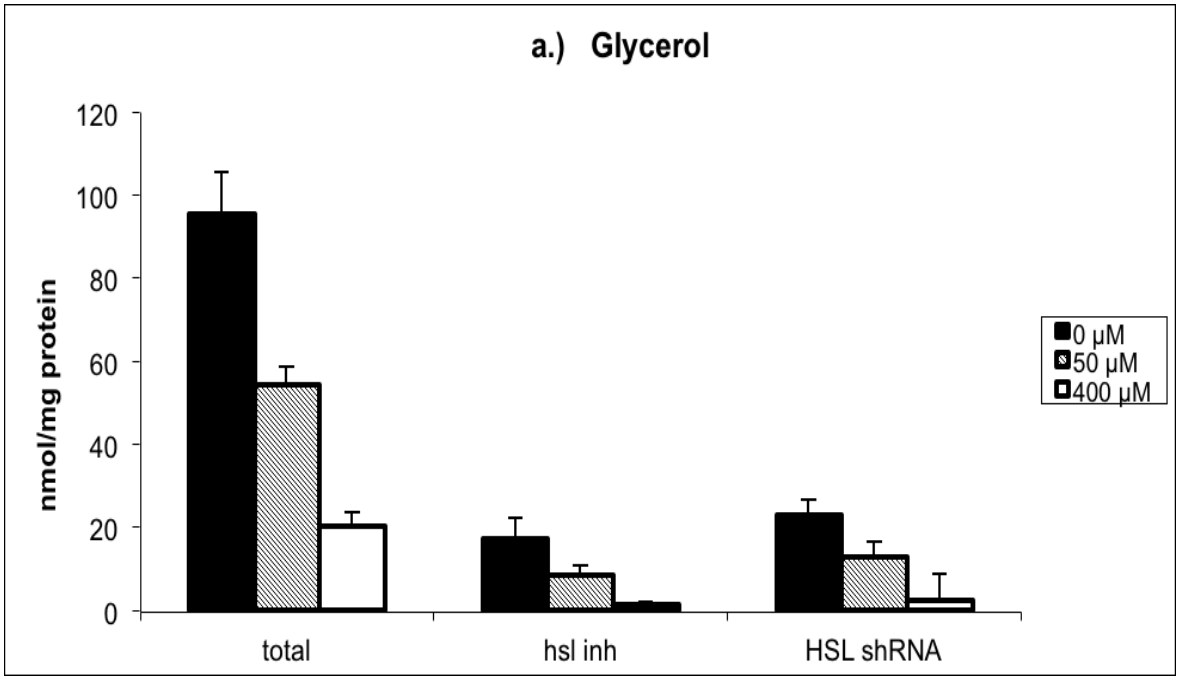


Figure 9 (a, b) Glycerol release was quantified as an indicator of lipolysis from basal (a) or forskolin stimulated (b) gonadal WAT explants of Wt, Atgl-/- and Hsl-/- mice after treatment with various concentration of olanzapine or DMSO as control. Olanzapine decreases the glycerol release in a dose-dependent fashion.

3T3-L1



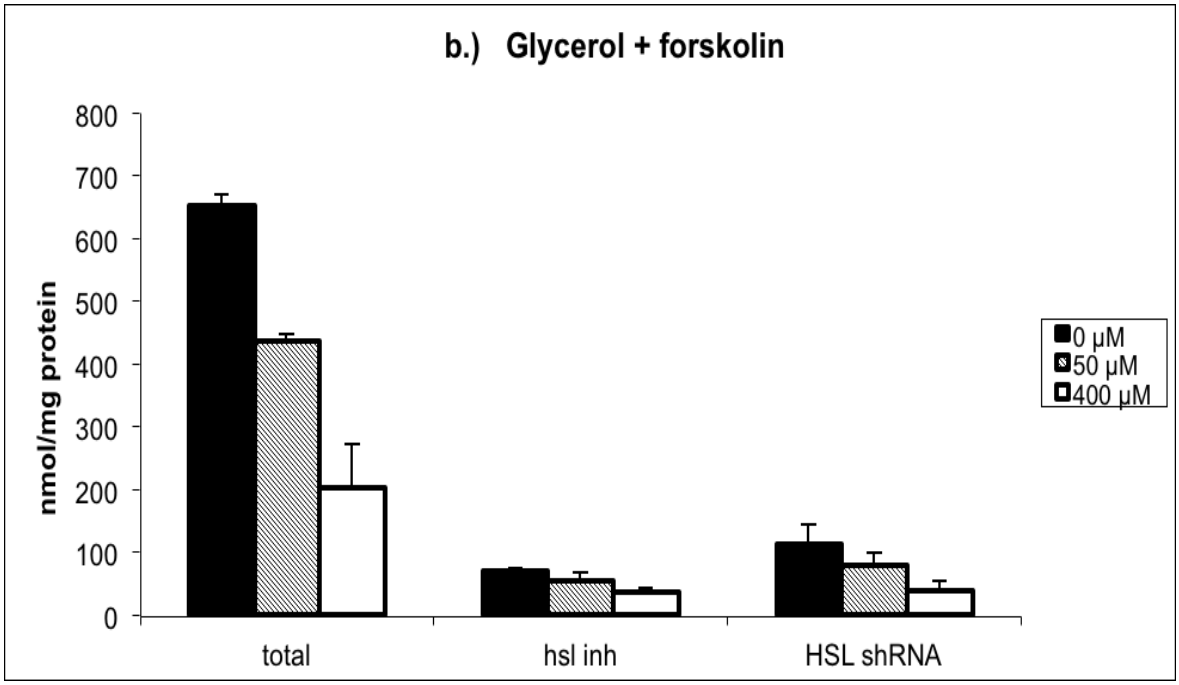


Figure 10 (a, b) Basal (a) and forskolin stimulated (b) glycerol release was measured from olanzapine or DMSO treated normal and HSL inhibited/downregulated 3T3-L1 adipocytes. Olanzapine decreases basal and forskolin stimulated glycerol release in a dose dependent manner in 3T3-L1 adipocytes, 3T3-L1 adipocytes treated with a HSL inhibitor and even if HSL was knocked-down by a shRNA approach.

