

REGULAR RESEARCH ARTICLE

One-Year Treatment with Olanzapine Depot in Female Rats: Metabolic Effects

Kari M. Ersland, Lene S. Myrmed, Even Fjære, Rolf K. Berge, Lise Madsen, Vidar M. Steen, Silje Skrede

The Norwegian Centre for Mental Disorders Research (NORMENT), Department of Clinical Science, University of Bergen, Norway (Drs Ersland, Steen, and Skrede); Dr. Einar Martens' Research Group for Biological Psychiatry, Department of Medical Genetics, Haukeland University Hospital, Bergen, Norway (Drs Ersland, Steen, and Skrede); Institute of Marine Research, Bergen, Norway (Drs Myrmed, Fjære, and Madsen); The Lipid Research Group, Section for Medical Biochemistry, Department of Clinical Science, University of Bergen, Bergen, Norway (Dr Berge); Department of Biology, University of Copenhagen, Copenhagen, Denmark (Dr Madsen)

V.M.S. and S.S. contributed equally to the present work.

Correspondence: Professor Vidar M. Steen, MD, PhD, Department of Clinical Science, University of Bergen, Bergen, Norway (vidar.martin.steen@helse-bergen.no).

Abstract

Background: Antipsychotic drugs can negatively affect the metabolic status of patients, with olanzapine as one of the most potent drugs. While patients are often medicated for long time periods, experiments in rats typically run for 1 to 12 weeks, showing olanzapine-related weight gain and increased plasma lipid levels, with transcriptional upregulation of lipogenic genes in liver and adipose tissue. It remains unknown whether metabolic status will deteriorate with time.

Methods: To examine long-term metabolic effects, we administered intramuscular long-acting injections of olanzapine (100 mg/kg BW) or control substance to female rats for up to 13 months.

Results: Exposure to olanzapine long-acting injections led to rapid weight gain, which was sustained throughout the experiment. At 1, 6, and 13 months, plasma lipid levels were measured in separate cohorts of rats, displaying no increase. Hepatic transcription of lipid-related genes was transiently upregulated at 1 month. Glucose and insulin tolerance tests indicated insulin resistance in olanzapine-treated rats after 12 months.

Conclusion: Our data show that the continuous increase in body weight in response to long-term olanzapine exposure was accompanied by surprisingly few concomitant changes in plasma lipids and lipogenic gene expression, suggesting that adaptive mechanisms are involved to reduce long-term metabolic adverse effects of this antipsychotic agent in rats.

Keywords: olanzapine, weight gain, diabetes, long-term, rat

Introduction

The global lifetime prevalence of schizophrenia, a serious psychotic disorder, approximates 0.7% (McGrath et al., 2008). Patients diagnosed with schizophrenia may experience different illness

trajectories with varying incidence of psychotic episodes, but a significant number of patients require long-term treatment with antipsychotic medication (Bowtell et al., 2017; Wunderink,

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Significance Statement

Patients suffering from schizophrenia often receive treatment with so-called antipsychotic medication for extended periods of time. While dampening symptoms, this medication can also have side effects such as weight gain and diabetes. In trying to reveal how antipsychotics induce such side effects, rat models are frequently used, but experiments are often unrealistically short (1–2 weeks). In this study, we tried to mimic the real-life situation, treating rats for over a year (corresponding to more than one-half of a rat's life span) with the antipsychotic olanzapine. In spite of gaining extra weight and showing early signs of diabetes, overall the rats adapted surprisingly well to the treatment. Healthy rats may provide a less reliable model for antipsychotic-induced side effects, and replacing them with rodents more vulnerable to drug-induced side effects could increase the relevance for humans.

2017). Many second-generation antipsychotic drugs that are commonly used have serious metabolic adverse effects such as weight gain, dyslipidemia, and type 2 diabetes (Correll et al., 2015). The second-generation antipsychotic drugs olanzapine and clozapine are associated with particularly high risk, but are also regarded as clinically efficacious and valuable drugs (Leucht et al., 2013; Correll et al., 2015). To prevent metabolic adverse effects and aid drug development, uncovering the underlying molecular mechanisms is pivotal. Propensity for weight gain correlates with affinity of antipsychotic agents for serotonin 5HT_{2C} and histamine H₁ receptors, while binding to peripheral muscarinic M₃ receptors has been linked to diabetes (Kroeze et al., 2003; Reynolds and Kirk, 2010). However, much remains unknown with regard to downstream and possible receptor-independent mechanisms.

Preclinical studies, which can be performed in a controlled environment and also grant access to biological samples not available in clinical studies, are valuable in this context. In the female rat, antipsychotic-induced metabolic adverse effects such as hyperphagia, weight gain, increased serum lipids, and glucose dysregulation have been extensively reproduced (Boyda et al., 2010; Benarroch et al., 2016). For some dysmetabolic features, molecular mechanisms have been suggested. For instance, olanzapine-induced hyperphagia has been linked to activation of hypothalamic AMP-activated kinase and upregulation of orexigenic neuropeptides in the hypothalamus (Kim et al., 2007; Ferno et al., 2011; Skrede et al., 2014). Although increased serum cholesterol and triglycerides correlate with activation of the key lipogenic transcription factors sterol regulatory element binding proteins 1 and 2 in peripheral tissues, causality has yet to be established (Skrede et al., 2012b; Skrede et al., 2014).

The gap between the observed metabolic adverse effects of antipsychotic drugs and the underlying molecular mechanisms still needs to be bridged. Establishing a valid animal model has proved challenging (Benarroch et al., 2016). As an example, the average half-life of olanzapine in rats is short—2.5 hours, compared with 33 hours in humans (Aravagiri et al., 1999). In many rat experiments, olanzapine has been administered 2 or 3 times a day, but markedly fluctuating drug concentrations throughout the day has represented a major confounder. Owing to the difficulty in achieving steady-state conditions, in addition to the significant workload associated with drug administration at least twice a day, most rat studies have lasted for 1 to 12 weeks (Han et al., 2008; Boyda et al., 2010; Skrede et al., 2012a). As an alternative to repeated daily drug administrations, previous experiments have also involved exposure to olanzapine for up to 8 weeks by means of subcutaneously implanted osmotic minipumps, ensuring stable delivery of drug amounts corresponding to clinically relevant dopamine D₂ receptor occupancy (Kapur et al., 2003; Mondelli et al., 2013; Calevro et al., 2018). Unfortunately, the duration of experiments is limited by

the fact that minipumps have to be exchanged every 2 weeks, with a limited number of pump exchanges regarded as ethically and practically feasible (Remington et al., 2011). We and others have recently described how the use of long-acting injections (LAI) of olanzapine can yield stable plasma concentrations and relevant metabolic phenotypes (Saeedi et al., 2007; Skrede et al., 2014; Ersland et al., 2015; Ferno et al., 2015; Horska et al., 2016). This development paves the way for long-term studies in the rat, which could clarify whether metabolic adverse effects of olanzapine will become more pronounced with prolonged exposure times.

