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## The a-typical effects of olanzapine on body weight regulation

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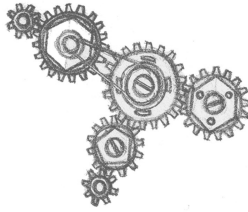
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## Chapter 3:

# Gastroenterology

**Olanzapine disturbs luminal glucose absorption via inhibition of gastrointestinal motility.**

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## Abstract

The second generation antipsychotic (SGA) Olanzapine (OLZ) is commonly used in the treatment of schizophrenia. OLZ is known for its weight gain and insulin resistance inducing effects, but also constipation is a common side-effect in patients treated with SGAs, like Clozapine and OLZ, having a high affinity for the M3 receptor. In addition, OLZ has a high affinity to the 5-HT receptors, which in the gastrointestinal tract is also involved in luminal absorption and peristalsis. We hypothesized that OLZ attenuates gastrointestinal motility and that this might disturb luminal glucose absorption and hinder an adequate insulin response. We show that OLZ reduces circulating insulin levels during an intragastric-glucose tolerance test (IG-GTT), which is a consequence of reduced glucose uptake from the gut. We show that after 60 minutes of intragastric glucose infusion glucose is present in the gut of OLZ treated animals up to the level of the ileum, whereas glucose is undetectable in the gut of the control treated group. In addition, we show that OLZ blocks acetylcholine and serotonin induced smooth muscle contraction *in vitro*, and *in vivo* reduces carmine red transport through the gastrointestinal (GI) tract. Via PET-scan we additionally show that OLZ reduces gastric glucose release and increases glucose levels in the duodenum. In conclusion, we show that OLZ affects glucose absorption from the gastrointestinal tract mainly via reducing GI peristalsis and hinders an adequate insulin response.

### Keywords:

Olanzapine, gastrointestinal tract, peristalsis, glucose absorption, acetylcholine, serotonin, rat

## Introduction

The second generation antipsychotic (SGA) Olanzapine (OLZ) is commonly used in the treatment of schizophrenia. OLZ is designed to have antagonistic properties primarily for dopamine ( $D_{1,2,4}$ ) and serotonin ( $5\text{-HT}_{2A/C,3}$ ) receptors, but also shows antagonistic affinity for the histamine ( $H_1$ ),  $\alpha$ -adrenergic ( $\alpha_1$ ), and muscarinic ( $M_{1,2,3,4}$ ) receptors [1]. In contrast to the first generation antipsychotics, which are highly selective for dopamine receptors, SGA's like OLZ are therapeutically favored because they induce less extrapyramidal side effects [2]. However, during the last decade, OLZ became notorious for its severe weight-gaining and diabetes-inducing properties. The mechanisms via which OLZ induces its metabolic side effects are still a topic of discussion, but certainly relate to altered food intake behavior [3,4] and insulin sensitivity [5-8]. Underexposed are the effects OLZ has on gastrointestinal functioning, especially because of the relation between gastrointestinal complications and psychiatric disorders [9]. Moreover, constipation is a common side-effect in patients treated with SGAs, like Clozapine [10,11] and OLZ [12], having a relatively high affinity for the M3 receptor. Multiple studies have shown the deteriorating effects of antimuscarinic agents on gastrointestinal functioning. For example, pretreatment of rats with the antimuscarinic agent propantheline (i.p.) delayed and decreased lead absorption [13] and the selective M3-antagonist darifenacin delays small bowel transition [14]. These studies show that smooth muscle gastrointestinal peristalsis is predominantly regulated via M3 receptor pathways. For that matter OLZ has been used in the treatment of irritable bowel syndrome [15]. It is thought that OLZ's effect on gastrointestinal functioning is predominantly a result of its antimuscarinic effect [12]. The  $5\text{-HT}_{2A/C,3}$  antagonistic properties of OLZ may provide additional or alternative routes of action. In fact, Nakajima *et al* show that 5-HT-induced smooth muscle contraction is blocked by the nonspecific 5-HT<sub>1A</sub> agonist/5-HT<sub>2C</sub> antagonist methysergide, and the specific 5-HT<sub>3</sub> antagonist ondansetron in the gastric antrum and duodenum, but not the ileum (which was only sensitive to the selective M3 antagonist atropine). Since these receptor affinity characteristics are combined in OLZ, this does point towards the possibility that OLZ indeed may target 5HT mechanisms in the GI tract. We hypothesized that OLZ attenuates gastrointestinal motility and that this might disturb luminal glucose absorption and hinder an adequate insulin response. We performed an *in vivo* intragastric-glucose tolerance test (IG-GTT) and measured circulating glucose and insulin levels, but also measured gastrointestinal (GI)-transport and the amount of unabsorbed glucose still present in the lumen of the GI-tract. We measured the effect of OLZ *in vitro* on duodenal and jejunal