Effect of olanzapine on triglyceride hydrolase activity with and without CGI-58

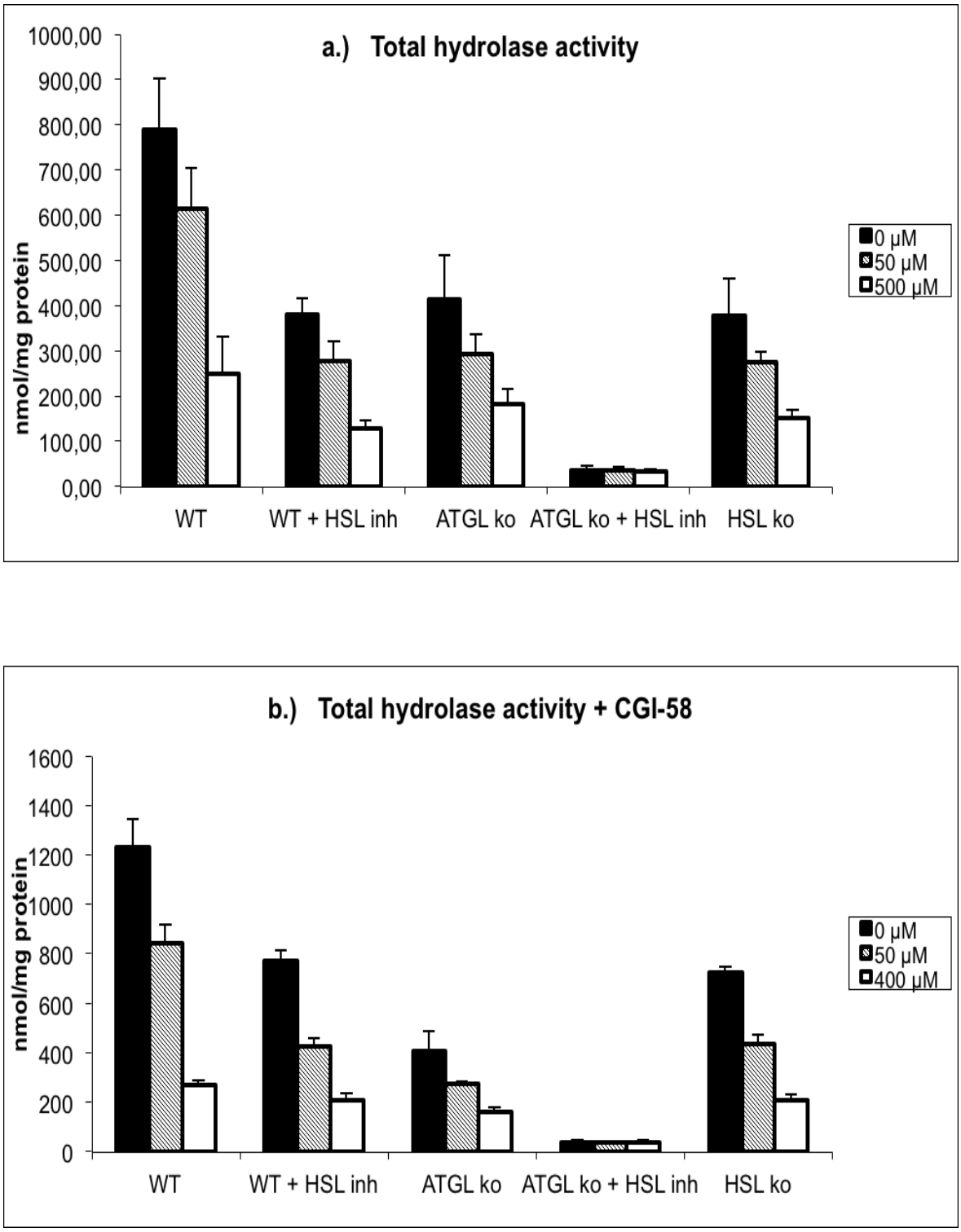
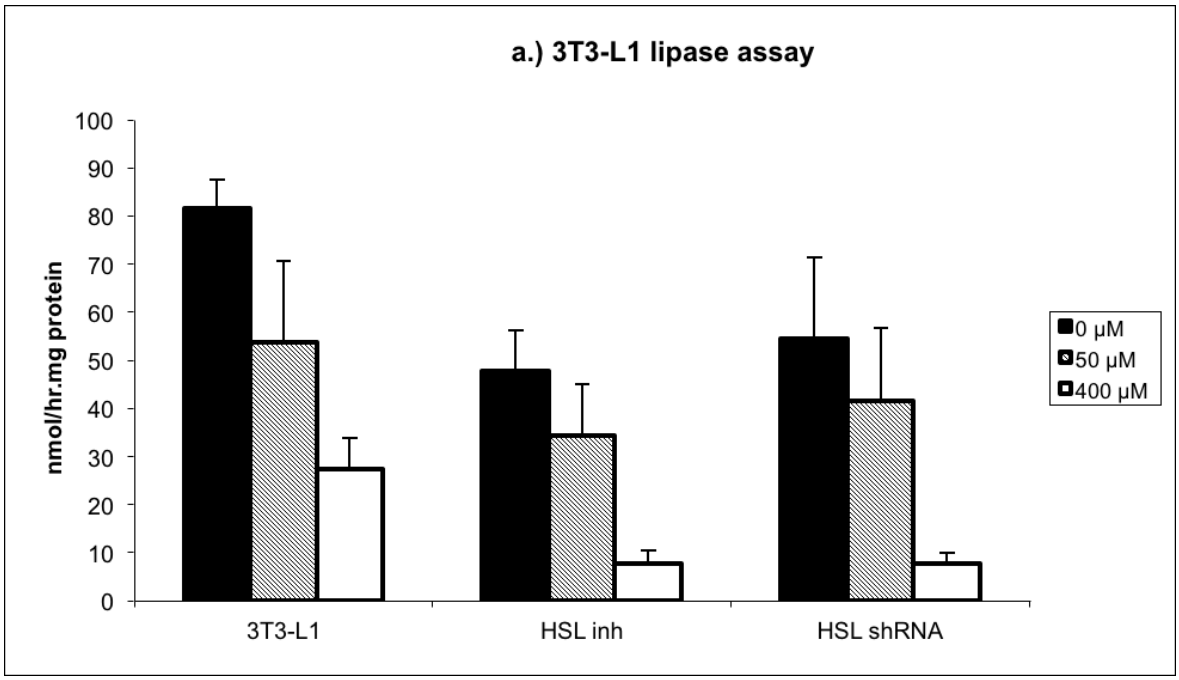


Figure 11 a) Total TG hydrolase activity measured from fat pads of Wt mice, mice treated with a HSL inhibitor (+76-0079), ATGL-/- mice with and withouth a HSL inhibitor and HSL-/- mice. TG hydrolase activity was decreased in a dose dependent manner when incubated with various concentration of olanzapine (0 µM, 50 µM and 400 µM). Total TG hydrolase activity in protein lysates of Atgl-/- (mostly HSL activity) and Hsl-/- (mostly ATGL activity) mice was remarkably reduced when treated with olanzapine. (b) Addition of CGI-58 recombinant protein increased total, HSL inhibited (+76-0079) TG hydrolase activity in WAT lysate of Wt mice. Nevertheless, olanzapine treatment blunts the activating action of CGI-58. Olanzapine treatment also reduced total TG hydrolase activity in protein lysates of Atgl-/- (mostly HSL activity) and Hsl-/- (mostly ATGL activity) mice when incubated with CGI-58.



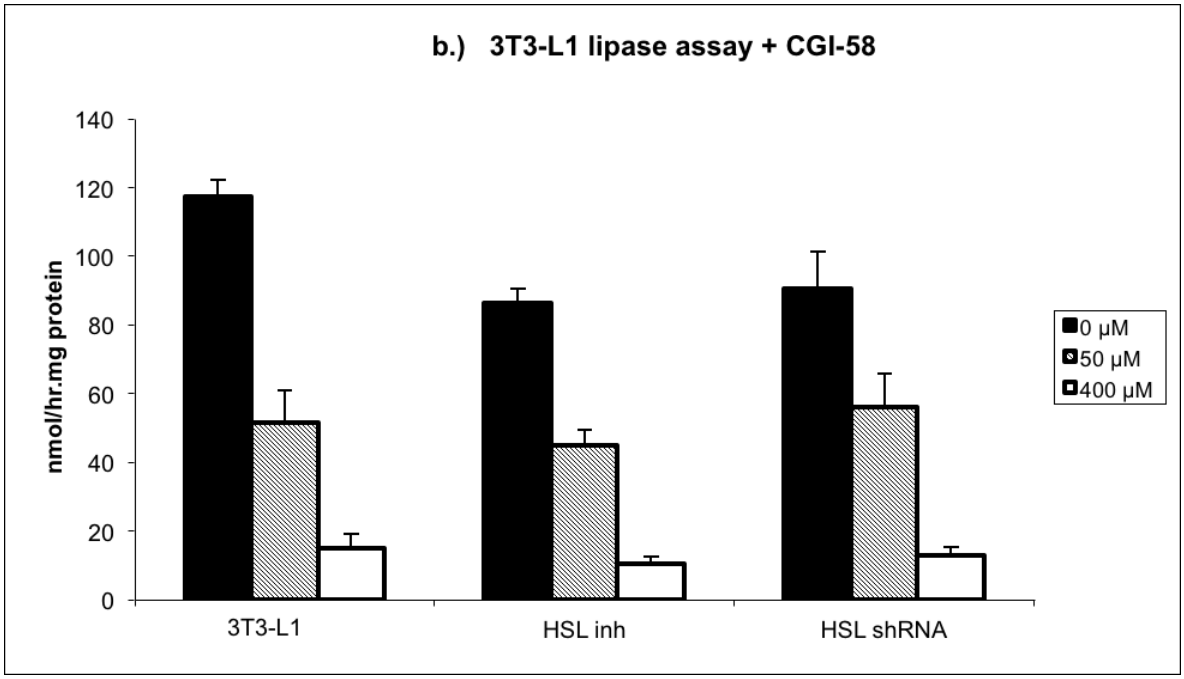


Figure 12 (a, b) Olanzapine (0 µM, 50 µM and 400 µM) treatment reduces basal (a) and CGI-58 stimulated (b) total TG hydrolase activity in lysates from 3T3-L1 adipocytes, 3T3-L1 adipocytes treated with a HSL inibitor and 3T3-L1 adipocytes subjected to gene knockdown with a HSL specific shRNA.