Using a treatment scheme with biweekly intramuscular injections of long-acting olanzapine, we treated female rats for 13 months, roughly corresponding to 26 years of human life (Sengupta, 2013), with frequent measurements of body weight throughout the experiment. Female rats were chosen due to the fact that the clinically relevant phenotype of weight gain is readily reproduced in female rats, unlike in male rats (Boyda et al., 2010; Ferno et al., 2015). In a long-term experiment, this facilitates noninvasive monitoring of the metabolic phenotype. Separate cohorts of rats were sacrificed after 1, 3, 6, and 13 months of treatment, followed by measurements of plasma olanzapine concentrations, plasma glucose, plasma lipids, and lipogenic gene expression in liver and visceral adipose tissue. Furthermore, glucose and insulin tolerance tests were performed 12 months into the experiment. In addition, to determine whether the previously described lipogenic activation only occurs in young rats, we examined the effects of a first-ever olanzapine injection 5 days prior to sacrifice in a subset of rats having been handled as controls for 13 months. To the best of our knowledge, this is the first study to report on the long-term effects of olanzapine exposure in the rat.

Methods

Animals

All experiments were approved by and carried out in accordance with the guidelines of the Norwegian Committee for Experiments on Animals (Forsøksdyrutvalget, FDU) with ID 2015–7661. Female outbred Sprague-Dawley rats (Møllegaard, Denmark) were kept under standard conditions with an artificial 12:12-hour light/dark cycle (lights on: 8:00 AM) and constant 48% humidity. Animals were housed 5 per cage during the experiment and allowed access to tap water and free (ad libitum) access to standard laboratory chow (Special Diets Services, Witham, UK) during the experimental period. Rats were weighed regularly, while food intake was not measured in this experiment. Care was taken to ensure minimal suffering of the animals at all stages of the experiments.

Treatment Schemes

The experimental design is shown in Figure 1. Rats ($n=110$) weighing 220 to 240 g at the start of the experiment received regular intramuscular injections of either commercially available long-acting olanzapine pamoate formulation (100 mg/kg body weight [BW] ZypAdhera, Eli Lilly, IN) or vehicle solution (injection volume 160 μ L/250 g BW) for up to 13 months. A drug dose of 100 mg/kg BW was chosen to reflect the amount of olanzapine received by patients exposed to LAI, which are approximately 15 times higher than oral formulations (Kane et al., 2010; Skrede et al., 2014). From the start of the experiment, rats received injections every 10th day. In rats, an injection interval between 10 to 14 days has previously been shown to reproduce metabolic effects observed in humans (Skrede et al., 2014; Ferno et al., 2015; Horska et al., 2016). However, no published study has so far exposed rats to olanzapine LAI for more than 8 weeks (Horska et al., 2016). When sedation and gradual weight loss observed in the rats after more than 8 weeks of drug exposure (see the Results section for further information), we performed drug monitoring that disclosed very high olanzapine concentrations in plasma. The injection interval was subsequently increased to 2.5 weeks (approximately 18 days) for the rest of the experiment. Olanzapine-treated animals ($n=10$) and vehicle-treated animals ($n=10$) were sacrificed 1, 3, or 6 months after the first injection (Figure 1a). For each of the 3 time points, rats received the last dose of vehicle or olanzapine 1 week prior to sacrifice and were fasted overnight to minimize potential variability in gene expression linked to differences in metabolic parameters. Due to the signs of possible drug toxicity (see above), samples from the 3-month time point were excluded from further analysis.

After 13 months, the remaining animals ($n=50$) were split into 5 subgroups (Figure 1b). Vehicle-treated rats were either

continuously treated with vehicle solution ($n=12$) or received a first-ever injection of olanzapine pamoate ($n=14$; olanzapine subchronic group). Of these latter animals, one-half ($n=7$) received chow ad libitum. The remaining animals ($n=7$) were pair-fed, that is, the group of animals received an amount of chow corresponding to that consumed by the group of control animals during the previous 24 hours. A time period of 5 days was selected to represent a subchronic treatment period, based on previous rat experiments of gene expression (Saeedi et al., 2007; Erslund et al., 2015; Ferno et al., 2015; Erslund et al., 2017). As for the animals already treated with olanzapine for 13 months ($n=24$), this group was divided into 1 ad libitum-fed group ($n=12$) and 1 pair-fed group ($n=12$). Six days after the last injection and group subdivision, rats were fasted overnight. Sacrifice was performed over 3 separate days (9 AM–1 PM) but always 6 days after the last injection. All rats were randomized to time of sacrifice to avoid confounding effects.

Dissection, RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Rats were anesthetized by isoflurane gas (Isoba vet, Schering-Plough, Denmark) and subsequently sacrificed by decapitation. Upon sacrifice, selected organs were rapidly weighed before tissue samples were harvested and flash frozen. Truncal blood collected in EDTA tubes was centrifuged at 3000 g for 10 minutes (4°C) to extract plasma, which was stored at -80°C . Tissue samples (~ 20 mg of liver tissue or ~ 100 mg of adipose tissue) were homogenized using a TissueLyser (Qiagen, Hilden, Germany). RNA extraction was performed using an ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA). DNase treatment was performed according to the manufacturer's protocol. Quality and amount of total RNA were measured using