smooth muscle contraction. In addition, we also measured luminal [ $F^{18}$ ]-FDG glucose expression in a small cohort of animals after intragastric administration using positron emission tomography (PET). Finally, we conclude that intragastric administered OLZ acutely attenuates the amount of luminal glucose entering the circulation via deceleration of gastric emptying and gastrointestinal motility, through 5-HT and M3 receptor antagonism.

## Methods

### *Experiment 1: Animals and surgery*

Eighteen male Wistar rats (Harlan, Horst, NL), weighing  $459 \pm 6$ g were used in these experiments. Upon arrival, they were housed individually in a 24x24x36cm Plexiglas cage with wood chip bedding, a wooden gnawing stick, paper nesting material, and maintained under a 12-12hr light-dark cycle (lights off at 11:00hr) with *ad libitum* access to standard chow (AB diets, Woerden, NL) and water, unless otherwise stated. One week after acclimatization, they underwent surgery (under high O<sub>2</sub>-low CO<sub>2</sub> isoflurane inhalation anesthesia), during which a silicon gastric cannula (1.40mm OD, 0.80mm ID) was inserted in the corpus of the stomach [16] and a second silicon cannula (1.40mm OD, 0.80mm ID) was inserted in the right jugular vein [17]. The jugular vein cannula allows stress free blood sampling and the intragastric cannula can be used for drug administration according to methodologies described elsewhere [18]. Both cannulas were subcutaneously guided to the head, where they were exteriorized via a bent 19G stainless steel metal sleeve, which was anchored to the skull by surgical stainless steel screws and dental cement. Post-surgery 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. Both exteriorized jugular vein and gastric cannulas were closed by plastic caps made of a piece of flame-sealed PE100 tubing, and were rinsed twice a week starting 2 days after surgery to prevent blockage. The gastric cannula was rinsed with 0.5 ml saline; patency of the jugular vein cannula was maintained by filling the cannula between blood sampling with a 55% PVP/heparin solution [17]. These and all other procedures were approved by the animal ethical committee of the University of Groningen.

### *Drug administration*

Pure powdered Olanzapine was kindly provided by Solvay Pharmaceuticals (Fournier Laboratory, France). Olanzapine was dissolved, after acidification using 1 M HCl, in 0.9% NaCl saline at a concentration of 0.51mg/ml (OLZ 10 mg/kg) and 1.02mg/ml

(OLZ 20mg/kg) and adjusted to pH 6.5-7 using 1 M NaOH. Animals were administered saline, 10, or 20 mg/kg Olanzapine in combination with 150 mg/ml glucose at the start of each study by a 9-min constant infusion (1ml/min) via the gastric cannula, while the animals were freely moving in their home cage.

#### *Intragastric Glucose Tolerance Test*

IG-GTTs were performed at the start of the dark phase (11AM). Prior to the start of an IG-GTT, animals (n=6) were fasted for 4 hours. Then animals were connected about 45 min before the start of the IG-GTT, and a baseline blood sample was drawn 15 minutes (t=-15) before the start (t=0) of the combined glucose and Olanzapine infusion. Blood samples (0.2 ml) were taken at time points -15, 0, 5, 10, 15, 20, 25, 30, 40, and 60 minutes. Blood samples were immediately put on ice during IG-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 ferricyanide method [19] 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, <sup>125</sup>I-Insulin Cat# RI-13K, Linco Research, Nucli Lab, NL).

