The a-typical effects of olanzapine on body weight regulation
Evers, Simon

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Chapter 5:

Reproductive System

Olanzapine disrupts estrous cyclicity in female Wistar rats and induces hyperprolactineamia; a possible cause for weight gain, increased food intake, and insulin desensitization.

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Abstract

The second generation antipsychotic OLZ is known to cause severe body weight gain and induce type II diabetes in humans irrespective of gender. Rat studies, on the other hand, show that only female rats are sensitive to OLZ-induced weight gain and insulin resistance. Based on previous experiments we hypothesized that OLZ disrupts estrous cyclicity in rats, and that this effect contributes to energy balance and metabolic derangements of OLZ in female rats. First, we demonstrated that multiple doses of OLZ (2, 4, and 10mg/kg/day) increase body weight (BW), but also disrupt the estrous cycle (assessed by vaginal smears). Importantly, Control rats show reduced food intake specifically at the day of estrous, and this reduction is absent OLZ-treated rats. In a second study, we found that OLZ changes circadian food intake and activity patterns, and also induces a hyperinsulineamic response during a glucose challenge. Finally, OLZ did not affect circulating β-estradiol levels, but apparently blocked estrous related anorexia which was present in the Control group. OLZ increased circulating prolactin levels, which is most likely a result of its antagonistic action on the D2 receptor. OLZ-induced hyperprolactineamia is a possible cause for the observed disrupted estrous cycle and specifically in female rats this can cause hyperphagia. Together with decreased locomotor activity this results in increased weight gain and a reduction of insulin sensitivity.

Keywords:

Olanzapine, glucose tolerance, insulin sensitivity, prolactin, β-estradiol, estrous cycle, locomotor activity, feeding behavior, rat
Introduction

The benefit of Olanzapine (OLZ), a second generation antipsychotic (SGA) drug, in the treatment of schizophrenia is primarily based on reduced extrapyramidal symptoms [1,2] and a reduction of cognitive impairment [3-6] compared to first generation antipsychotics. Olanzapine results in enhanced tolerability and compliance in treating schizophrenia [7,8], but unfortunately also induces a variety of metabolic side effects, e.g. increased food intake, increased body weight, hyperglycaemia, hyperinsulinaemia, increased corticosterone/cortisol levels, hypothermia, and reduction of locomotor activity [9-14]. Of these negative side effects, the diabetogenic liability and severe body weight gain [15-19] associated with OLZ treatment are of major concern, although the underlying mechanisms are still not fully understood.

The broad spectrum of side effects related to OLZ treatment is caused by its atypical nature and its wide spread distribution of putative action sites. While OLZ is primarily designed to antagonize 5-HT2A/C and dopamine receptors (especially D2), it also has antagonistic properties on histaminergic, muscarinic, and α-adrenergic receptors [20-22]. These transmitter systems are –besides influencing cognitive and affective functions- intimately involved in neuroendocrine, ingestive behavioral, and metabolic regulatory systems, both in rodents and humans.

In a previous study [12] we showed that 10 mg/kg/day OLZ increased body weight after 14 days of treatment in female Wistar rats. We concluded that the increase of body weight (BW) was predominantly a result of reduced locomotor activity and thermogenesis, while no difference was observed in food intake. Another observation we made in that study was that OLZ-treated animals appeared to lack cyclicity in their feeding or BW regulation. This is different from the control situation in which female rats reduce their food intake and BW [23] and at the same time increase running wheel activity [24] during estrous, which repeats every 4-5 days. This observation was interesting, however, at that time we felt that it was beyond the particular scope of the study.

Still, OLZ may have some effects on the estrous cycle. Cooper et al [25,26] previously showed that the OLZ-induced changes of spontaneous activity and food intake in female Han Wistar rats is associated with changes in circulating sex hormones. OLZ treatment caused decreased levels of estradiol in female rats, and a dose dependent increase of prolactin [25,26]. Therefore, in the present study, we hypothesize that OLZ may affect the estrous cycle, and inhibit estradiol induced anorexia and hyperactivity, which may be one of the causes of OLZ-induced weight
gain, specifically in female rats. To evaluate the specificity of OLZ on cyclicity we administered OLZ (2, 4, and 10 mg/kg/day) for 14 days and took vaginal smears on 5 consecutive days to examine estrous stage and concomitantly measured food intake and fluctuations in body BW. From this set of data we determined that 4 mg/kg/day OLZ increased food intake and BW gain most effectively and decided to use this dose to do a follow-up study measuring circulating β-estradiol and prolactin levels, circadian home cage activity, food intake behavior, as well as glucose and insulin regulation. The data revealed that OLZ disrupts estrous cyclicity in female Wistar rats, which may, in part, explain the metabolic effects of OLZ.

Materials and Methods

2.1 Animals

All procedures involving animal care and experimentation were approved by the Animal Experimentation Committee of the University of Groningen. Female Wistar rats (211 ± 1.3 g; on arrival), obtained from Harlan (Horst, NL) were individually housed in clear Plexiglass cages (25 x 25 x 30 cm) with wood chip bedding. Room temperature was controlled at 22 ± 2 °C, under a 12:12 hr light-dark cycle (lights off at 11:00AM; CT=12). Baseline measurements started one week after surgery when all animals had surpassed their pre-surgical body weight. Animals had ad libitum access to standard chow (3.8 kcal/g) and water, unless otherwise specified.