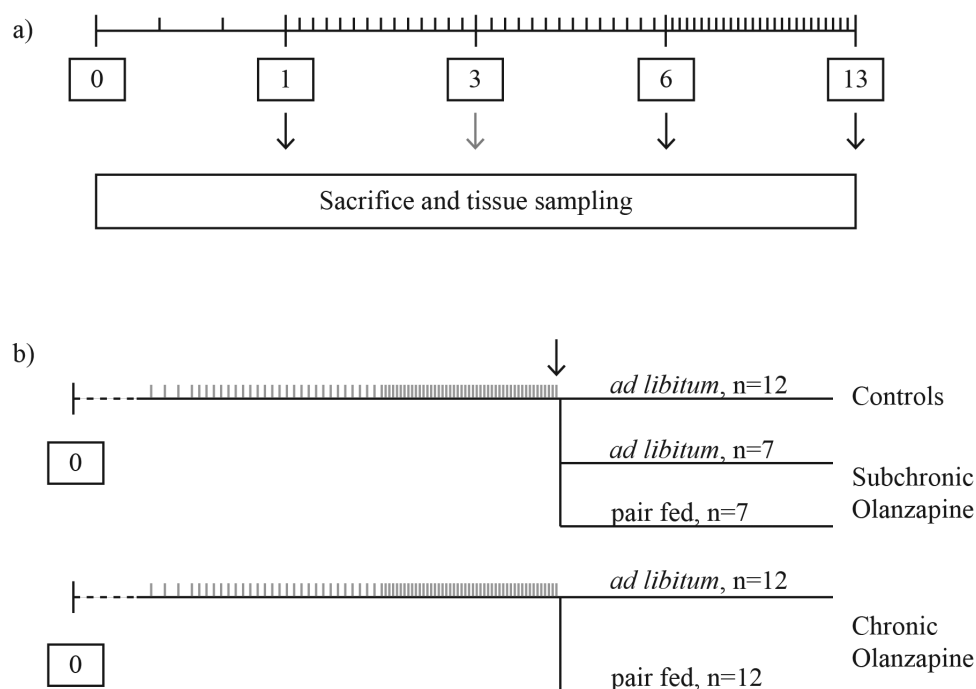


Figure 1. Schematic overview of the experimental setup. The study initially included 56 control rats and 54 olanzapine-treated rats. (a) Long-acting injections (LAI) of olanzapine are indicated by vertical bars, and time points of sacrifice are illustrated by number of treatment months. Olanzapine-treated rats ($n=10$) and vehicle-treated rats ($n=10$) were sacrificed at each indicated time point. (b) After 13 months of olanzapine treatment, the remaining 50 rats were subdivided into 5 groups: ad libitum-fed vehicle-treated rats ($n=12$), ad libitum- and pair-fed (both $n=7$) subchronic olanzapine-treated rats, and ad libitum- and pair-fed (both $n=12$) chronic olanzapine-treated rats. Grey vertical bars illustrate LAI during the experiment, and the black arrow indicates the last injected dose.

the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA synthesis and quantitative real-time PCR (qPCR) were performed as previously described (Ferno et al., 2009). Relative gene expression levels were determined using the comparative C_t method, using β -actin and ribosomal protein lateral stalk subunit P0 as endogenous controls.

Plasma Measurements of Lipids, Glucose, and Olanzapine

Fasted glucose and lipid parameters (total cholesterol, HDL cholesterol [HDL-C], LDL cholesterol [LDL-C], free fatty acids, phospholipids, and triglycerides [TG]) in plasma samples from each time point were measured enzymatically using the Hitachi 917 system (Roche Diagnostics, Basel, Switzerland). Plasma levels of olanzapine were determined by means of high performance liquid chromatography by the Agilent 1290 Infinity Binary LC coupled to an Agilent 6490A triple quadrupole mass spectrometer using positive electrospray ionization (Agilent Technologies, Santa Clara, CA).

Glucose and Insulin Tolerance Tests

After 12 months of treatment with either LAI olanzapine or vehicle and 1 week after the most recent injection, a subgroup of randomly selected olanzapine ($n=10$) and vehicle ($n=10$) rats underwent a glucose tolerance test (GTT). Blood glucose from all rats was measured before the initiation of the test (i.e., “fed” blood glucose). Rats were fasted for 2 hours, and blood glucose was measured once more (i.e., “fasted” blood glucose). After fasting, the rats received 2 mg glucose per gram of total body mass by oral gavage. During the test, ~ 1 μ L and ~ 20 μ L whole blood was collected from the severed distal tip of the animal’s tail at 5 time points (0, 15, 30, 60, and 120 minutes). One μ L was used for direct measurement of glucose level using a glucometer (Ascensia Contour, Bayer, Norway). For subsequent insulin level measurements in plasma, 20 μ L whole blood was collected in EDTA tubes, immediately centrifuged and 5 μ L plasma was extracted and analyzed using an Ultrasensitive Rat Insulin ELISA kit (CrystalChem, Elk Grove Village, IL). To maintain animal well-being, the 2 tests were separated in time by 3 weeks. Two weeks after the GTT, rats received an intramuscular injection of olanzapine in accordance with the general treatment protocol. One week later, the same 10 rats underwent an insulin tolerance test (ITT). Similar to the GTT, blood glucose was measured before the initiation of the test and after 6 hours of fasting. ITT was performed by i.p. injection of 0.75 U insulin per kilogram of body weight (Actrapid, Denmark), blood was sampled at 0, 15, 30, 45, 60, 75, and 90 minutes, and blood glucose was measured as described for the GTT.

Statistical Analysis

Body weight gain was analyzed using repeated-measures ANOVA (SPSS statistics, SPSS Inc, Chicago, IL). Pearson’s correlation analysis and Kaplan-Meier survival analysis were performed in SPSS. All other analyses were performed in GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA). Alteration in the composition of plasma lipids and glucose over time was assessed by linear regression. Results from glucose measurements during GTT and ITT were analyzed using 2-way repeated-measures ANOVA (with time and treatment as independent factors), using Sidak’s multiple comparisons test. Plasma insulin levels during GTT were analyzed using mixed-effects model due to 1 missing value. Alterations between fasted and fed blood glucose were analyzed by the 2-sided Student’s t test. For differential gene expression,

statistical significance was determined by the 2-sided Student’s t test (when 2 groups were compared) or 1-way ANOVA (when 3 groups were compared, i.e., subchronic groups) followed by post hoc Dunnett’s multiple comparisons test. Two-sided Student’s t test was used to assess alterations in liver and periovarian white adipose tissue (WAT) weights. Uncorrected- or Dunnett’s adjusted P values $<.05$ were considered significant. Data are reported as mean \pm SEM.

Results

In the following, data for up to 13 months of olanzapine exposure are presented. Initially, rats were exposed to LAI of olanzapine every 10th day. This dosing interval was chosen due to previous observations of weight gain during the first 10 days after olanzapine LAI injections, with subsequent deceleration of weight gain (Skrede et al., 2014; Ersland et al., 2015). However, after more than 8 weeks of exposure, rats gradually displayed signs of drug toxicity, with sedation, weight loss, and high olanzapine plasma concentrations. Based on these observations, blood and tissue samples from the 3-month time point were not included in the analyses. For purposes of clarity, data from rats treated subchronically (i.e., for 5 days) with olanzapine in the final stages of the 13-month experiment are presented separately at the end of the results section.

Body Weight Gain in Olanzapine-Treated Rats Sustained Throughout the Experiment

Average body weight in the 2 groups was similar at baseline (average \pm SEM: control group, 235.6 \pm 0.48 g; olanzapine group, 237.1 \pm 1.2 g; P value: n.s.). Rats receiving injections of olanzapine every 10th day rapidly gained more weight than control animals (Figure 2). More than 8 weeks into the experiment, clinically obvious sedation in olanzapine-treated rats coincided with pronounced, unexpected weight loss (Figure 2). Blood samples from a selection ($n=10$) of the rats showed very high plasma olanzapine levels (234.6 \pm 73.9 ng/mL). Due to poor sample quality, these results have to be regarded as uncertain, but correspondingly high plasma olanzapine levels were likely present in many of the rats at this stage of the experiment. After a period of 5 weeks without further injections, sedation and weight loss receded and treatment was re-initiated, followed by injection interval adjusted to approximately 2.5 weeks (18 days). Subsequent to this adjustment, that is, from approximately day 90, each olanzapine injection was generally followed by a short period of accelerated weight gain, which then decelerated, followed by renewed increase in body weight gain after the next injection (Figure 2). As expected, control rats gained weight throughout the experiment. Cumulative body weight gain in the control and olanzapine treatment groups remained statistically different throughout the entire experiment (repeated-measures ANOVA).