#### *In vivo gastrointestinal motility and luminal glucose transport*

Indigestible carmine red dye (10mg/ml; BUFA bv, Uitgeest, NL) was added to the Olanzapine/glucose infusate, and administration of it was identically performed as in the above-mentioned IG-GTT, however no blood samples were taken. At 60 min post-infusion animals were sacrificed by jugular infusion of 0.25ml pentobarbital. Immediately thereafter, the abdominal cavity of each rat was opened sagittally by surgical scissors, and the whole gastrointestinal tract was removed and divided into 7 segments by ligation with surgical silk. The segments were divided into stomach (0cm), duodenum1 (0-5cm), duodenum2 (5-10cm), jejunum1 (10-30cm), jejunum2 (30-50cm), jejunum3 (50-70cm), jejunum4 (70-90cm), ileum (90-110cm). The content of each segment was collected in 10ml vials by rinsing the lumen of each segment with demineralized water; 1ml for stomach and duodenum segments; 4ml for jejunum and ileum segments. Vials were centrifuged for 10min (1500rpm, 4°C) and 1ml supernatant was collected for analyses of carmine red and glucose concentration, results were corrected for dilution factor. Glucose concentrations were measured in an auto analyzer as described above and are expressed as mg/ml, carmine red concentration was determined using spectrophotometry with peak absorbance at 529nm (E580) wave length and expressed in mg/ml.

### *Experiment 2: In vitro gastrointestinal motility*

Eight male Wistar rats (Harlan, Horst, NL) were anesthetized by isoflurane inhalation after which the duodenum + proximal jejunum (app. 16 cm) were removed and transferred to a Krebs–Henseleit (KH) buffer solution (composition in mM: NaCl 117.5, KCl 5.6, MgSO<sub>4</sub> 1.18, CaCl<sub>2</sub> 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.28, NaHCO<sub>3</sub> 25.00 and D-glucose 5.55; pre-gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>; pH 7.4) at 34°C. The duodenum was carefully separated from adipose and connective tissue. Segments of approximately 2cm were mounted for isometric recording, using Grass FT-03 transducers, in 50 ml water-jacketed organ baths (34°C) containing KH solution. During a 60min equilibration period, with washouts every 15 min, resting tension was gradually adjusted to  $\sim 5 \times 10^{-4}$  Newton. Subsequently, the preparations were exposed to 40 mM KCl to obtain maximal contraction. Following two washouts, maximal relaxation was established and tension was readjusted to  $\sim 5 \times 10^{-4}$  Newton, immediately followed by two changes of fresh KH-buffer [20]. After another equilibration period of 30 min we added 0,5ml 10mM (3.12mg/ml) OLZ solution (or saline) to reach a 100 $\mu$ M OLZ concentration, cumulative concentration response curves (CRCs) were constructed to stepwise increasing concentrations of acetylcholine (ACh;  $10^{-8}$  mM –  $1.33 \cdot 10^{-4}$  mM), and serotonin (5HT;  $10^{-8}$  mM –  $10^{-5}$  mM). Per animal eight segments were collected and divided over four experimental groups: ACh (control vs OLZ 100 $\mu$ M), and 5HT (control vs OLZ 100 $\mu$ M). Contraction induced by ACh and 5HT are expressed as a percentage of KCl induced maximal contraction (%KCl<sub>max</sub>).

### *Experiment 3: [<sup>18</sup>F]-FDG small animal positron emission tomography*

As a proof of principle small animal positron emission tomography (PET) with the glucose analog [<sup>18</sup>F]-FDG was used to determine the effect of Olanzapine on luminal glucose uptake after intragastric infusion. Rats received a 9-minute infusion (1ml/min) of glucose (150 mg/ml) mixed with  $21.3 \pm 3.0$  MBq of [<sup>18</sup>F]-FDG. Olanzapine (5mg/kg; n=2) or control (saline, n=3) was administered intragastrically 30 minutes prior to the start of glucose infusion. After the infusion the rats were anaesthetized by isoflurane (5% induction, 2% maintenance, Pharmachemie BV, The Netherlands) that was mixed with medical air at a flow of 1 ml/min. Following anesthesia the rats were positioned in the small animal PET camera (Focus 220, Siemens Medical Solutions USA, Inc.) in transaxial position with their stomach in the field of view. A dynamic emission scan of 90 minutes was started 30 minutes after the initiation of glucose infusion, 60 minutes after OLZ or saline administration. Following the emission scan a transmission scan with a Co-57 point source was obtained for the correction of attenuation and scatter by tissue.