2.2 Drugs

Olanzapine (as powder) was kindly provided by Solvay Pharmaceuticals (Fournier Laboratory, France). To obtain a dosage of 1, 2, or 5 mg/kg, OLZ was diluted to 0.5, 1, and 2.5 mg/ml resp. in 0.9% NaCl saline. Olanzapine was first dissolved in saline using 1M HCl and adjusted to pH 6.5 using 1M NaOH. Animals were administered intragastrically 2ml/kg OLZ solution or saline twice a day, prior to the dark phase (circadian time (CT) =11.5) and 6 hrs after lights turned off (CT=18).

2.3 Surgical Procedures

All surgical procedures were performed under isoflurane-O₂/N₂O gas-anesthesia. Animals were equipped with a permanent gastric catheter for intragastric drug administration. A silicon catheter (1.40-mm OD, 0.80-mm ID) was inserted through the gastric wall at the level of the corpus, extending 0.5 cm into the gastric lumen. The catheter was drawn subcutaneous towards the head where it was fixed to the skull with dental acrylic [27]. Double jugular vein cannulation was performed according to the methods described by Steffens [28]. The jugular vein cannula allows
stress free blood sampling according to methodologies described previously [29]. Analgesia (0.1 mg/kg Finadyne diluted in 0.1 ml/kg saline) was administered s.c. 15 minutes before animals were taken off anesthesia.

Exteriorized jugular vein and gastric cannulas were extended by a bent metal sleeve (20G), and closed by plastic caps made of a piece of flame-sealed PE100 tubing. Patency of the cannulas was checked twice a week starting 2 days after surgery to prevent blockage; i.e., the gastric cannula was rinsed with 0.5 ml saline; obstruction of the jugular vein cannulas was prevented by a 55% PVP solution (0.09ml sampling cannula, 0.06ml infusion cannula).

2.4 Exp. 1: OLZs dose response effect on body weight and food intake regulation

Sixteen female Wistar rats were equipped with a permanent gastric catheter and divided in four groups (Control, OLZ 2, OLZ 4, OLZ 10) matched for body weight at day -7 (222±1.4g). During baseline measurements, starting at day -7, all animals received twice daily intragastric injections of 2 ml/kg saline at CT12 and CT18, and food, water, and body weights were recorded every morning right before intragastric administration. Treatment started at day 0; per administration (2 ml/kg) animals received 2 ml/kg saline (Control), 1 mg/kg OLZ (OLZ 2), 2 mg/kg OLZ (OLZ 4), 5 mg/kg OLZ (OLZ 10). From day -1 animals had ad libitum access to a western style diet (4.7 kcal/g; 45% fat, 35% carb., 20% prot., Arie Blok Diets, Woerden, NL) to stimulate weight gain. All rats had ad libitum water access throughout the whole experiment.

At days 5 to 9 vaginal smears were collected between CT 11.5 and 12, by gently rotating a sterilized 4mm stainless steel oese app. 2cm into the vaginal cavity. The collected cells were deposited into a drop of regular tap water on a glass slide. Per animal two smears were collected per day. After drying slides at room temperature the smears were stained with Giemsa blue for 10 minutes. The slides were rinsed by keeping them with the back side up under a gentle stream of deminerzalized water, after which the slides were dried at room temperature again. The stage of the estrous cycle was determined by counting and identifying the cells under a light microscope (Olympus BH-2, 100x magnification). Three different cell types could be identified in the smear: 1) epithelial cells, 2) keratinized epithelial cells lacking a nucleus and mucous, 3) leukocytes (neutrophils and granulocytes).

Animals were sacrificed at day 14 by CO2 inhalation, after which abdominal adipose tissue depots (parametrial and retroperitoneal) were carefully removed and weight.

2.5 Exp 2: OLZ’s effect on glucose tolerance

Based on data from Exp.1, OLZ 4 was selected as the optimal dose to test OLZ’s effect
on glucose tolerance. Twenty-two female Wistar rats were equipped with a permanent gastric catheter for drug administration and a double jugular vein cannula for stress free blood sampling (described below). Animals were divided into two body weight matched groups (216±2.1g): Control and OLZ 4. Both groups followed the same feeding and administration protocol as described above. At day 14 an IV-GTT was performed, from day sixteen 8 animals were individually housed in a continuous food and activity registry cage (details below); and 10 animals were used for measuring circadian β-estradiol levels (details below, four animals were not used due to blockage of intravenous cannula). Animals were sacrificed at treatment day 21; animals were sedated via CO2 inhalation and decapitated, after which trunk blood was collected. Blood samples were immediately put on ice in vials containing 10μl EDTA (0.09 g/ml), after which they were centrifuged (15min, 2500 rpm, 4°C) and plasma was collected and stored at -20°C until further analysis. Visceral adipose tissue depots (parametrial and retroperitoneal) were carefully removed and weight.