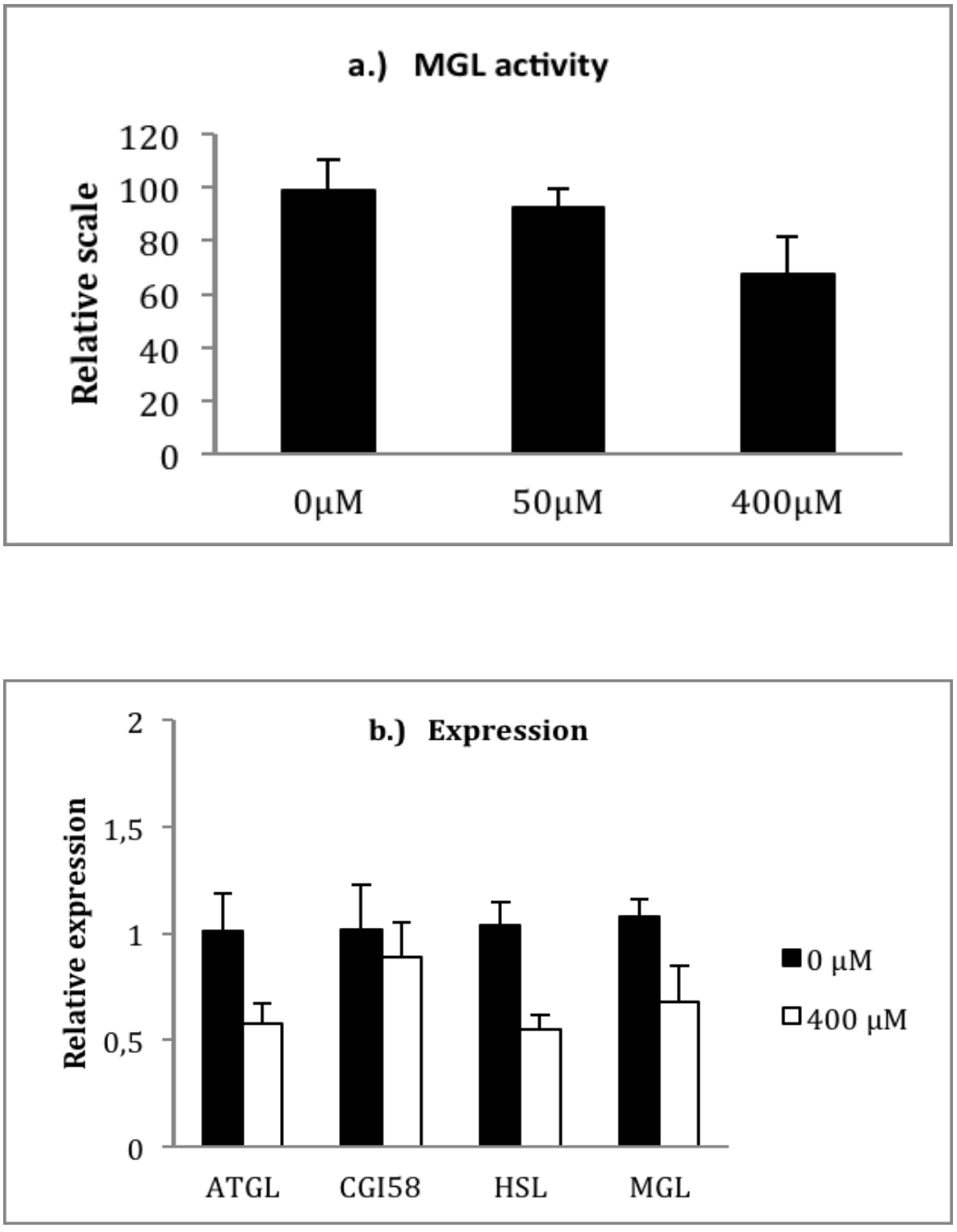


Figure 13 (a) Olanzapine treatment reduced MGL activity in WAT lysates from Wt mice compared to DMSO (control) treatment. (b) ATGL, HSL and MGL expression is significantly reduced at mRNA level with treatment of 400 μM olanzapine compared to DMSO treated 3T3-L1 adipocytes. No significant differences could be observed on CGI-58 expression at mRNA level after olanzapine treatment.

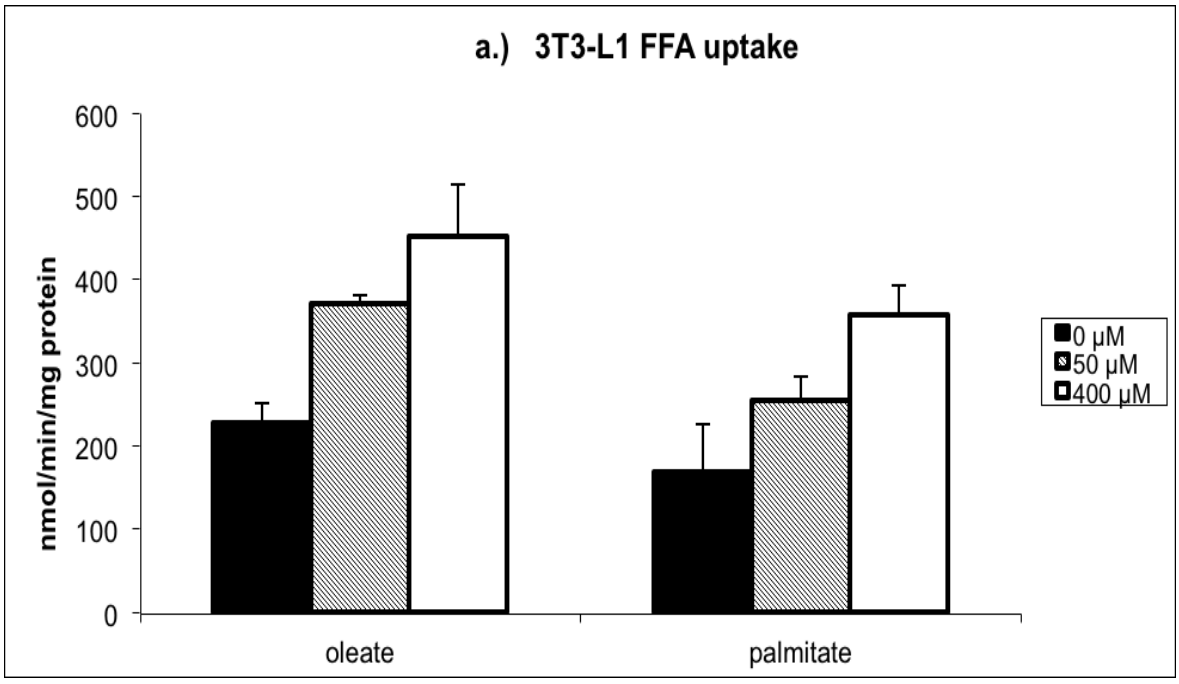
3.2 Olanzapine increases FFA uptake and TG accumulation

Our analyses have shown that lipase activity of both ATGL and HSL are strongly influenced by olanzapine. We next asked whether the inhibition of these lipases can cause accumulation of TG in cells, which could explain the reported fat accumulation in murine models. Indeed, olanzapine increased the TG concentration in 3T3-L1 cells. Total TG content of in 3T3-L1 cells was increased 2 fold when cells were treated with 50 µM olanzapine for 48 hours. 400 µM olanzapine increased TG contend 3 fold (Figure 15b). Olanzapine treatment also increased the uptake of saturated and unsaturated FFA into cells. The uptake of C^{14} labelled oleate was increased by 1.6 fold when treated with 50 µM olanzapine and 2 fold when treated with 400 µM olanzapine. The C^{14} palmitate uptake was also elevated by 1.5 fold when treated with 50 µM olanzapine and 2.1 fold when treated with 400 µM olanzapine (Figure 14a).