The list-mode data of the emission scan was separated into 6 frames of 15 minutes. Emission sinograms were iteratively reconstructed (OSEM2d, 4 iterations) after being normalized, corrected for attenuation and decay of radioactivity. The PET images were analyzed using PMOD 3.3 (PMOD Technologies Ltd., Zurich, Switzerland). Regions of interest were manually drawn around the stomach, duodenum and small intestines. Time-activity curves of the average [ $^{18}\text{F}$ ]-FDG uptake were generated for each region of interest. [ $^{18}\text{F}$ ]-FDG uptake was expressed as the standardized uptake value (SUV), which was defined as: [tissue activity concentration (MBq/cm $^3$ )]/[([injected dose (MBq)]/body weight (g)]. It was assumed that 1 cm $^3$  of gastrointestinal tissue and contents equals 1 gram.

### *Analysis and significance*

All data are expressed as averages  $\pm$  sem and analyzed for significance using SPSS 20.0 by repeated measures-, or Oneway- ANOVA LSD post hoc test, correlations were determined using Pearson's 2-tailed test. Differences are considered significant when  $P < 0.05$ . Graphs were designed using Graphpad Prism 5.

## **Results**

### *In vivo intragastric glucose tolerance test.*

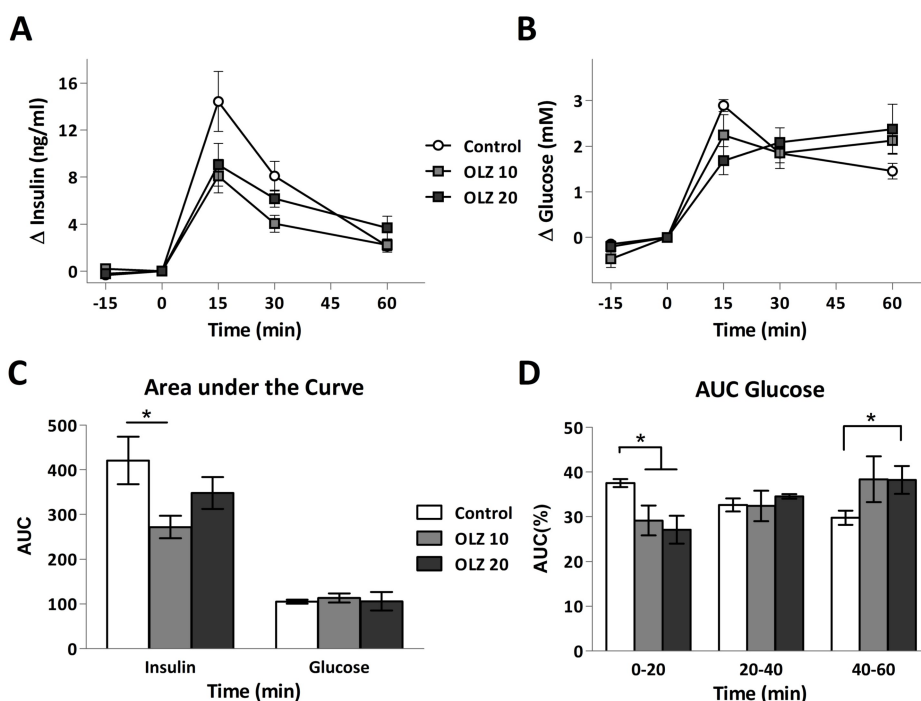
Plasma insulin and blood glucose levels during the IG-GGT are shown in figure 1, and are expressed as differences from the levels assessed at baseline taken at  $t=0$  min (prior to infusion of the glucose/Olanzapine solution). RM-ANOVA showed that delta plasma insulin levels (fig. 1A) were significantly affected by treatment over time ( $F_{16,120}=2.015$ ,  $P < 0.05$ ), and post-hoc analysis revealed that only OLZ10 differed significantly from Control. Plasma insulin levels in the OLZ10 group were lower in the Control group at 15, 20, and 30 minutes (post hoc oneway-ANOVA,  $*P < 0.05$ ). Fig. 1C shows the area under the curve (AUC) of plasma insulin levels, which is lower in OLZ10 ( $P < 0.05$ ), but not OLZ 20, compared to Control. Blood glucose during the IG-GTT were influenced by a main effect of treatment ( $F_{16,120}=1.793$ ,  $P=0.04$ ), however, no significant difference between delta glucose curves of the OLZ treated groups or the control group was found (post hoc rm-ANOVA: *n.s.*). Neither was there a difference in the total glucose AUC between groups (fig. 1C). When the glucose AUC is divided into three periods of 20 minutes (fig. 1D) the percentage of the AUC of the control group was higher in the first 20 minutes compared to both OLZ10 and OLZ20 (post hoc oneway-ANOVA:  $P < 0.05$ ). No difference between groups was seen during the period of 20-40 minutes; during 40-60 minutes AUC was higher in OLZ20 compared to Control (post hoc oneway-ANOVA;  $P < 0.05$ ). In addition, both the control