Equal Long-Term Mortality in Control and Olanzapine-Exposed Rats

Kaplan-Meier survival analysis showed no significant difference in mortality between vehicle-treated rats and rats treated with olanzapine during the 13-month experiment (supplementary Figure 1). One olanzapine-treated rat died spontaneously 3 weeks after the initiation of treatment, with postmortem examination revealing signs of paralytic ileus (supplementary Figure 1). Age-related adenomas are common in the rat and occurred with equal prevalence in control rats ($n=6$) and

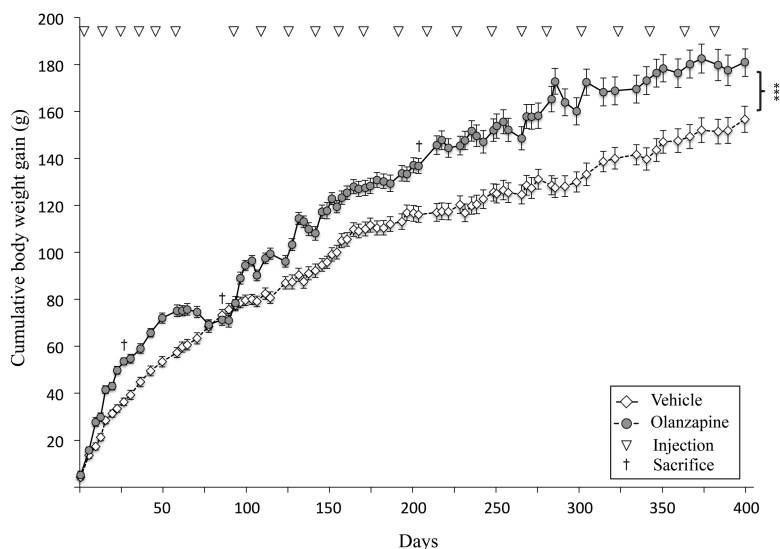


Figure 2. Cumulative body weight gain. Average weight gain in grams (\pm SEM). Data for all rats alive at any given time point are included. Note that for each time point where rats were sacrificed (marked with †), these rats were eliminated from the weight estimates. A drop in cumulative weight gain for olanzapine-exposed animals can be observed around day 75, due to possible signs of toxicity, e.g., sedation. Repeated-measures ANOVA was used to analyze differences in cumulative body weight gain between olanzapine- and vehicle- exposed animals: *** $P \leq .001$, compared with vehicle.

Table 1. Plasma Olanzapine Concentrations

Treatment Group	Plasma Olanzapine (ng/mL)
1 month	59.5 \pm 9.8
6 months	25.1 \pm 2.1
13 months chronic	84.8 \pm 7.9
13 months subchronic ad lib	18.8 \pm 2.6
13 months subchronic pair-fed	17.2 \pm 3.6

Plasma olanzapine measures are given as average \pm SEM ng/mL. ad lib, ad libitum; subchronic, 5-day treatment.

olanzapine-treated rats ($n=5$) in our study (Okada et al., 1981). Rats developing adenomas were euthanized.

Plasma Concentrations of Olanzapine

In the 10 olanzapine-treated rats sacrificed after 1 month of treatment, that is, 1 week after a third intramuscular injection of olanzapine, the average plasma concentration of olanzapine was 59.5 \pm 9.8 ng/mL (Table 1). At 6 months the concentration was 25.1 \pm 2.1 ng/mL, while at 13 months it had increased to 84.8 \pm 7.9 ng/mL. Former control rats administered with olanzapine for the first time 5 days prior to sacrifice at the 13-month time point (subchronic group) had an average plasma olanzapine concentration of 18.0 \pm 2.2 ng/mL (all rats, independent of nutritional status). With the exception of the subchronically exposed rats, where plasma olanzapine was relatively low, plasma concentrations at all other time points (1, 6, and 13 months) remained within a range described as therapeutic and nontoxic in humans (i.e., 22.77 \geq 100 ng/mL) (Robertson and McMullin, 2000; Lu et al., 2016; Tveito et al., 2018).

Olanzapine Does Not Increase Plasma Lipids or Glucose in the Long Term

Plasma lipids (free fatty acids, TG, phospholipids, total cholesterol, LDL-C, and HDL-C) and plasma glucose were measured

at each time point of sacrifice (1, 6, and 13 months). As previously described, linear regression analysis revealed that levels of phospholipids and cholesterol increased with age in both olanzapine- and vehicle-exposed animals (Table 2a–b; supplementary Figure 2) (Uchida et al., 1978). Levels of TG increased only in olanzapine-exposed animals, while HDL-C increased only in control animals, over time. Contrary to our expectations, plasma lipids were not further increased after long-term olanzapine treatment compared with control rats. The only observed alteration was a lower amount of plasma LDL-C in olanzapine-exposed animals, compared with controls, after 1 month of treatment (2-sided Student's *t* test, uncorrected *P* value: <.05) (Table 2). Levels of plasma glucose in olanzapine-exposed rats significantly decreased over time, whereas control animals did not show any alteration (Table 2c; supplementary Figure 2).

GTT and ITT at 12 Months: Olanzapine-Induced Hyperinsulinemia

A subgroup of vehicle-treated ($n=10$) and olanzapine-treated ($n=10$) rats underwent a separate GTT and ITT 1 week after the last injection, approximately 12 months into the experiment. Fasted or fed blood glucose was not different between vehicle- and olanzapine-treated rats (2-sided Student's *t* test, uncorrected *P* value: n.s.) (Figure 3a).

Using 2-way repeated-measures ANOVA, time was found to have the main effect on glucose levels in rats during the GTT ($F(2.303,41.45)=60.24$, $P < .0001$) (Figure 3b), regardless of whether they were exposed to olanzapine. Further analysis using Sidak's multiple comparisons test revealed no significant difference between the 2 groups, which was also evident from the area under the curve (AUC) (Figure 3c).

In accordance with the glucose measurements, time was also found to be the main effector on insulin levels in plasma taken during the GTT ($F(2.256,40.05)=33.53$, $P < .0001$), while treatment had no apparent effect (Figure 3d). Using Sidak's multiple comparisons test, significantly higher plasma insulin was found in olanzapine-treated rats compared with vehicle-treated

Table 2a. Composition of Plasma Lipids: Free Fatty Acids, Triglycerides, and Phospholipids

Month	FFA		TG		PL	
	VEH	OLZ	VEH	OLZ*	VEH***	OLZ***
1	0.47±0.04	0.46±0.06	0.56±0.03	0.66±0.10	1.86±0.08	1.98±0.06
6	0.70±0.04	0.63±0.09	1.64±0.25	1.08±0.21	2.76±0.18	2.46±0.10
13	0.55±0.06	0.55±0.06	1.19±0.17	1.43±0.32	3.14±0.06	2.76±0.06

Table 2b. Composition of Plasma Lipids: Cholesterol

Month	TC		HDL-C		LDL-C	
	VEH***	OLZ***	VEH***	OLZ	VEH**	OLZ***
1	2.08±0.12	2.08±0.10	1.89±0.12	1.91±0.09	0.31±0.03	0.21±0.02#
6	2.96±0.28	2.62±0.13	2.08±0.09	2.20±0.10	0.25±0.03	0.28±0.02
13	3.45±0.24	2.95±0.18	2.58±0.12	2.19±0.09	0.54±0.12	0.38±0.04

Table 2c. Plasma Levels of Glucose

Month	Glucose	
	VEH	OLZ***
1	7.90±0.23	8.81±0.30
6	8.10±0.17	8.43±0.18
13	8.04±0.18	7.38±0.23

Abbreviations: FFA, free fatty acids; HDL-C, HDL-bound cholesterol; LDL-C: LDL-bound cholesterol; OLZ, olanzapine-treated rats; PL, phospholipids; TG, triglycerides; VEH, vehicle-treated rats.