3.3 Olanzapine treatment transforms the transcriptome of fat cells

We next investigated the effect of olanzapine on the expression of genes, which play an important role in lipid metabolism. Therefore, we treated 3T3-L1 cells with either 0 µM or 400 µM olanzapine for about 30 hours and evaluated the mRNA levels of selected genes by qRT-PCR. This analysis revealed that olanzapine downregulates the expression of ATGL by 25%, HSL by 50% and MGL by 25% when treated with 400 µM (+/- standard deviation). LPL expression was not influenced (Figure 13a & b, 14b, 15a). No significant differences could be observed on CGI-58 expression, an activator of ATGL, at mRNA level after olanzapine treatment. FASN and CD36 two important FFA transporters showed an increased expression of about 2 fold when treated with 400 µM olanzapine. Corresponding to our findings of TG accumulation in cells, the fatty acid binding proteins, FABP4 and SCD-1 were upregulated by olanzapine. mRNA levels of PPAR-γ, a nuclear transcription factor important for regulation genes involved in lipid uptake and adipogenesis, was also upregulated 1.7 fold when treated with olanzapine (Figure 15 a & c).

3.3.1 FFA uptake, *de novo* lipogenesis and TG accumulation



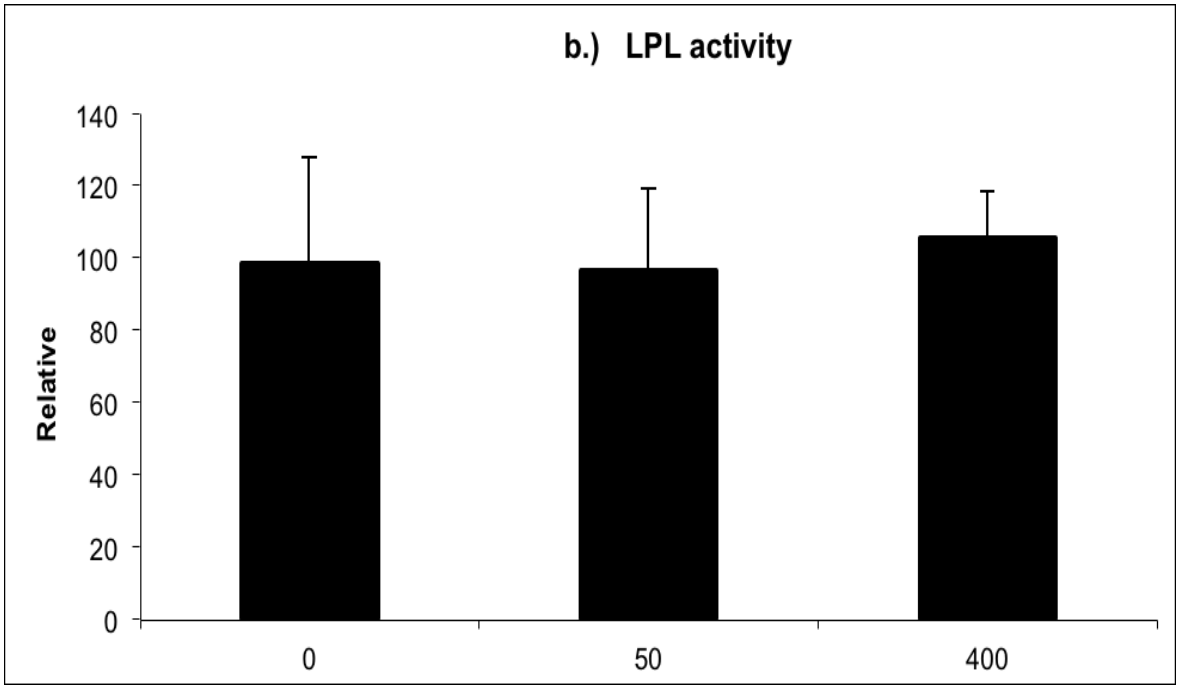
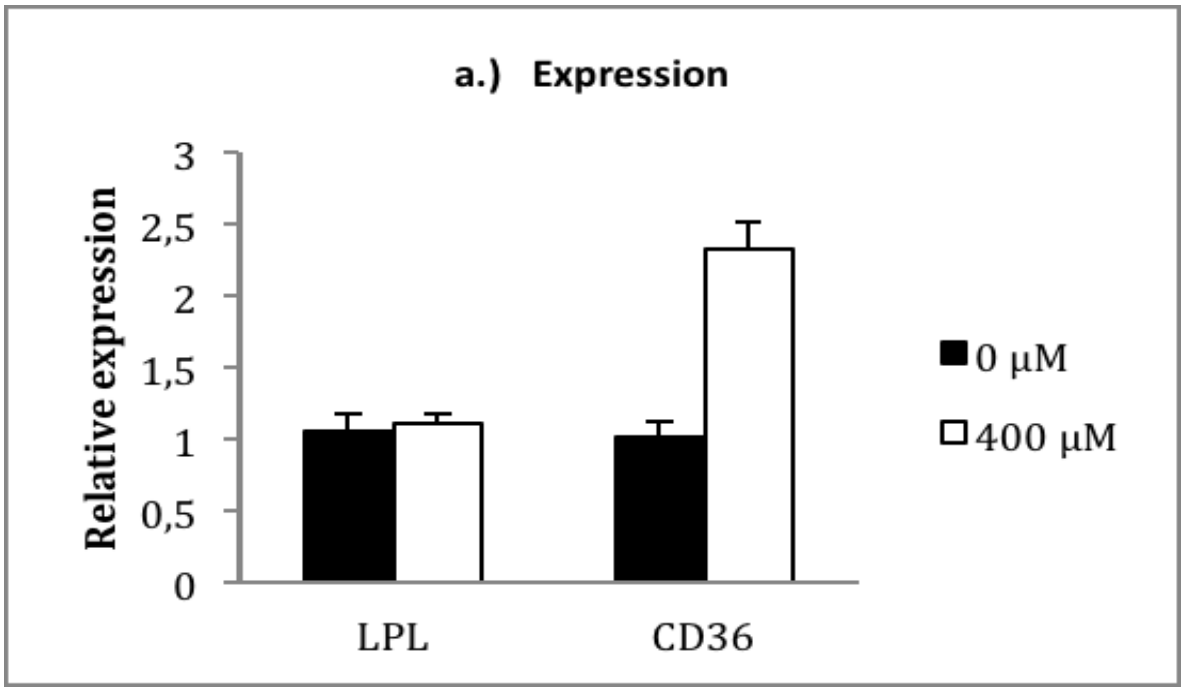
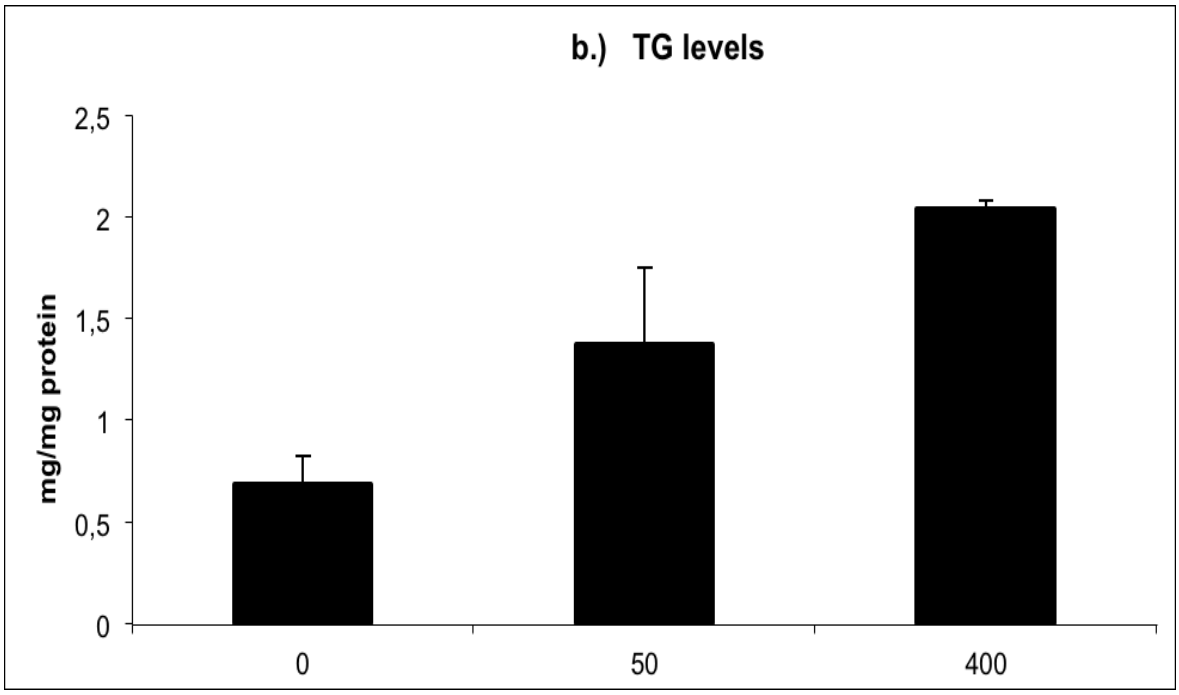