group ( $P < 0.01$ ,  $r = 0.663$ ) and the OLZ10 group ( $P < 0.01$ ,  $r = 0.514$ ) showed a correlation between circulating insulin and glucose levels, whereas no correlation was found in the OLZ20 group.

*In vivo luminal glucose absorption and transport.*

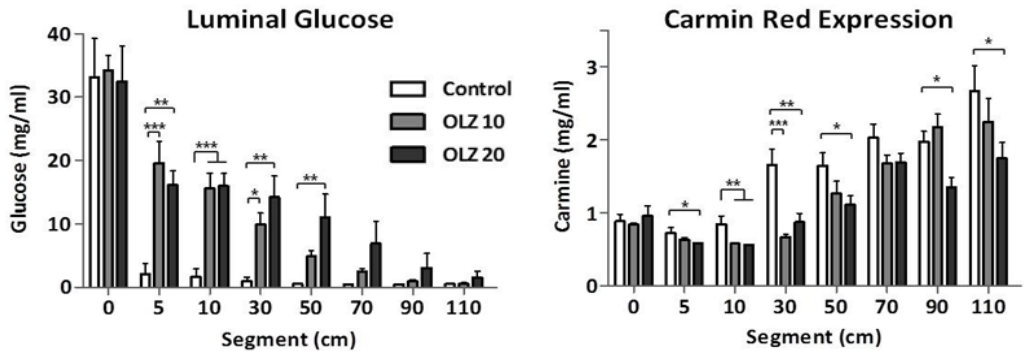
Figure 2A shows the concentration of glucose in the lumen of the small intestine 60 minutes after intragastric glucose infusion. RM-ANOVA revealed a main effect of treatment. The total glucose curve was lower in the Control compared to OLZ10 (post hoc rm-ANOVA:  $P < 0.01$ ) and OLZ20 (post hoc rm-ANOVA:  $P < 0.01$ ), but not between



**Fig. 1:** Circulating insulin and glucose levels after a 9 minute intragastric glucose and OLZ infusion. A) Circulating insulin levels are reduced by OLZ (10 and 20 mg/kg) compared to controls. B) Circulating glucose levels. The graph shows that the peak glucose level at 15 minutes post the start of infusion is reduced by OLZ, while circulating glucose levels are increased at 60 minutes in the OLZ treated groups. C) Area under the curve of glucose and insulin response. Total AUC of insulin is reduced in OLZ 10 group compared to control. Total AUC of glucose is not different between groups. D) Area under the curve of glucose response divided into periods of 20 minutes. AUC of 0-20min is reduced in OLZ 10 and OLZ 20 compared to Control; AUC 40-60min is increased in OLZ 20 compared to Control. \* $P < 0.05$ , post hoc Oneway-ANOVA. Light grey square/bar is 10 mg/kg OLZ, dark grey square/bar is 20 mg/kg OLZ, and open circle/bar is Control.