**2.6 Intravenous-Glucose Tolerance Test (IV-GTT)**

IV-GTT was performed at the start of the dark phase (CT12). Prior to the start of IV-GTT, animals (N=19) were fasted for 4 hours. A baseline blood sample was drawn 60 minutes (t=-60) before starting the intravenous glucose infusion (t=0). A single dose of 2 mg/kg OLZ was intragastrically administered immediately after the baseline blood sample was drawn at t=-60 minutes. Blood samples (0.2 ml) were taken at time points -60, 0, 5, 10, 15, 20, 25, 30, 40, 60, and 120 minutes. Glucose (150mg/ml) was infused via the infusion cannula starting at t=0min at a rate of 0.1 ml/min and stopped at t=30min. Blood samples were immediately put on ice during IV-GTT in vials containing 10μl EDTA (0.09 g/ml). Whole blood samples of 50μl diluted in 450μl 2% heparin solution were stored at -20°C until analysis of glucose concentrations by the ferry-cyanide method[30] in a Technicon auto analyzer. The remaining blood samples were centrifuged (15min, 2500 rpm, 4°C) and plasma was collected and stored at -20°C until insulin determination. Plasma insulin levels were measured in duplicates using a commercial radioimmunoassay kit (Rat Insulin, 125I-Insulin Cat# RI-13K, Linco Reasearch, Inc., St. Charles, MO, USA).

**2.7 Circadian food intake and locomotor activity**

At day 16, eight animals (Control/OLZ: n=4/4) were individually housed in specialized cages (TSE Systems GmbH, Bad Homburg, Germany) for continuous food intake and locomotor activity registration. These Plexiglas cages (40*23*15cm) consisted of a sensitive weight balanced food station (stainless steel food container for standard size food pellets) for continuous food intake registration; water bottles were weighed
daily prior to dark phase. OLZ administration was continued twice a day at the above mentioned regimen.

2.8 Estradiol and prolactin measurement

From day 16 blood samples (0.2 ml) were drawn twice a day at CT0 and CT12 for 5 consecutive days in 10 animals (Control/OLZ: n=5/5). Blood samples were immediately stored on ice, in vials containing 10μl EDTA (0.09 g/ml), sample were centrifuged (15min, 2500 rpm, 4°C) and plasma was collected and stored at -20°C until plasma β-estradiol levels were analyzed using a commercial ImmuChem™ Double Antibody 17β-Estradiol 125I RIA kit (ICN Biomedical, Inc., Costa Mesa, CA, USA).

Prolactin was analyzed from plasma using a commercial rat PRL [125I] RIA KIT (MP Biomedicals Germany GmbH, Eschwege, D).

2.9 Data Analysis

All data are expressed as averages ± sem. Changes in BW (ΔBW in g) are presented as a difference compared to day 0 of treatment. FI is presented as caloric intake.

Vaginal smears are presented as the amount of cells collected, because smears of the OLZ treated animals lacked a sufficient amount of cells (<50cells) to detect estrous stage. In control animals estrous was defined as 70-100% of the cells were keratinized epithelial cells, proestrous was defined as 40-100% of the cells were nuclear epithelial cells; dioestrous was defined as 40-100% of the cells were leucocytes.

Because no synchronization was found between animals in the circulating β-estradiol levels, we determined per animal the peak β-estradiol level, the lowest β-estradiol level, the delta between the peak and lowest level, and the average circulating β-estradiol level excluding the peak level (average/4 days).

Circadian activity and feeding data are analyzed as an average per hour over consecutive days per individual. The first 24hrs in the food and activity registration cage was considered as a habituation period and was not used in the assessment of circadian food and locomotor activity patterns.

Statistical analyses were performed using repeated measures (rm)ANOVA between-subjects for time dependent analyses, oneway-ANOVA for between group analyses (post hoc LSD), and t-test for within group analyses. All statistical analyses were performed in SPSS20, outcomes were regarded significantly different when P<0.05. Graphs were designed using Graphpad Prism 5.0.
Results

Exp. 1a: dose response effect on body weight and food intake regulation

During the first experiment OLZ2, OLZ4, and OLZ10 doses were tested for effects on ΔBW over 14 days of treatment relative to Control treatment. Groups’ starting BWs at day 0 for Control, OLZ2, OLZ4 and OLZ10 groups were respectively 220±4g, 216±4g; 219±5g; and 221±7g. Figure 1a shows that over 14 days of treatment all dosages increased ΔBW compared to Control (rmANOVA: F_{42,168} = 5.675, Control vs. OLZ 2: P<0.01, OLZ 4: P<0.01, OLZ10: P<0.05). The ΔBW increase was only accompanied by an increase in the cumulative adipose tissue weights of the parametrial and retroperitoneal fat pad in the OLZ4 group compared to the Control group (P<0.05). The difference in weight gain was, in part, explained by the increased total caloric intake during drug treatment, because particularly the OLZ4 (P<0.01), and to a lesser extend the OLZ10 (P<0.05) animals showed an increase in total food intake compared to controls. No differences in water intake were found between groups.