Figure 14 (a) Olanzapine treatment enhanced the uptake of unsaturated (oleic acid) and saturated (palmitic acid) fatty acids in a dose dependent manner in 3T3-L1 adipocytes. (b) LPL activity remained unchanged with treatment of 50 µM or 400 µM olanzapine.


a.) Expression
Relative expression
3
2,5
2
1,5
1
0,5
0
LPL
CD36
0 µM
400 µM


b.) TG levels
mg/mg protein
2,5
2
1,5
1
0,5
0
0
50
400

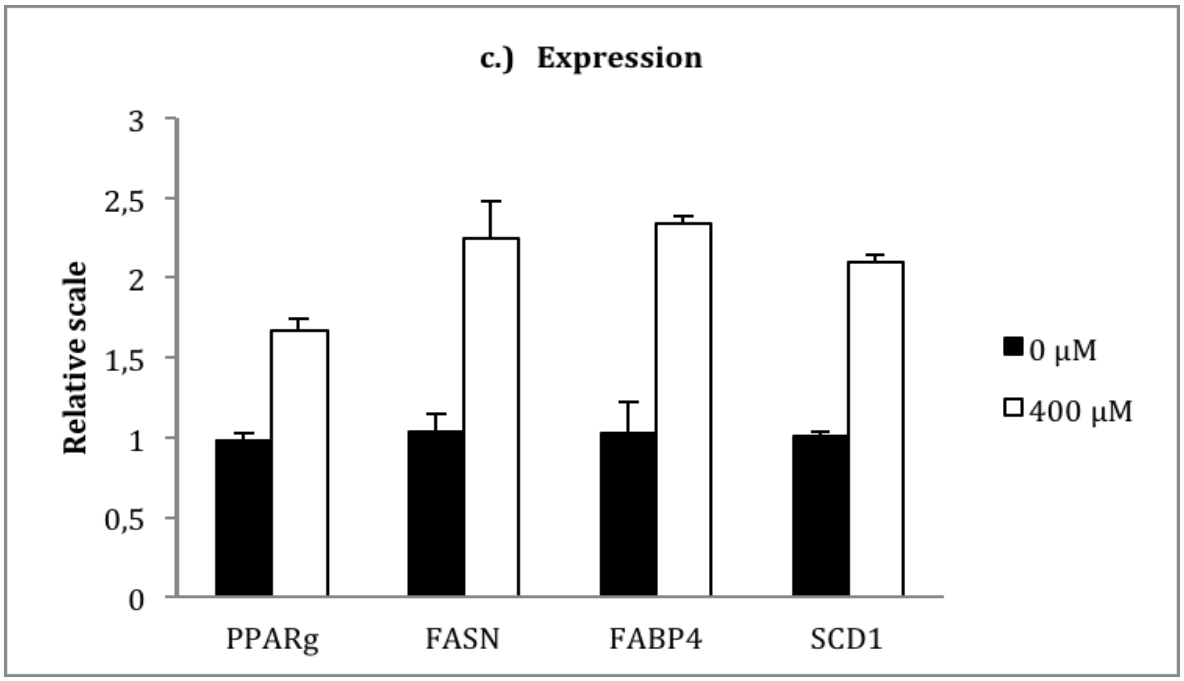
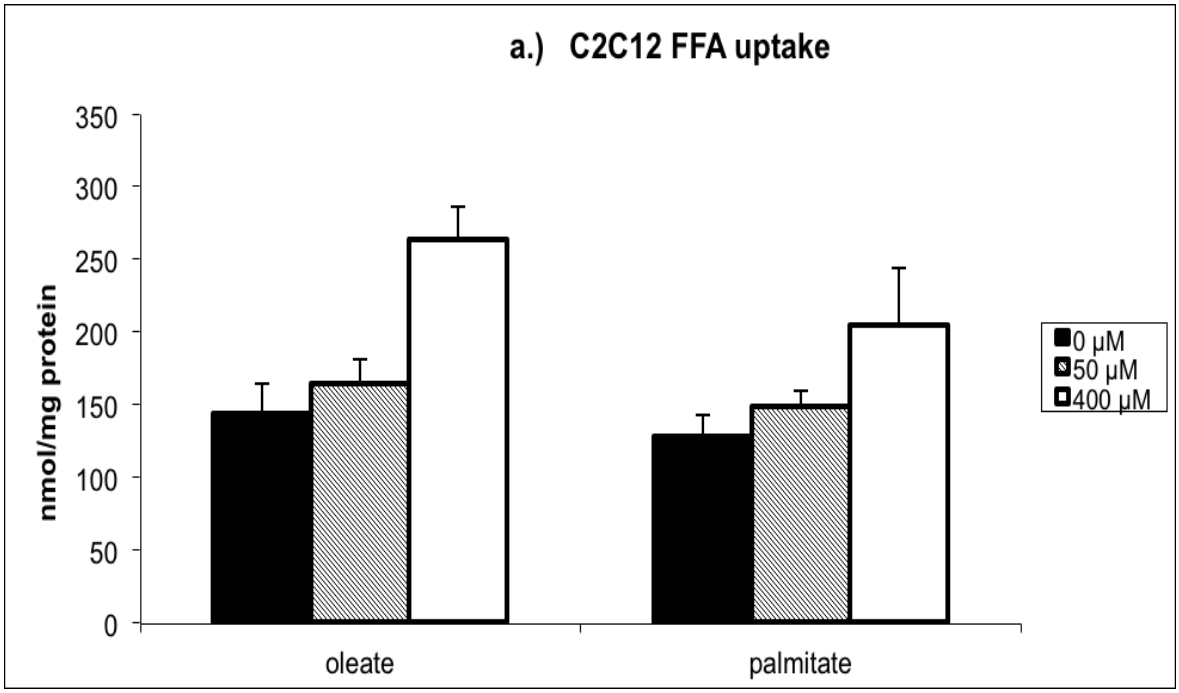


Figure 15 (a) *LPL* expression is unchanged at mRNA level whereas *CD36* expression is significantly increased at mRNA level when treated with 400 µM olanzapine (b) Olanzapine treatment at 50 µM and 400 µM concentration significantly increased TG levels in 3T3-L1 adipocytes. (d) Expression of *PPAR-gamma*, *FASN, FABP4* and *SCD1* is induced significantly in 3T3-L1 cells when treated with olanzapine.

3.4 Effect of olanzapine on C2C12 myotubes

Besides adipose tissue, muscle tissue is also important in the metabolism of lipids (Albaugh et al. 2011). Therefore, we used C2C12 myotubes and studied their behavior in lipid and glucose metabolism while they were treated with olanzapine. Treatment of C2C12 myotubes with 400 µM olanzapine increased oleate uptake 1.8 fold and palmitate uptake 1.6 fold, a finding previously observed in 3T3-L1 adipocytes (Figure 16a). The mRNA expression of the fatty acid transporter FATP1 was increased by 1.8 fold in C2C12 myotubes as seen in figure 16b. Under high concentrations of olanzapine (400 µM) the mitochondrial ß-oxidation of both saturated and unsaturated free fatty acids was reduced by 67% measured with palmitate and 67% measured with oleate (Figure 17a). Glucose uptake was increased under olanzapine treatment in a dose-dependent manner in C2C12 myocytes. Interestingly high concentrations of olanzapine (400 µM) blunt glucose uptake in C2C12 myotubes stimulated with 100 nM insulin (Figure 17b). In addition, 400 µM of olanzapine increased the expression of the glucose importer, Glut4 1.4 fold (Figure 16b).