OLZ treated groups. In the duodenal and proximal jejunal segments, 5<sup>\*\*</sup>, 10<sup>\*\*\*</sup> and 30<sup>#</sup> cm, glucose concentration was higher in both OLZ10 and OLZ20 groups compared to Control (post hoc oneway-ANOVA: <sup>\*\*</sup>P<0.01; <sup>\*\*\*</sup>P<0.001; <sup>#</sup>OLZ 10: P<0.05, OLZ 20: P<0.01). At 50cm glucose was higher in OLZ20 treated animals compared to Controls (post hoc oneway-ANOVA: P<0.01), no significant differences were found between groups at 70 and 110cm. Figure 2B shows that at 60 minutes after infusion the amount of carmine red present in the GI tract was different between the OLZ10 and OLZ20 group (post hoc rm-ANOVA: P<0.05) and both compared to Controls (post hoc rm-ANOVA: P<0.01). Carmine red concentrations in OLZ10-treated animals were lower compared to Controls in segment 10cm (post hoc mANOVA: P<0.01) and 30cm (post hoc oneway-ANOVA: P<0.05) and higher compared to OLZ20-treated animals in segment 110cm (post hoc oneway-ANOVA: P<0.05). Carmine red concentration in OLZ20-treated animals was lower compared to Controls in segment 5cm<sup>\*</sup>, 10cm<sup>\*\*</sup>, 30cm<sup>\*\*</sup>, 50cm<sup>\*</sup>, 90cm<sup>\*</sup>, and 110cm<sup>\*</sup> (post hoc oneway-ANOVA: <sup>\*\*</sup>P<0.01, <sup>\*</sup>P<0.05), and lower compared to OLZ 10-treated animals at 110cm (post hoc oneway-ANOVA: P<0.05).

The average carmine red concentration in the small intestine was significantly lower in animals treated with OLZ compared to control (AVG: Control=1.801±0.061mg/ml, OLZ 10=1.434±0.027mg/ml, OLZ 20=1.221±0.064mg/ml, post hoc Oneway-ANOVA: P<0.001), and between OLZ-treated groups (post hoc Oneway-ANOVA: P<0.05).



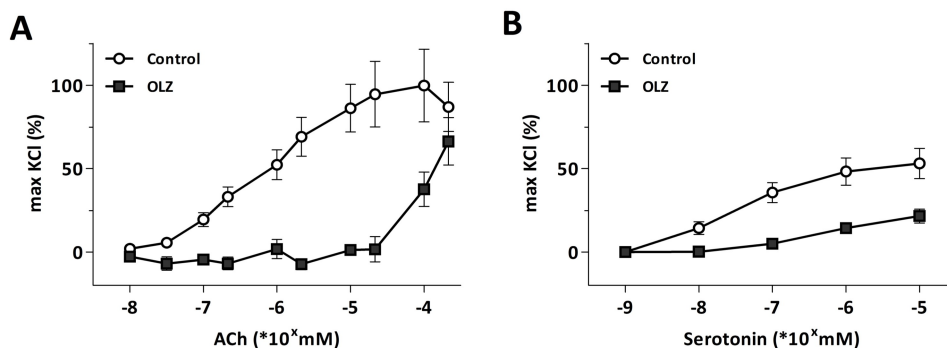
**Fig. 2:** A) Luminal glucose concentration 60 minutes after intragastric glucose and OLZ (10 and 20mg/kg) administration. OLZ (10 and 20) show higher amounts of glucose within the lumen of the gastrointestinal tract compared to control (mANOVA post hoc LSD: <sup>\*</sup>P<0.05, <sup>\*\*</sup>P<0.01, <sup>\*\*\*</sup>P<0.001). B) Carmine red concentration in the gastrointestinal tract 60 minutes after intragastric glucose and OLZ (10 and 20mg/kg) administration. OLZ (10 and 20) decrease expression compared to control in multiple segments of the small intestine (mANOVA post hoc LSD: <sup>\*</sup>P<0.05, <sup>\*\*</sup>P<0.01, <sup>\*\*\*</sup>P<0.001). Segment: 0cm= stomach; 5-10cm=duodenum; 30-90cm= jejunum; 115cm= ileum.

*In vitro smooth muscle contraction.*

To study OLZ's effect on gastrointestinal motility we performed an *in vitro* experiment using segments ( $\pm 2$ cm) of duodenum and proximal jejunum exposed to increasing concentrations of ACh and 5HT.

Figure 3A shows that OLZ was a strong inhibitor of acetylcholine-induced gastrointestinal smooth muscle contraction (rm-ANOVA:  $F_{9,180}=6.036$ ,  $P<0.001$ ). Whereas  $1 \cdot 10^{-5}$  mM ACh induced almost complete muscle contraction in control segments ( $86.32 \pm 14.37 \% KCl_{max}$ ), this response was completely inhibited by  $100 \mu\text{M}$  OLZ ( $1.19 \pm 2.25 \% KCl_{max}$ ). Only at the highest concentration of ACh,  $3 \cdot 10^{-4}$  mM,  $100 \mu\text{M}$  OLZ was not sufficient of significantly reducing smooth muscle contraction (Control= $89.38 \pm 13.96 \% KCl_{max}$ ; OLZ= $66.37 \pm 14.21 \% KCl_{max}$ ).