Exp. 1b: Vaginal smears cell count

The average number of cells counted in a representative image of the smear was: Control=400±43, OLZ 2=53±25, OLZ 4=18±9, OLZ 10=22±5 cells (Control > OLZ 2/4/10; F_{3,15}=53.655, P<0.001, oneway-ANOVA post hoc LSD; fig. 2A). Although we were able to identify all the different cell types expected in a vaginal smear of Control and OLZ treated animals, none of the smears of the OLZ-treated animals showed a clear domination by mucous and keratinized epithelial cells (fig. 2B). Thus, in contrast to the Control rats, the low numbers and absence of clear variation between smears on consecutive days in OLZ rats made it impossible to identify estrous cyclicity in the OLZ-treated animals. This contrast heavily with the Control group (fig. 2C) where the average percentage of cell types per stage of the estrous cycle could be clearly indicated. In addition, in the Control group daily food intake during one estrous cycle was decreased at the day of estrous compared to the two days previous to estrous, diestrous-2 (P<0.01, t_6=3.830, independent t-test 2-tailed) and proestrus (P<0.05, t_6=2.892, independent t-test 2-tailed), and also compared to diestrous-2 (P<0.05, t_6=2.876, independent t-test 2-tailed) subsequent to estrous, but not on diestrous-1 (fig. 2D).
Fig 1: A) Body Weight: All doses of Olanzapine increase body weight compared to control over 14 days of drug treatment ($F_{42,168} = 5.675; \ P<0.01$ rm-ANOVA post hoc LSD: Control vs OLZ2, OLZ10: $P<0.05$; Control vs OLZ4: $P<0.01$). From day 4 till day 14 BW was significantly higher in OLZ4 compared to Control, from day 8 OLZ2 and OLZ10 showed significantly higher BWs compared to Control. B) Adipose tissue: No significant differences were found between parametrial and retroperitoneal fat pad weights of OLZ treated and Control groups. Only when both fat pads are add up together OLZ 4 shows increased adiposity compared to Control ($F_{3,15} = 3.287, P<0.05$ oneway-ANOVA post hoc LSD). C) Daily caloric intake: Daily caloric intake over 14 days of drug treatment was changed in OLZ4 and OLZ10 compared to control ($F_{42,168} = 1.598, P<0.05$ rm-ANOVA post hoc LSD: Control vs OLZ4: $P<0.01$; Control vs OLZ10: $P<0.05$). D) Cumulative food intake: Cumulative caloric intake over 14 days of drug treatment was increased in OLZ4 and OLZ10 compared to Control ($F_{3,15} = 5.107, P<0.05$ oneway-ANOVA post hoc LSD: Control < OLZ 4: $bP<0.01$; Control < OLZ 10: $cP<0.05$). Statistics: Control vs OLZ2: $aP<0.05$; A=P<0.01; Control vs OLZ4: $bP<0.05$; B=P<0.01; Control vs OLZ10: $cP<0.05$; C=P<0.01, oneway-ANOVA post hoc LSD)
Exp. 2a: OLZ4 body weight and food intake regulation

Based on the data in Experiment 1, we selected the OLZ4 dose in a follow-up study to investigate the effect of OLZ on BW homeostasis and glucose regulation. Fig. 3a shows that OLZ4-treated rats had significantly elevated BWs compared to Controls over the course of 14-day treatment (rm-ANOVA: $F_{14,266} = 6.488$, $P<0.0001$). Weight trajectories in the OLZ4 groups of both experiments 1 and 2 were comparable. Unlike experiment 1: compared to Control cumulative 14 day food intake was not increased in the OLZ4 treated group (Control=954±23kcal; OLZ4=1011±27kcal). Rm-ANOVA did show a difference between daily caloric intake curves (rmANOVA: $F_{13,247}=2.441$, $P<0.01$). Compared to Control in the OLZ4 group daily caloric intake was

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**Fig. 2:** A) Total cell count vaginal smears. Compared to the control group, OLZ treated groups showed a decrease of total amount of cells per smear (Control vs OLZ2/4/10: oneway-ANOVA post hoc LSD, $P<0.001$). B) Representative picture of a vaginal smear of a rat in estrous (200x magnification) stained with Giemsa blue. The smear consists of non-nuclear keratinized epithelial cells and mucous (black arrow, blue staining), epithelial cells with a nucleus (black arrow head, purple staining), and leukocytes (white arrow head, purple staining). C) Estrous cycle in control animals, based on the percentage of different cell types (lymphocytes, epithelial cells with a nucleus, and epithelial cells without a nucleus) per smear.
decreased at day 0 (oneway-ANOVA: $F_{1,19}=7.348, P<0.05$) and day 1 ($F_{1,19}=10.894, P<0.01$), whereas it was increased at day 5 ($F_{1,19}=4.654, P<0.05$), day 6 ($F_{1,19}=8.787, P<0.01$), day 8 ($F_{1,19}=8.783, P<0.01$), and day 9 ($F_{1,19}=6.786, P<0.05$).