3.4.1 Olanzapine increases FFA uptake, blunts insulin induced glucose uptake and FFA oxidation in C2C12 myotubes



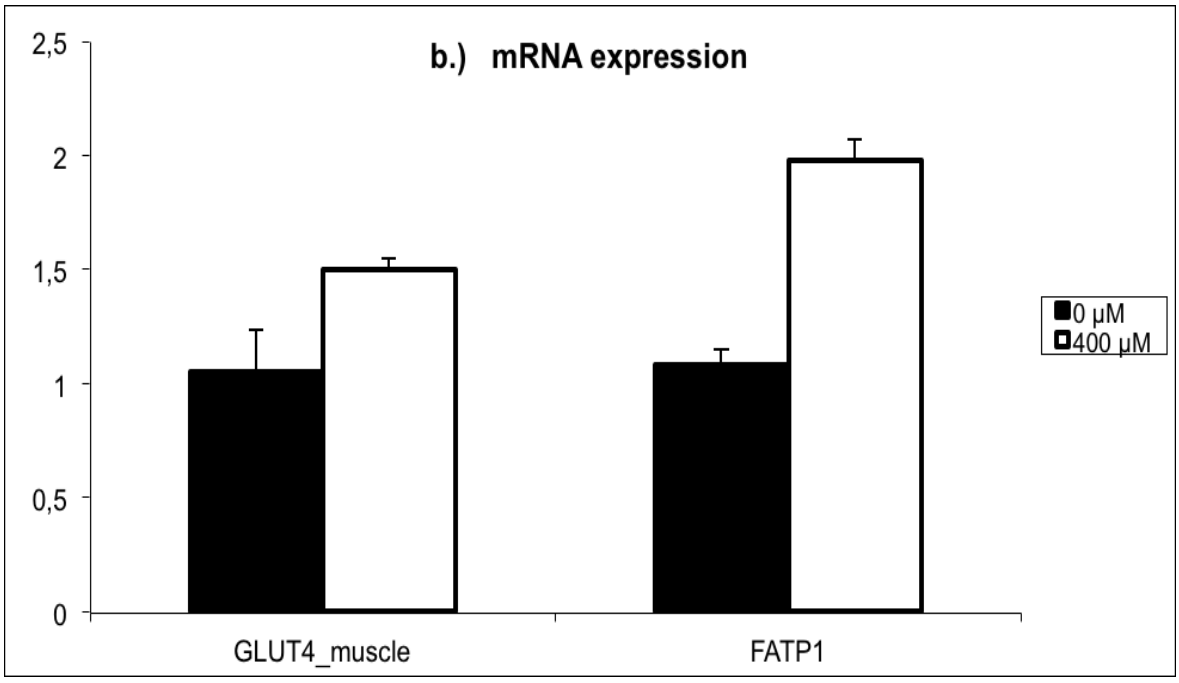


Figure 16 (a) oleic acid and palmitic acid uptake is increased under olanzapine treatment. (d) 400 µM olanzapine increases mRNA expression of *Glut4* and *Fatp1*.