Figure 3B shows that also 5HT-induced smooth muscle contraction was blocked by OLZ (rm-ANOVA:  $F_{4,56}=11.419$ ,  $P<0.001$ ). Except for the lowest 5HT concentration ( $1 \cdot 10^{-9}$  mM), which did not induce any contraction,  $100 \mu\text{M}$  OLZ was capable of reducing 5HT-induced smooth muscle contraction at every concentration tested (oneway-ANOVA:  $P<0.01$ ). The highest 5HT concentration ( $1 \cdot 10^{-5}$  mM) induced a contraction, which was  $53.17 \pm 9.01 \%$  of  $KCl_{max}$  in the control condition, whereas  $100 \mu\text{M}$  OLZ reduced smooth muscle contraction to  $21.57 \pm 4.14 \%$  of  $KCl_{max}$  (oneway-ANOVA:  $P<0.01$ ).



**Fig 3:** Gastrointestinal smooth muscle contraction. a) Olanzapine ( $1 \mu\text{M}$ ) inhibits acetylcholine induced smooth muscle contraction ( $P<0.0001$  rm-ANOVA). b) Olanzapine ( $1 \mu\text{M}$ ) inhibits serotonin induced smooth muscle contraction ( $P<0.001$  rm-ANOVA).

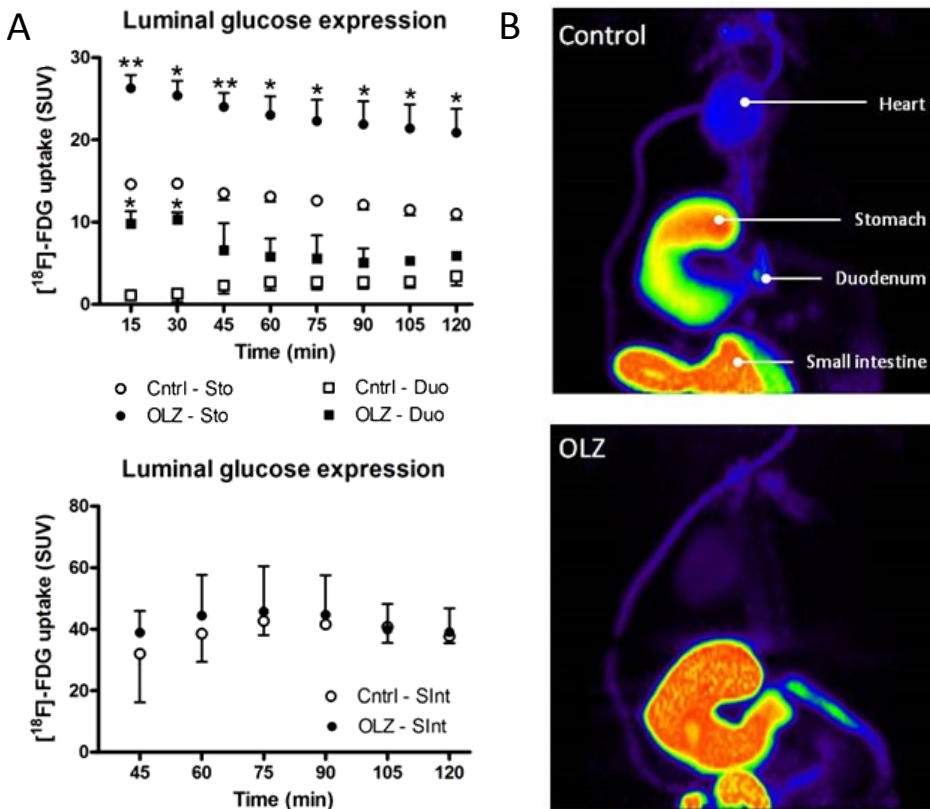
### *Glucose uptake measured with [<sup>18</sup>F]-FDG PET.*