Measurements of plasma lipids and glucose (mean ± SEM, mmol/L).

Linear regression analysis was used to assess change over time in the lipid parameters and glucose measurements: * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$. Alterations in lipid parameters and glucose levels at 1, 6, or 13 months, between VEH and OLZ animals, were assessed by the Student's *t* test; # uncorrected $P < .05$.

rats 60 and 120 minutes after an oral glucose load ($P = .0376$ and $.0286$, respectively), indicating that olanzapine-treated rats were hyperinsulinemic.

During the ITT, 2-way repeated-measures ANOVA revealed that both time ($F(2,018,36.32) = 33.21$, $P < .0001$) and treatment ($F(1,18) = 4.675$, $P = .0443$) significantly affected glucose levels in the rats (Figure 3e). Furthermore, an interaction effect was observed between time and treatment ($F(6, 108) = 3.114$, $P = .0075$), indicating that olanzapine exposure had an effect on glucose levels over time. This was also reflected at 60 and 75 minutes, where olanzapine-exposed rats had significantly higher blood glucose levels than vehicle-treated rats (Sidak's multiple comparisons test $P = .0354$ and $.0195$, respectively) and in an increased AUC (Figure 3f).

Increased Liver and Periovarian WAT Weights at 1 Month

At each time point of sacrifice (1, 6, and 13 months), liver and periovarian WAT were weighed, with the addition of skeletal muscle tissue (gastrocnemius muscle) at 13 months. For purposes of comparison, data at 13 months include ad libitum-fed rats only. At 1 month, liver and periovarian WAT weights (in percentage of body weight) were significantly higher in olanzapine-treated rats than vehicle rats (2-sided Student's *t* test, uncorrected $P = \leq .05$ and $.001$, respectively) (Figure 4a–b). At the later time points, this was not the case. At 13 months, cumulative body weight gain was positively correlated with periovarian

WAT weight (Pearson's correlation analysis, $r = 0.58$, $P = .04$), but not with relative weight of the liver or gastrocnemius muscle (data not shown).

Transiently Increased Expression of Genes Encoding Lipid Biosynthesis Enzymes

The expression of lipogenic genes in liver and periovarian WAT was examined using qPCR. At 1 month, hepatic transcription of the key lipogenic genes *Acetyl-CoA carboxylase 1 (Acc1)*, *Fatty acid synthase (Fasn)*, and *HMG-CoA reductase (Hmgcr)* was upregulated approximately 2-fold in olanzapine-treated rats compared with control rats (2-sided Student's *t* test, uncorrected $P = .024$, $.033$, and $.022$, respectively) (Figure 5a–c). A similar upregulation was not found in periovarian WAT (Figure 5d–f). At the later time points, no transcriptional differences were found between the treatment groups in these tissues (Figure 5).

Lipogenic Activation After Subchronic Exposure to Olanzapine in Older Control Rats

Subchronic (5-day) treatment with olanzapine did not result in alterations of plasma lipid levels or organ weights in rats that had previously been part of the vehicle-treated group for more than 12 months (data not shown). The short-term treatment did, however, significantly affect the transcription of selected lipogenic genes both in liver and periovarian WAT. In the liver, *Acc1* and *Hmgcr* expression levels were significantly upregulated by olanzapine in the pair-fed group (Dunnett's adjusted $P = .0001$ and $.0022$, respectively) (Figure 6a,c), while expression of *Fasn* was unaffected (Figure 6b). No upregulation was observed in the liver in the ad libitum-fed group. In periovarian WAT, the *Fasn* level was upregulated, most prominently in the ad libitum-fed rats (Dunnett's adjusted $P = .0081$) (Figure 6e). Expression of *Acc1* and *Hmgcr* was unaltered by olanzapine treatment in this tissue, regardless of feeding regime (Figure 6d,f).

Discussion

We performed a 13-month experiment, which to our knowledge constitutes the most extensive exposure to olanzapine described in the rat in an academic setting. Comparing the lifespan of rats to that of humans, 2 weeks of a rat's life corresponds to approximately 1 human year (Sengupta, 2013). According to