Fig 3: a) Body Weight: OLZ changes BW gain significantly over 14 days compared to control (rm-ANOVA: $P<0.001$). Only at day 10 and 13 of treatment BW in OLZ 4 is significantly higher compared to Control (oneway-ANOVA: *$P<0.05$). b) Daily caloric intake: At day -1 animal were put on a western style diet. Daily caloric intake was significantly changed during 14 days of OLZ treatment (rm-ANOVA: $P<0.05$). At day 0 of treatment caloric intake was increased in the Control group compared to the OLZ 4 group, at days 5, 6, 8, and 9 of treatment daily caloric intake was increased in the OLZ treated group (oneway-ANOVA: *$P<0.05$, #$P<0.01$).

Exp 2b: Glucose tolerance and insulin response

At day 14 of treatment an IV-GTT was performed (fig. 4). No difference in baseline glucose or insulin levels between groups at $t=-60$ min were observed. Immediately after $t=-60$ min OLZ4 or vehicle were intragastrically administered as part of their daily dosing. The intragastric administration did not affect circulating glucose and insulin levels in the second baseline blood sample ($t=0$). The ensuing changes in blood glucose levels in the OLZ4 and Control group during IV-GTT were comparable (fig. 4a), resulting in similar areas under the curve (Control=279±39, OLZ=234±29). However, a major increase in the insulin response was observed in the OLZ4 rats compared to Control rats (rm-ANOVA; $F_{9,126}=7.640, P<0.001$). Insulin levels were increased at every time point during the 30 min-infusion and dropped to control levels at 45 min after the start of glucose infusion (see fig. 4b). This resulted in an increased AUC in OLZ-treated rats compared to Controls (Control=295±68, OLZ=642±127; oneway-ANOVA: $F_{1,16}=6.209, P<0.05$).
Fig. 4: Intravenous – Glucose Tolerance Test. A) Glucose response: OLZ does not affect the glucose response during an IV-GTT after 14 days of drug treatment compared to control (Control: n=9, OLZ: n=9; F_{9,144} = 0.502  rm-ANOVA; ns). B) OLZ increases circulating insulin during an IV-GTT after 14 days of drug treatment (Control: n=8, OLZ: n=8; F_{9,126} = 7.640  rm-ANOVA; P<0.001).

Exp 2c: Circadian food intake and locomotor activity

From the 16th till the 20th day of treatment 8 animals (Control: n=4, OLZ 4: n=4) were housed in continuous food and activity registration cages. OLZ 4 changed the circadian food intake pattern over 24 hour registration (rm-ANOVA: F_{23,138}=2.396, P<0.01). Food intake was increased significantly in the OLZ 4 group compared to Controls between CT8 and CT11 (see fig. 5a). At CT0, however, food intake in OLZ 4 rats was significantly decreased compared to Controls (see fig. 5a). Average 24 hour food intake was not changed during the period animals were housed in these continuous registration cages (Control = 12.4±0.7g, OLZ 4 = 12.50 ± 0.9g; F_{1,7}=0.005, P=0.944). However, when food intake was divided into light and dark phase; OLZ4 increased food intake during the light phase compared to Controls (Control = 3.8±0.2g; OLZ 4 = 5.4±0.4g; oneway-ANOVA: F_{1,7}=13.618, P<0.05), whereas no significant difference was observed between dark phase food intakes of Control and OLZ-treated rats (Control = 8.6±0.5g; OLZ 4 = 7.1±0.5g; oneway-ANOVA: F_{1,7}=4.985, P=0.067).

The circadian locomotor activity patterns (fig. 5b) of Control and OLZ4 differed significantly (rm-ANOVA: F_{23,138}=5.900, P<0.01), and had comparable fluctuation patterns as food intake. Locomotor activity was increased in OLZ 4 rats compared to Controls during the light phase at CT 6, 10, and 11, while it was decreased in the OLZ4 rats at CT 0, CT 18, 19, and 20. Total 24 hour locomotor activity was decreased in the
OLZ4 group (53.0±5.1) compared to the Control group (70.2±9.4; \( F_{1,7}=6.889, \ P<0.05 \), oneway-ANOVA), however, during the light phase locomotor activity was increased in the OLZ4 group (30.2±2.1) compared to the Control group (22.3±2.3AU; \( F_{1,7}=6.507, \ P<0.05 \)), whereas the locomotor activity was decreased in the OLZ4 group during the dark phase (Control=47.9±7.4 OLZ 4=22.8±3.7; oneway-ANOVA: \( F_{1,7}=9.414, \ P<0.05 \)). This was reflected in the percentage of activity spent in the dark phase: Control=68.2±2.4%, OLZ 4=43.3±3.7% (oneway-ANOVA: \( F_{1,7}=31.841, \ P<0.001 \)).