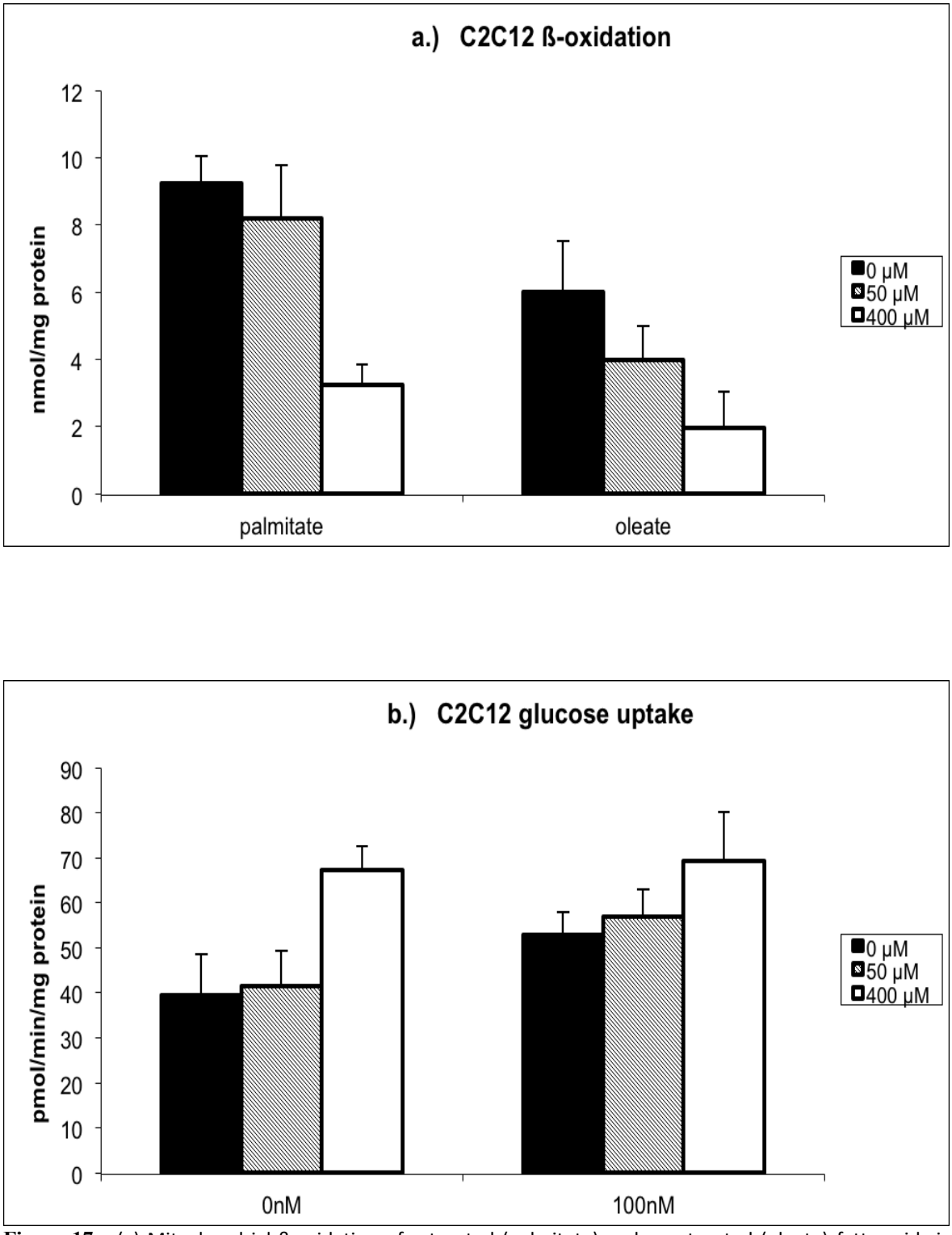


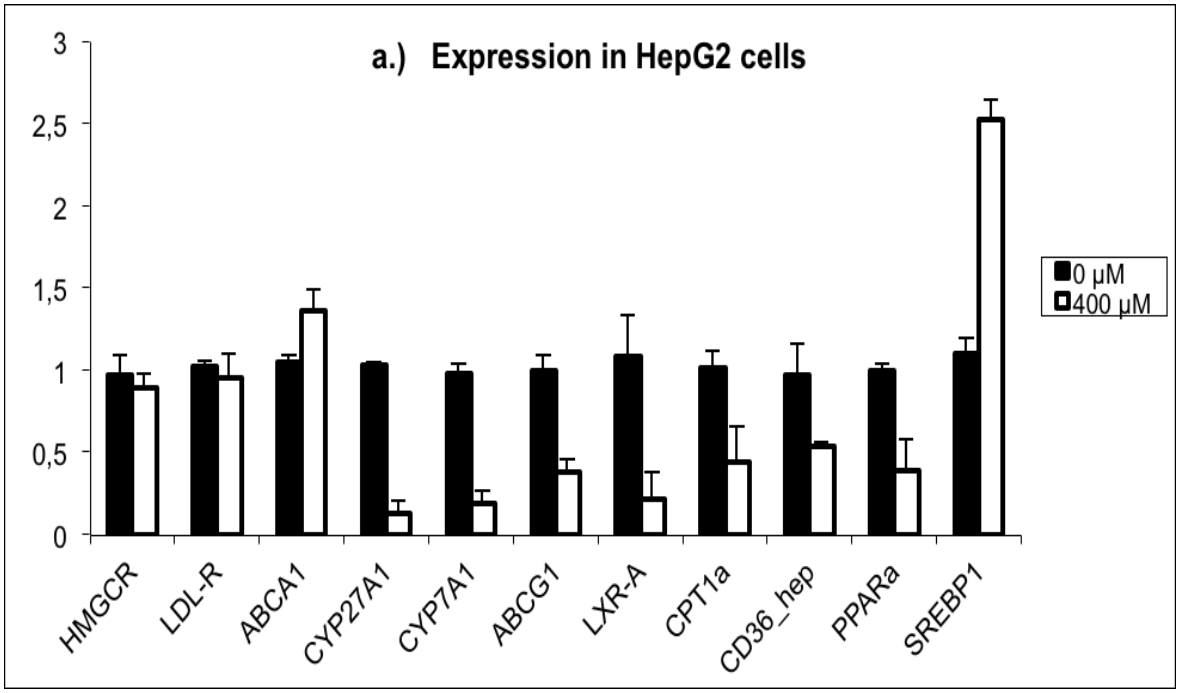
Figure 17 (a) Mitochondrial ß-oxidation of saturated (palmitate) and unsaturated (oleate) fatty acids is hampered after treatment with olanzapine. (b) Olanzapine treatment increases basal glucose uptake, however blunts insulin induced (100 nM) glucose uptake in C2C12 myotubes.

3.5 Effects of olanzapine on liver cells.

One of the most important organs in metabolism is the liver. Due to this vital role, many side effects of drugs arise in the liver and harm its functionality. Therefore we investigated the effect of olanzapine on liver cells. We treated HepG2 cells with either 0 or 400 µM of olanzapine for about 30h and measured cholesterol and FFA metabolism. Moreover, qRT-PCR was used to investigate the effect on gene expression. Treatment with 400 µM olanzapine reduced the expression of CYP7A1 by 81%, of CYP27A1 by 88%, of ABCG1 by 62% and of LXR- by 81% whereas the expression of SREBP1 was increased by 56% compared to untreated conditions (Figure 18a). No significant difference in the expression of HMGCR, LDL-r and ABCA1 could be observed (Figure 18a).

CPT-1 α expression was significantly decreased by 58% and PPAR-α expression was significantly decreased by 63% under treatment with 400 µM olanzapine, which possibly results in a decreased mitochondrial FFA import and leads to an affected ß-oxidation. Comparable to the findings in 3T3-L1 cells, CD36 expression was decreased 1.8 fold in HepG2 cells when treated with 400 µM olanzapine. Olanzapine treatment also resulted in cholesterol accumulation. Primary murine hepatocytes isolated from C57BL6 mice were treated with 50 µM and 400 µM olanzapine for 30 hrs, respectively, and showed an 1.2 fold increase of the cellular cholesterol content if treated with with 50 µM olanzapine and an 1.7 fold increase of the cellular cholesterol content if treated with 400 µM of olanzapine (Figure 18b).

3.5.1 Effect of olanzapine on the expression of genes important for lipid metabolism in HepG2 cells. Olanzapine induced intracellular cholesterol accumulation.



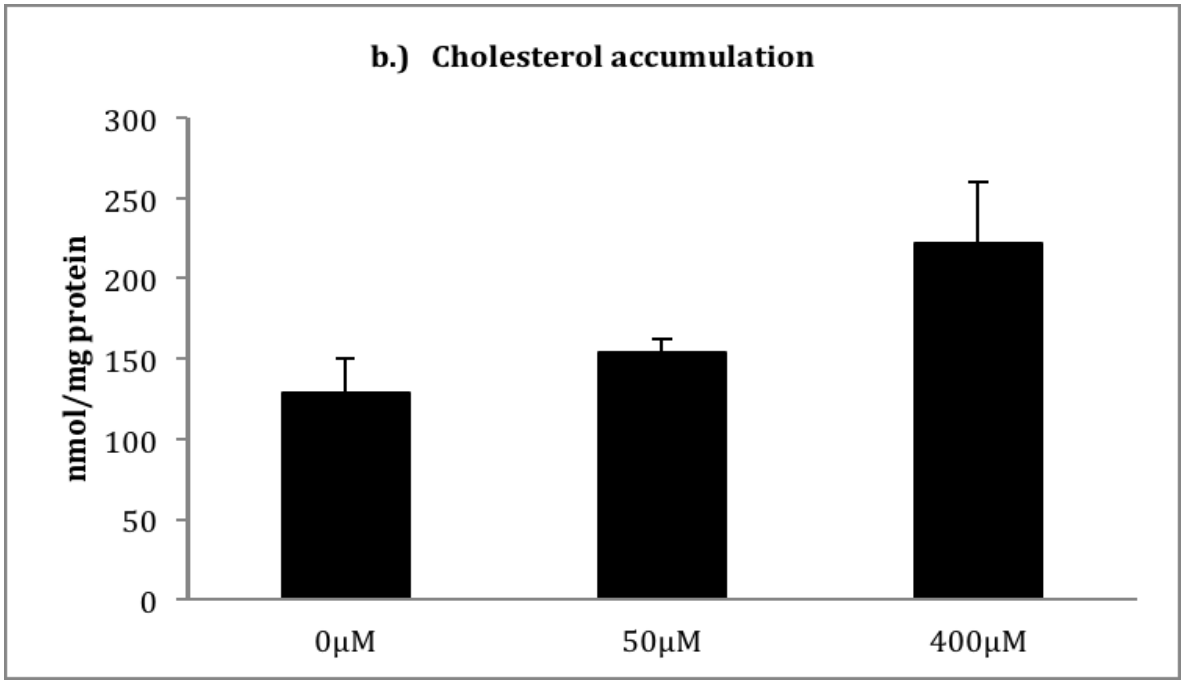


Figure 18 (a) Olanzapine increased mRNA expression of SREBP1 and ABCA1 whereas decreased expression of CYP7a1, CYP27a1, ABCG1, LXR-a, CPT1a, CD36 and PPAR-alpha. HMGCoA-r and LDL-r did not show a significant changed mRNA level when treated with 400 µM olanzapine compared to DMSO (control) in HepG2 cells. (b) Olanzapine treatment caused intracellular cholesterol accumulation in primary murine hepatocytes.

4 Discussion

The treatment of schizophrenia and bipolar I disorders relies heavily on olanzapine, one of the most prescribed SGA's nowadays, although it is known to cause metabolic de-regulation, obesity and diabetes. Earlier findings, such as lipid accumulation in adipocytes, decreased lipolysis, blunted insulin sensitivity and increased intra-cellular cholesterol can be confirmed by our results. (Vestri et al. 2007) (Minet-Ringuet et al. 2007) (Albaugh et al. 2010) (Wirshing et al. 2002) To investigate the underlying mechanism on a basic level we tried to identify the molecular pathways affected by olanzapine. The novelty lies in the fact that we investigated olanzapine's effects on the whole TAG degradation process, including the rate-limiting enzyme of lipolysis, ATGL, which was discovered in 2004 (Zimmermann et al. 2009).

4.1 Olanzapine decreases lipolysis

Our results support previous findings, stating that olanzapine decreases total lipase activity in a dose-dependent manner. (Figure 7 to 12) (Albaugh et al. 2010) (Vestri et al. 2007, Minet-Ringuet et al. 2007) Our findings replenish the field of interest by adding evidence that not only HSL and MGL activity is decreased, but also ATGL activity is significantly inhibited both in basal and forskolin stimulated conditions, without affecting LPL activity. (Figure 7 to 12) Interestingly if ATGL and HSL are absent the decrease in lipase activity is abrogated as seen in Figure 11. This does not necessarily mean that MGL lipase activity is not affected because it lacks its substrates normally provided by ATGL and HSL. However MGL activity was inhibited due to olanzapine treatment in a separate assay.