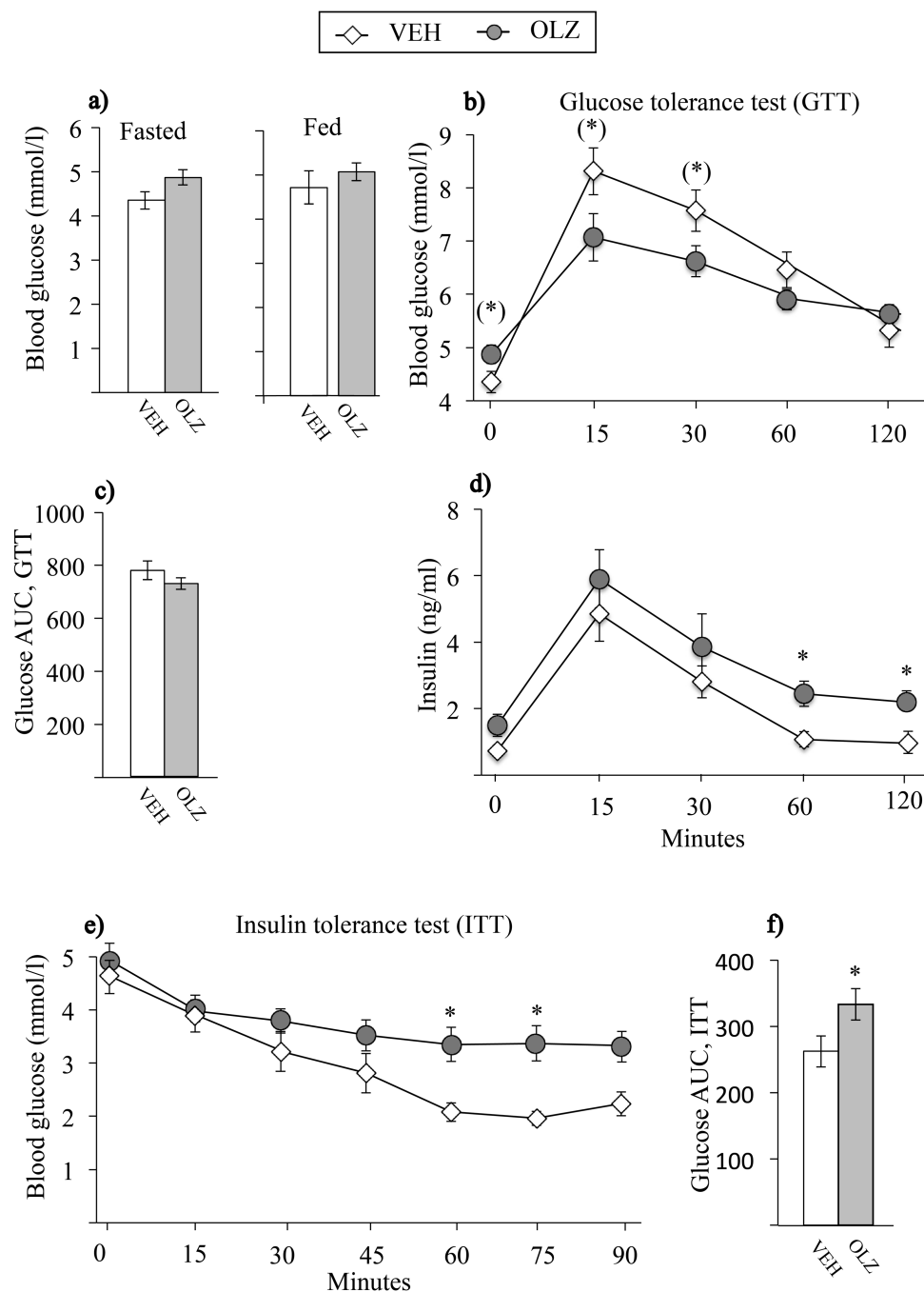


Figure 3. Glucose and insulin tolerance. Glucose and insulin tolerance tests (GTT/ITT) were performed 3 weeks apart in the same rats treated either with vehicle (VEH; n=10) or olanzapine LAI (OLZ; n=10). (a) Fed and fasted blood glucose was measured from whole blood before and after a 2- (GTT) or 6- (ITT) hour fast, respectively. (b) GTT showing whole blood glucose concentrations during the first 2 hours after administration of 2 mg glucose per gram of total body mass by oral gavage. (c) Area under the curve (AUC) calculated from blood glucose concentrations during the GTT. (d) Plasma insulin concentrations during the GTT. (e) ITT showing blood glucose during the first 90 minutes after an i.p. injection of 0.75 U/kg BW insulin. (f) AUC calculated from blood glucose concentrations during the ITT. Statistically significant change between fasted and fed blood glucose was assessed using the 2-sided Student's t test. Two-way repeated-measures ANOVA or mixed-effects model, and Sidak's multiple comparison test was used to analyze the glucose measurements during the GTT and ITT, in addition to insulin measurements taken during the GTT. * $P \leq .05$.

this estimate, rats sacrificed at 13 months had been treated with olanzapine for what would amount to 26 human years.

The pharmacokinetics of olanzapine LAI in the rat is largely unknown. An olanzapine dose of 100 mg/kg was chosen due to the fact that patients treated with olanzapine LAI receive doses approximately 15 times as high as patients treated with oral formulations (Kane et al., 2010). We and

others have previously observed hyperphagia and weight gain in female rats treated with oral administration of 5 to 10 mg/kg olanzapine daily (Goudie et al., 2002; Kalinichev et al., 2005; Skrede et al., 2012b). The chosen dose of 100 mg/kg corresponds to a daily dose of 6.67 mg/kg multiplied by 15.

Repeated administration of olanzapine LAI 100 mg/kg every 10th day initially resulted in rapid and sustained body weight

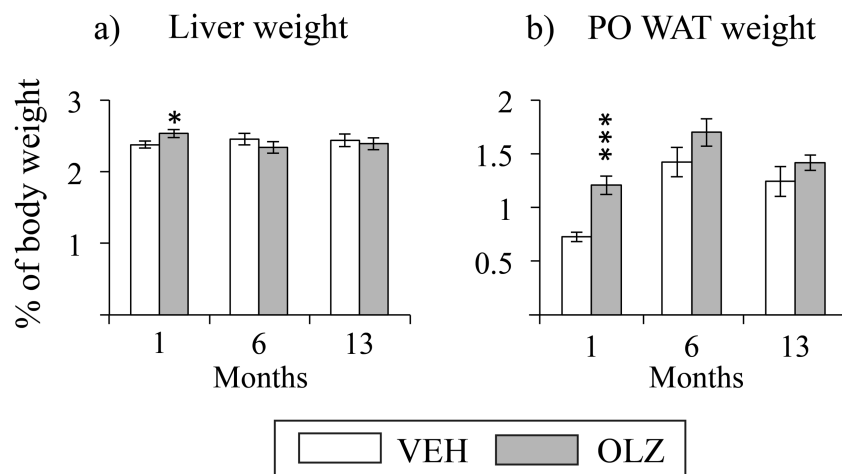


Figure 4. Liver and periovarian white adipose tissue weight. Weights are given as percentage body weight (\pm SEM) at the 4 different time points of sacrifice. OLZ, olanzapine; VEH, vehicle. Data for ad libitum-fed olanzapine-treated rats are shown at 13 months. The 2-sided Student's *t* test was used to assess statistically significant alterations in weight: *uncorrected $P \leq .05$, ***uncorrected $P \leq .001$, compared with vehicle.