Fig. 5: Circadian food intake and locomotor activity. On the horizontal axis is the circadian time, light go on at CT 0 and turn off at CT 12; the arrows show the time of drug administration at CT 12 and CT 18. Both graphs represent the average per hour on day 16 till 20 of treatment. A) Food intake: Total 24hr food intake is not different between OLZ and Control. OLZ changes the circadian food intake pattern compared to control (\( F_{23,138}=2.396, \ P<0.01 \), rm-ANOVA) and increases food intake at CT8 (\( F_{1,7}=6.033, *P<0.05 \)), CT9 (\( F_{1,7}=27.417, *p<0.01 \)), CT10 (\( F_{1,7}=33.456, *p<0.01 \)), and CT11 (\( F_{1,7}=18.025, #p<0.01 \)); whereas food intake is decreased at CT 0/24 (\( F_{1,7}=6.441, P<0.05 \), oneway-ANOVA). B) Locomotor activity: OLZ changes the circadian activity compared to control (\( F_{23,138}=5.900, \ P<0.01 \), rm-ANOVA) according to the administration paradigm (administration at CT12 and CT18). Compared to Control, OLZ decreases total 24hr activity (\( F_{1,7}=6.889, P<0.05 \) oneway-ANOVA), decreases dark phase activity (\( F_{1,7}=9.414, P<0.05 \), oneway-ANOVA), but increases light phase activity (\( F_{1,7}=6.507, P<0.05 \), oneway-ANOVA). Per hour locomotor activity is increased in the OLZ group compared to Control at CT6 (\( F_{1,7}=6.944, *P<0.05 \)), CT10 (\( F_{1,7}=8.103, *P<0.05 \)), and CT11 (\( F_{1,7}=28.368, *P<0.01 \)); whereas activity is decreased in the OLZ compared to Control at time points CT0/24 (\( F_{1,7}=11.915, *P<0.05 \)), CT18 (\( F_{1,7}=81.289, #P<0.01 \)), CT19 (\( F_{1,7}=30.321, #P<0.01 \)), and CT20 (\( F_{1,7}=8.898, *P<0.05 \)).
Exp. 2d: Circulating β-estradiol levels and food intake.

From day 16 till 20 blood samples were collected at time points CT0 and CT12 to assess circulating β-estradiol. After analyzing the samples we decided the peak level of every individual animal over the 5-day period and averaged the other samples as a baseline value (average/4 days). Table 1 shows that there were no differences between Control and OLZ treated animals in the peak β-estradiol levels (Controlpeak=90.90±15.93pg/ml; OLZpeak=98.34±15.01; oneway-ANOVA: F1,9=0.116, P=0.743), nor did we find any difference in the lowest circulating β-estradiol level (Controllow=47.50±9.07pg/ml; OLZlow=51.80±4.19; oneway-ANOVA: F1,9=0.185, P=0.678), the delta between peak and lowest levels (Controldelta=43.40±10.45pg/ml; OLZdelta=46.54±15.96; oneway-ANOVA: F1,9=0.026, P=0.875), or the average circulating β-estradiol level excluding the peak levels (Controlaverage=60.18±7.04pg/ml; OLZaverage=59.65±4.60; oneway-ANOVA: F1,9=0.001, P=0.977). Food intake was measured daily at CT12, total food intake was not significantly different during the 5 days of blood sample collection between Control (66.40±4.63g) and OLZ 4 (76.20±2.15g; oneway-ANOVA: F1,9=3.680, P=0.091). On the day circulating β-

Table 1: Circulating β-estradiol levels and related food intake.

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<th>Estradiol levels (pg/ml)</th>
<th>Control</th>
<th>OLZ</th>
<th>P-value</th>
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<td><strong>Peak</strong></td>
<td>90.90±15.93</td>
<td>98.34±15.01</td>
<td>0.743</td>
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<td><strong>Lowest</strong></td>
<td>47.50±9.07</td>
<td>51.80±4.19</td>
<td>0.678</td>
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<tr>
<td><strong>Delta</strong></td>
<td>43.40±10.45</td>
<td>46.54±15.96</td>
<td>0.875</td>
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<tr>
<td><strong>Average/4 days</strong></td>
<td>60.18±7.04</td>
<td>59.65±4.60</td>
<td>0.977</td>
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<table>
<thead>
<tr>
<th>Food Intake (g)</th>
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<tbody>
<tr>
<td><strong>Peak</strong></td>
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<td>16.00±0.55</td>
<td>0.005*</td>
</tr>
<tr>
<td><strong>Lowest</strong></td>
<td>13.90±0.62a</td>
<td>15.00±0.45</td>
<td>0.188</td>
</tr>
<tr>
<td><strong>Average/4 days</strong></td>
<td>13.75±0.89b</td>
<td>15.05±0.44</td>
<td>0.228</td>
</tr>
</tbody>
</table>