The fact that stimulation with CGI-58 does not affect lipolysis in ATGL-knockout cells, (Figure 11) but in HSL-knockout cells supports CGI-58's described affinity for ATGL. (Zimmermann et al. 2009) Interestingly at a dose of 400 μM olanzapine, CGI-58 seems to be unable to further increase ATGL's lipolytic activity. This leads to the hypothesis that CGI-58 and olanzapine may compete for ATGL suggesting a similar area of interaction. Olanzapine's lipophilic character may play a role in these interactions. This point has to be

further investigated. Looking at the FFA release we can report that without ATGL the FFA release is less than without HSL, demonstrating the importance of ATGL in lipolysis. Considering olanzapine's role we assessed that under concentrations of 400 µM olanzapine the effect on the FFA release in the range between 0 and 400 µM is much more pronounced in the HSL knockout cells compared to ATGL knockout. This leads to the assumption that the effect of olanzapine on ATGL is more prominent than on HSL. Considering the results derived from wild type cells (Figure 7a), which show also an impaired FFA release with similar values like in ATGL-knockout cells, one possible explanation for this phenomenon is that olanzapine's affinity is higher towards HSL than ATGL but more severe when HSL is absent due to its strong interaction with ATGL.

To that end, the glycerol release without HSL is lower than without ATGL due to the fact that HSL and MGL can still degrade triglycerides although at reduced levels. ATGL cannot break down DG without the action of HSL resulting in the inability to produce the final product glycerol (Figure 9).

Moreover, assays performed with 3T3-L1 adipocytes, and cells wherein HSL was downregulated or inhibited by using gene knock-down or inhibition with a selective inhibitor, support the results found in the ex vivo lipolysis assays. Importantly, we could not assess a significant effect of olanzapine on LPL expression or LPL activity.

4.2 Olanzapine increases FFA uptake and TG accumulation

Our results show that olanzapine decreases the total lipase activity by blocking both ATGL and HSL, which are the two major lipases regulating the lipolytic pathway as shown above. Therefore, we were wondering if the inhibition of lipases could cause the accumulation of TG in cells, which could explain the reported fat accumulation in patients and murine models. (Vestri et al. 2007) Indeed treatment with 50 µM olanzapine for 48 hrs resulted in a 2 fold increase and treatment with 400 µM olanzapine caused a 3 fold increase in the TG content of 3T3-L1 adipocytes. Comparable to VL. Albaugh et. al.'s results, along with suppressed lipolytic activity, olanzapine treatment increased saturated and unsaturated FFA uptake in 3T3-L1 adipocytes and C2C12 myotubes.

Although, LPL mRNA levels were not affected by treatment with various olanzapine concentrations, mRNA expression of the FFA transporter, CD36 was significantly increased suggesting that olanzapine modulates the active FFA uptake into adipocytes. Increased insulin sensitivity of adipocytes after olanzapine treatment, leading to increased intracellular glucose levels also leads to the increased flux of FFA in fat depots and due to increased glycerol levels synthesized out of glucose TAG's can be synthesized and therefore accumulate in adipocytes. Furthermore increased glycerol levels inhibit lipolysis and lead to TAG accumulation.

4.3 Olanzapine treatment transforms the transcriptome of fat cells

Our mRNA expression data supports the impairment of lipolysis by showing reduced expression levels of *atgl, hsl* and *mgl.* (Alternative: "genes encoding for ATGL, HSL and MGL"), resulting in the inability to break down TAG's. Moreover, expression levels of FFA transporters CD36 and FASN were increased, explaining and supporting the increased FFA uptake into 3T3-L1 cells. Also the fatty acid binding proteins FABP4 and Stearoyl-CoA desaturase-1 (SCD-1) were significantly upregulated, resulting in an additional mechanism of TAG accumulation. Upregulated PPAR-gamma supports the increased glucose uptake levels shown in C2C12 myotubes, as well as might explain general effects of dysregulation in the lipid metabolism of olanzapine treated patients. To that end we can conclude that olanzapine directly or indirectly increases the expression of lipogenic genes to boost up FFA synthesis in the presence of carbohydrate precursors. All together, the increased lipogenesis, FFA uptake, and the hindered lipolysis due to blockage of ATGL, HSL and MGL, lead to the increased fat accumulation in cells.

4.4 Effect of olanzapine on C2C12 myotubes

The side effects of olanzapine are not only restricted to adipose tissue. Skeletal muscle is also affected, at least in some patients (Albaugh et al. 2010). We used differentiated C2C12 cells for our in vitro model to investigate the effects of olanzapine on the FFA and glucose metabolism of skeletal muscle. The results show that in C2C12 myotubes, FATP1 and correspondingly the FFA uptake was increased similarly as seen in 3T3-L1 adipocytes. The increased expression of FATP1 might be the driving force of an increased FFA uptake in C2C12 myotubes. The increased FATP1 expression could also be a direct reaction to olanzapine or it could be the result of decreased FFA circulation levels due to inhibited lipolysis. Decreased FFA levels in the cell could also be the reason for increased FFA influx.

Like Albaugh et. al. we mentioned a blunted glucose uptake under insulin stimulated conditions into C2C12 myotubes treated with 400 µM olanzapine, suggesting some kind of insulin resistance due to high doses of olanzapine. (Albaugh et al. 2010) We measured also elevated Glut4 mRNA expression, presumambly leading to the increased glucose uptake observed. (Minet-Ringuet et al. 2007) This finding suggests that olanzapine might hinder the translocation of Glut4 from the cytoplasm to the membrane of cells in response to insulin. This has to bee further investigated.

Interestingly, the mitochondrial ß-oxidation of both saturated and unsaturated free fatty acids was decreased. There is a known correlation between lipolysis and ß-oxidation of FFA in which the decreased expression of lipases and therefore the reduced TG hydrolase activity also results in a significant reduction of mitochondrial oxidation of saturated (palmitate) and unsaturated (oleate) FFA oxid. Thus, the combination of decreased FFA oxidation and increased FFA uptake increases the TG concentration muscle tissue due to olanzapine treatment.

4.5 Effect of olanzapine on liver cells

The liver represents the major metabolic organ, which is also important in drug metabolism, thus most side effects of drugs concern the liver. In order to study the effect of olanzapine on liver tissue and function, we treated HepG2 cells with various concentrations of olanzapine. As described previously in literature, we can report increased SREBP1 expression resulting in stimulated lipid synthesis. (Lauressergues et al. 2010) We can also report a hindered FFA transportation by decreased CPT-1a and PPAR-a levels resulting in the decreased B-oxidation as we assessed in our study. Supporting our findings that cholesterol is accumulated in C57BL6 WAT cells we can report a decrease in CYP27A1 and CYP7A1 resulting in impaired cholesterol degradation to bile acids and therefore accumulation of cholesterol in cells.

In addition, we found also a decreased expression of ABCG1, representing an important cholesterol transporter, together with decreased cholesterol conversion to bile acid. Interestingly, ABCA1 expression is slightly increased due to olanzapine treatment. Both transporters are regulated by LXR-alpha, however, ABCG1 acts as the major transporter of cholesterol and ABCA1 has a modest role in cholesterol efflux. To that end, LXR-alpha levels were significantly reduced due to treatment with olanzapine, which might explain the decreased ABCG1 expression levels. Expression levels of HMGCR, the rate limiting enzyme of cholesterol/isoprenoid synthesis, were not affected by olanzapine treatment.

In summary, our experiments showed that olanzapine interferes with the lipid metabolism by de-regulating the expression of multiple important genes involved in lipid metabolism and also by impairing lipase activity. Olanzapine decreases lipolysis, increases FFA uptake and FFA synthesis and therefore increases the accumulation of TAG in a dose dependent manner in adipose and muscle tissue. Olanzapine also induces insulin resistance and decreases ß-oxidation in C2C12 myotubes. Furthermore, olanzapine impairs expression levels of enzymes important for the conversion of cholesterol to bile acids resulting in increased intracellular cholesterol levels. All these effects might result in metabolic dysregulation and obesity with its severe side effects found in patients treated with olanzapine. The evidence that ATGL is impaired due to olanzapine treatment adds important knowledge on olanzapine's actions on lipid homeostasis. Hopefully one day we will be able to use our knowledge to diminish SGAS side effects and improve patient's quality of life.

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