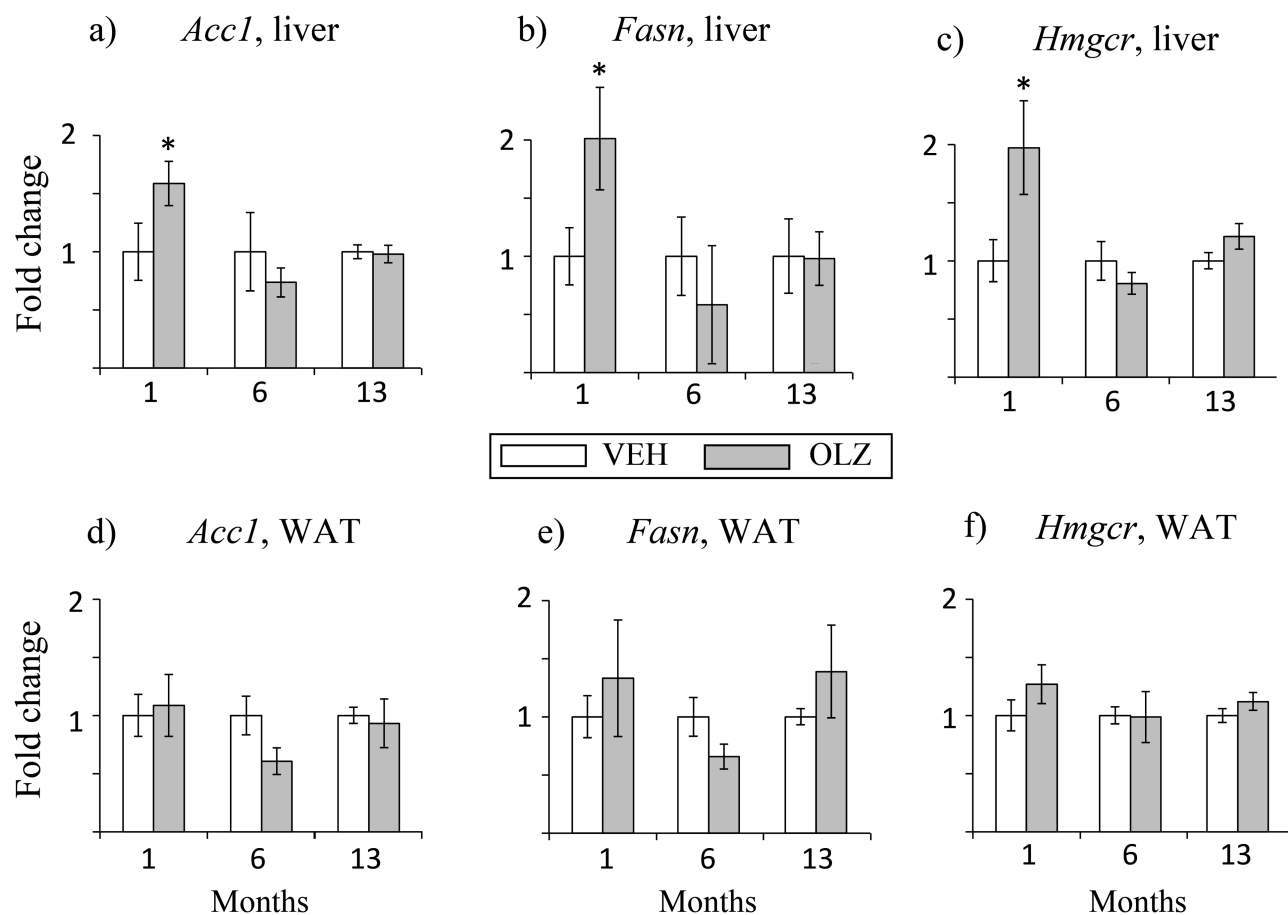


Figure 5. Expression of genes encoding rate-limiting lipogenic enzymes in liver and periovarian white adipose tissue. At each time point, quantitative real-time PCR (qPCR) was performed. Fold change is expressed relative to vehicle, which is always normalized to 1. Fold changes are given as mean \pm SEM. *Acc1*, acetyl-CoA carboxylase 1; *Fasn*, fatty acid synthase; *Hmgcr*, HMG-CoA reductase. The 2-sided Student's *t* test was used to assess statistically significant alterations in expression levels: *uncorrected $P \leq .05$, compared with vehicle.

gain, as previously described (Skrede et al., 2014; Horska et al., 2016). However, as treatment continued using injections every 10th day, a gradual cessation in weight gain appeared. Previous studies have only analyzed the effects of LAI of olanzapine

(injections every 10th–14th day) for up to 8 weeks. Following almost 3 months of drug exposure, the selected treatment scheme resulted in very high plasma olanzapine levels. It is likely that frequent LAI led to accumulation of olanzapine

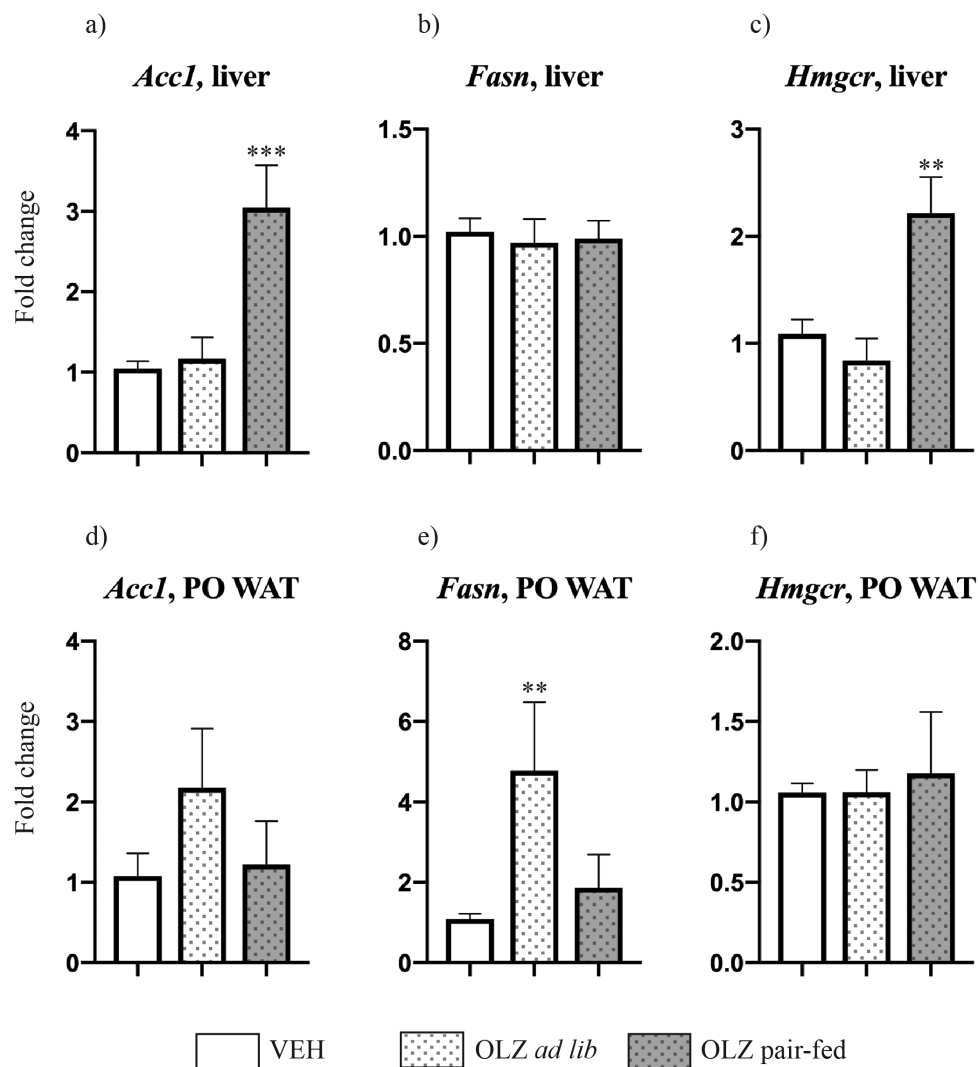


Figure 6. Expression of genes encoding rate-limiting lipogenic enzymes in liver and periovarian white adipose tissue 5 days after a first-ever injection of olanzapine LAI in rats treated as controls for 12.5 months. *Acc1*, acetyl-CoA carboxylase 1; *Fasn*: fatty acid synthase; *Hmgcr*: HMG-CoA reductase; OLZ ad lib: olanzapine-treated rats with free access to chow; OLZ pair-fed: olanzapine-treated rats with restricted access to chow; VEH, vehicle. Fold changes are given as mean \pm SEM. One-way ANOVA, followed by Dunnett's multiple comparisons test, was used to assess statistical significance. **Dunnett's adjusted $P \leq .01$, ***Dunnett's adjusted $P \leq .001$.