*Peak: Day with highest circulating β-estradiol level measured. Lowest: Day with lowest circulating β-estradiol level measured. Delta: the difference between the highest (peak) and lowest circulating β-estradiol level measured. Average/4 day: the average of the 4 days outside the day β-estradiol peaked. No differences have been measured between β-estradiol levels between control and OLZ treated animals. At the day β-estradiol peaks Control animals show a lowered food intake compared to OLZ treated animals (*P<0.01 oneway-ANOVA; F1,9=14.493). Within-subject analysis shows food intake in the Control group is decreased at the day β-estradiol peaked compared to the day the lowest β-estradiol was measured (P<0.05, paired T-Test) and to average 4 days food intake (P<0.01, paired T-Test).
estradiol level peaked food intake was lower in the Control compared to OLZ4-treated group (Control peak = 11.40±1.07g; OLZ peak = 16.00±0.55; oneway-ANOVA: F_{1,9} = 14.493, P<0.01), no significant differences were found on the average food intake between Control and OLZ4-treated group besides the β-estradiol peak day (see table 1). Within-subject analyses showed that in the Control group food intake was decreased at the day β-estradiol peaked compared to the day the lowest circulating β-estradiol was measured (P<0.05, t_{4} = -3.835, paired t-test), and compared to the average 4 day food intake (P<0.01, t_{4} = -10.136, paired t-test). No within-subject differences in food intake on the day β-estradiol peaked versus the average 4 day intake (P=0.099, t_{4} = 2.144, paired t-test) or compared to the day the lowest circulating β-estradiol was found (P=0.210, t_{4} = 1.491, paired t-test) in the OLZ4-treated group (see table 1).

**Exp. 2e: Post mortem prolactin levels.**

Animals were sacrificed after 21 days of treatment via decapitation, trunk blood was collected and plasma prolactin levels were measured. Prolactin was significantly increased in the OLZ4-treated group compared to Controls (fig.6; Control=29.1±6.85 ng/ml, OLZ 4=91.3±1.49 ng/ml; oneway-ANOVA: F_{1,10} = 84.225, P<0.001).

![Prolactin](image)

**Fig. 6:** Post mortem plasma prolactin levels. After 21 days of drug treatment, compared to control, prolactin levels are increased in the OLZ4 treated group (**P<0.001, oneway-ANOVA).
Conclusions and Discussion

Exp. 1: OLZs dose response effect on body weight, food intake and estrous cyclicity.

All doses of OLZ (2, 4, and 10 mg/kg/day) administered via a permanent gastric catheter during the dark phase increased body weight over 14 days of treatment. The increase in body weight seen in all three treatment groups was only partly explained by changes in food intake because only the OLZ4 and OLZ10 groups increased food intake significantly compared to the Control group. This suggests that a reduction in metabolic rate induced by OLZ was, at least in part, responsible for an increased body weight gain. In a previous study [12], we already showed that OLZ dosed at 10mg/kg/day in a similar administration protocol decreased body temperature and locomotor activity. In that study, body weight gain was increased in the OLZ10 group as well, despite the fact that food intake was not increased compared to Controls. This suggests that reduced metabolic rate, either or not directly related to reduced locomotor activity, is a contributing factor to OLZ-induced weight gain. The increased weight gain was reflected only in the OLZ4 group by an increased sum of parametrial or retroperitoneal adipose tissue weight, suggesting that other depots contributed as well to the general effect of OLZ treatment to increase body weight.

From day 5 till day 9 of treatment we collected vaginal smears to assess the stage of the estrous cycle for each animal. Whereas the animals from the Control group offered smears from which a clear estrous cyclicity could be deduced, we were not able (except for one animal in the OLZ2 group) to observe estrous cyclicity in the OLZ treated rats due to 1) a low level of cell counts, and 2) irregular presence of different cell types over time.

Exp.2: OLZ increases insulin response and increases circulating prolactin level, but does not affect cyclic β-estradiol levels.

Based on the body weight and adiposity data from Exp.1, we decided to assess the effects of 4 mg/kg/day OLZ on the glucose and insulin responses during an IV-GTT after 14 days of treatment. At day 10 and day 13 of treatment body weight gain was significantly increased in the OLZ4 group compared to Controls in a comparable fashion as in Exp 1. However, unlike in Exp. 1, OLZ4 did not increase cumulative 14-day food intake. Nevertheless, OLZ4 caused a significant hyperinsulineamic response during the IV-GTT (fig. 4b), suggesting that 14 days of OLZ4 treatment is indeed sufficient to decrease insulin sensitivity.

Under the current administration protocol, from day 16 to 20, a very distinctive
The circadian pattern of the rats’ food intake and locomotor activity could be observed (see fig. 5). Particularly striking are the strong reductions in locomotor activity after the consecutive OLZ infusions, and a more subtle reduction in food intake observed only after the second OLZ infusion. These inhibitory effects were probably due to the sedative actions of OLZ [31]. Interestingly, these OLZ-induced suppressions were followed during the 4 hours prior to the onset of dark phase (CT12), by a profound increase in food intake. One explanation for this delayed hyperphagia may be that chronic OLZ treatment induces a powerful hyperphagic drive, which is transiently obstructed by OLZ’s immediate sedative effects. Alternatively it may be hypothesized that, animals might have adapted to the OLZ administration protocol by anticipating the sedative effects of OLZ [12]. Either way, decreased locomotor activity together with a transient increase in food intake were probably a major cause of OLZ-induced weight gain.

In a second group of animals we measured circulating β-estradiol levels at CT0 and CT12 for 5 consecutive days (day 16-20). In most of the animals of both the Control and OLZ treated group a clear peak in circulating β-estradiol was found at one single time-point during the 5 consecutive days of sampling. For each animal we determined the β-estradiol peak and found that OLZ did not affect the height of the circulating β-estradiol peak, nor did we find a difference of average circulating β-estradiol besides the peak. Thus, the results in Exp.1, in which vaginal smears of OLZ-treated animals showed a disruption of estrous cyclicity, was not reflected by derangements in the circulating β-estradiol pattern. However, when we consider the suppression of food intake that normally occurs at the peak day of β-estradiol levels [32,33] - which we clearly observed in the Controls of the present study - a, this suppression was not observed in the OLZ-treated animals during the day their β-estradiol levels peaked or at any other day. Besides a reduction in food intake, others have reported that the β-estradiol peak coincides with an increase in locomotor activity [32,33], but we unfortunately were unable to assess locomotor activity in addition to food intake in the same animals that were checked for oestrous cyclicity and β-estradiol levels. This would have been very interesting in light of the findings by Ohtani et al [34], who reported that spontaneous locomotor activity is decreased in ovariectomized (OVX) rats and restored by estradiol replacement. Furthermore, OVX animals have a blunted hyperactivity response and lower striatal dopamine release to methamphetamine administration compared to controls, which was also found to be restored by chronic estradiol replacement. Collectively, these data may be interpreted to indicate that estradiol stimulates the sensitivity of the dopaminergic system to tune oestrous cyclicity and energy balance, but it remains to be investigated whether such an effect is blocked by OLZ.
In contrast to the unaltered levels of β-estradiol, OLZ4 induced a 3-fold increase of plasma prolactin after 21 days of treatment relative to the levels found in Controls. Prolactin has been shown, by Naef et al [35], to increase food intake when administered chronically for 10 days into the lateral ventricle (5μg/h). Chronic infusion of prolactin in the cerebral ventricles reduced the efficacy of centrally administered leptin to reduce food intake or body weight. The reduction in leptin responsiveness due to prolactin treatment was found to be caused by an inhibition of the leptin signaling cascade in hypothalamic nuclei. Interestingly, this down-regulation of leptin signaling per sé by central prolactin was not causative to the diminished oestrus cyclicity [35]. Instead, Noel et al [36] reported that only prolactin administered systemically, and not centrally, disrupts estrous cyclicity. Accordingly, OLZ-induced increase in food intake, body weight, and disruption of estrous cyclicity seen in the experiments described here might be a result of increased circulating prolactin by distinct central and peripheral mechanisms. This also might be a key factor why in rats only female, and not male, animals gain weight on chronic OLZ treatment [25]. Hence, studies performed by Heil et al [37,38] showed that prolactin only increased food intake in female rats and not in male rats. This increase in food intake by s.c. administered prolactin (2mg/kg/day), was most prominent at 5-10 days of treatment, which was also the case in the study performed by Noel et al [36], where s.c. administered prolactin (1 and 3mg/kg) increased food intake, body weight, and disrupted estrous cyclicity. Similarly, our studies show that the increase in body weight gain and food intake, and the disruption of estrous cyclicity in the OLZ group occurs within a comparable time period.

While the OLZ effect to increase prolactin levels may seem quite dramatic, they may be still rather weak compared to other antipsychotic agents. In fact, Kinon et al [39,40] extensively showed that conventional antipsychotics in schizophrenic patients (both male and female) cause an increase in prolactin levels, which are considerably higher than those found with OLZ treatment. Hyperprolactinemia in these studies was especially found in females (between 65.6% and 45.1% depending on reproductive age), and less across males (42.1%). Melkersson et al [41] found that indeed risperidone induces hyperprolactinemia in 89% of the subjects, but still 24% of OLZ treated subjects were considered hyperprolactineamic. The OLZ induced hyperprolactineamia in Melkersson’s study was also more common in female subjects than men, which may also point to gender-specific effect of OLZ treatment in humans, analogous to that in rats.

In conclusion, we report here that doses ranging from 2-10 mg/kg/day of OLZ increase body weight over 14 days of treatment and disrupt estrous cyclicity in
female Wistar rats. OLZ (4mg/kg/day) increases the insulin response, reduces locomotor activity acutely, and increases food intake especially during the light phase as a response to the drug administration protocol. Furthermore, OLZ increases circulating prolactin levels, but does not influence circulating β-estradiol levels. However, OLZ does show to block β-estradiol’s anorectic effect. We suggest that, besides the reduction of locomotor activity, increased prolactin is one of the factors by which especially female rats increase body weight, food intake, and decrease insulin sensitivity due to OLZ treatment. Other harmful effects of hyperprolactineamia induced by antipsychotic drug treatment include symptoms of impotence, menstrual disturbances, decreased libido, and infertility [39-41], and decreased bone mineral density and osteoporosis in both humans [42] as well as rats [43]. Understanding the mechanisms by which OLZ interacts with central and peripheral pathways may eventually lead to a strategy to diminish the side effects associated with OLZ treatment.

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