over time, resulting in toxic effects and arrested weight gain. As a consequence of this situation, the injection rate was decreased to every 18th day, which subsequently resulted in reappearance of weight gain. Notably, at 1, 6, and 13 months, plasma levels of olanzapine were within the range measured in postmortem and therapeutic drug monitoring samples (Robertson and McMullin, 2000; Lu et al., 2016; Tveito et al., 2018). According to clinical recommendations, therapeutic drug monitoring samples during olanzapine LAI treatment are drawn at an estimated trough value 0 to 2 days before the next scheduled injection (10–28 days after the last injection). In the rats, samples were taken approximately 7 days after the last injection. Thus, plasma levels in the rats are not trough levels and thus not directly comparable with data from patients. Bearing this and other limitations of a direct comparison between rats and humans in mind, the measured plasma levels of olanzapine combined with the reappearance of weight gain during the subsequent stages of the experiment nevertheless indicate that the animals were not subject to toxic carryover effects during the remaining experimental period.

In the event of a new, similar study, the data from the present experiment should nevertheless lead to consideration of lower doses or an increased injection interval. Considering examples from experiments involving osmotic minipumps, a dosing regimen based on dopamine D2 receptor occupancy should be developed (Kapur et al., 2003).

In spite of significant weight gain, olanzapine treatment did not result in significant elevation of plasma lipids or fasting glucose throughout the experiment. This is in agreement with a recent study performed by Horska et al., where a lack of alteration in serum lipids at 4 separate time points during a 2-month experiment in female rats treated with olanzapine LAI was demonstrated (Horska et al., 2016). One possible cause for the lack of plasma lipid increase could be the presence of various homeostatic feedback mechanisms. In an acute setting, with regular measurements from 30 minutes until 48 hours after a single dose of olanzapine or clozapine, we previously described an initial, potent transcriptional increase in genes involved in lipid and carbohydrate biosynthesis, followed by marked compensatory downregulation (Ferno et al., 2009; Jassim et al., 2012).

Similar processes may be in operation during long-term drug exposure. Of note, in the present experiment separate cohorts of rats were sacrificed and sampled at each time point, and this may have influenced results. Ideally, the same rats should have been sampled at each time point, but this would limit plasma volume available for analysis and would not allow for tissue sampling.

After 1 month of treatment, but not at the later time points, hepatic expression of the rate-limiting lipogenic genes *Acc1*, *Fasn*, and *Hmgcr* was upregulated by olanzapine. Corresponding upregulation was observed in a subchronic context in treatment-naïve, 13-month-old rats that received their first-ever injection of olanzapine, but not 7 days after the last injection in rats having already been treated with olanzapine for 13 months. These results illustrate 2 points. First, while the majority of previous experiments have been performed in very young rats, young age is not a prerequisite for rats to be sensitive to the dysmetabolic potential of olanzapine. Secondly, long-term treatment diminished the lipogenic transcriptional response, which could be interpreted as a sign of desensitization to the lipogenic effects of olanzapine.

Notably, the upregulation of lipogenic genes was not accompanied by increased plasma lipids. *Acc1*, *Fasn*, and *Hmgcr* are involved in the early stages of fatty acid and sterol metabolism. To understand the full lipogenic impact of olanzapine in the liver and adipose tissues, transcription of downstream genes in complex lipid and lipoprotein biosynthesis and assembly should be examined, in addition to analyses of relevant protein and lipid levels.

In contrast to the analyses performed on dissected tissues, GTT and ITT were performed *in vivo*. After 1 year of olanzapine LAI treatment, these tests demonstrated the presence of olanzapine-induced hyperinsulinemia and pointed towards a reduction in insulin sensitivity, supported by the lack of increased fasting glucose levels in olanzapine-treated rats during the experiment. The association between olanzapine treatment and type 2 diabetes is well established in patients (Correll et al., 2017). The propensity to induce diabetes is correlated with affinity to muscarinic 3 receptors and could be due to inhibited insulin secretion, but several pathophysiologic mechanisms have been suggested (Reynolds and Kirk, 2010). In animal models, findings of glucose intolerance with concomitant decrease in insulin sensitivity and/or increased hepatic gluconeogenesis have been reproduced in short-term experiments with olanzapine as well as other atypical antipsychotics (Houseknecht et al., 2007; Chintoh et al., 2008). The GTT/ITT performed during this experiment did not allow us to determine the cause of hyperinsulinemia, but clearly demonstrated that the metabolically unfavorable olanzapine effects previously demonstrated in short-term experiments were also present at this chronic stage of treatment.

The choice of healthy rats as a model system may have influenced our findings, both with regard to metabolic disruption and major psychotic disorders. Lipid metabolism differs significantly in humans and rats, leaving rats far less vulnerable to dyslipidemia and atherosclerosis (Russell and Proctor, 2006). Dietary modification or use of genetically modified rodents are frequently used in animal studies on the metabolic syndrome (Aleixandre de Artinano and Miguel Castro, 2009). Psychotic disorders are also notoriously difficult to model in rodents, with pharmacological models potentially interfering with the effects of, for example, antipsychotic agents. Other approaches, such as the neurodevelopmental polyI:C model, have been successfully employed to examine metabolic effects of the antipsychotic

agent aripiprazole (Horska et al., 2017). The use of rats with a propensity to metabolic disruption and/or phenotypes reminiscent of schizophrenia would clearly increase the translational impact of experiments.

The choice of outcomes is also likely to have affected our results. In previously published studies, the use of functional testing such as oral lipid tolerance tests and isoproterenol challenges to examine lipolytic capacity has enhanced the understanding of metabolic derangements induced by short-term olanzapine exposure (Albaugh et al., 2011, 2012). As demonstrated through the GTT/ITT procedures performed during our experiment, metabolic challenges may also yield a more comprehensive impression of metabolic functioning during long-term treatment with olanzapine and should be included in future experiments.

To conclude, future experiments should include improved dosing regimens and ideally be performed in rats with phenotypes relevant for metabolic or psychiatric disorders. In addition, a true longitudinal design, with samples taken from the same rats at several time points, would be valuable. Finally, findings should be validated in the male rat. Still, bearing these limitations in mind, this work clearly shows that the use of olanzapine LAI in female rats facilitates long-term experiments, overcoming some of the previous obstacles in the field. Substantial weight gain, with accompanying signs of hyperinsulinemia, was maintained throughout the 1-year treatment period. Furthermore, *in vivo* tests supported the presence of reduced insulin sensitivity. The results from this proof-of-principle experiment could facilitate further unravelling of the molecular underpinnings of olanzapine-induced metabolic adverse effects.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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Statement of Interest

None.

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