As a proof of principal we used [<sup>18</sup>F]-FDG to show that the absorption rate of intragastric administered glucose was attenuated by OLZ. Fig. 4a (*upper panel*) shows that the amount of [<sup>18</sup>F]-FDG glucose was increased in the stomach of the OLZ treated animals at every time point measured over the duration of 90 minutes ( $P < 0.01$ ,  $F_{8,16} = 9.397$ , rm-ANOVA), i.e. 60-150 minutes post drug administration. In the duodenum, [<sup>18</sup>F]-FDG uptake was increased in the OLZ treated rats at the start of the measurement till 30 minutes and showed a slow decline over time. The control animals had lower [<sup>18</sup>F]-FDG expression in the duodenum at the start of measurement, which inclined over the duration of the measurement. The changes in [<sup>18</sup>F]-FDG expression over time in the duodenum shows to be affected by OLZ ( $P < 0.05$ ,  $F_{8,16} = 3.716$ , rm-ANOVA) and was increased by OLZ at  $t = 15$ min ( $P < 0.05$ ,  $F_{1,3} = 68.863$ ), and  $t = 30$ min ( $P < 0.05$ ,  $F_{1,3} = 93.740$ , oneway-ANOVA) during scan, which is presented in fig 4b. [<sup>18</sup>F]-FDG expression in the small intestines increased over time over a duration of 45 min, i.e. 115 minutes post-infusion, after which it decreased again. Small intestine [<sup>18</sup>F]-FDG expression was not affected by OLZ treatment (fig 4a *lower panel*).

## **Conclusions and Discussion**

Euglycemic clamp studies have shown that OLZ causes a decrease in insulin sensitivity [6,22,23]. The result of our study, in which OLZ was acutely administered intragastrically together with glucose does not appear to result in a hyperinsulinemic response indicative of decreased insulin sensitivity. On the contrary, judging from the circulating glucose and insulin responses during the IG-GTT (fig. 1A/B) insulin levels are in fact lower in the OLZ-treated groups, compared to the Control treatment. While we do not dismiss the possibility that OLZ induces insulin resistance, the underlying mechanism of lower insulin levels as a result of the IG-GTT caused by OLZ seems to arise from gastro-intestinal mechanisms. First, we showed that the amount of glucose that is found within the lumen of the ileum is dramatically increased in OLZ-treated rats compared to Controls following an IG-GTT, which indicates that the intragastrically infused glucose that reaches the circulation is lowered by OLZ treatment (fig 2A). The lower circulating insulin level corresponds with a decreased amount of glucose entering the circulation in the OLZ treated groups (fig 1A). The combined data of circulating glucose levels and AUCs from 0-20min and 40-60min of the glucose response (fig. 1D) suggests altered glucose absorption by OLZ and the presence of luminal glucose 60 minutes after intragastric glucose infusion confirms that OLZ delayed glucose absorption (fig. 2A). In addition, these data relate to the

increased circulating glucose levels we see during IG-GTT 60 minutes post infusion. Surprisingly, during IG-GTT insulin levels in the OLZ20 group do not correlate to glucose levels, especially at 60 minutes, where low insulin levels coincide with high glucose levels. It is possible that OLZ at 60 minutes post administration reduces glucose induced insulin secretion at the pancreatic  $\beta$ -cell [24] adding to the increased levels of circulating glucose (fig. 1B). To investigate whether OLZ alters intestinal transport, we tracked the indigestible carmine red compound along the gastrointestinal tract, and found that OLZ reduced its level, primarily in the jejunal segments (fig. 2B). The higher carmine red concentration in the distal parts (90-




**Fig 4:** Luminal glucose uptake, measured with  $[^{18}\text{F}]$ -FDG PET. A) *upper panel:* Uptake of  $[^{18}\text{F}]$ -FDG is increased in the stomach of the OLZ treated animals (OLZ-Sto) compared to saline treated animals (Cntrl-Sto) during 70-160 minutes post infusion. An increase of luminal  $[^{18}\text{F}]$ -FDG is also observed in the duodenum of OLZ (OLZ-Duo) animals, but only until 100 minutes post infusion (Control < OLZ:  $**P<0.01$ ,  $*P<0.05$ , oneway-ANOVA). *Lower panel:* No difference in  $[^{18}\text{F}]$ -FDG uptake was observed for the intestines. B) PET scans (Mean Intensity Projection (MIP)) of control and OLZ treated animals, in which stomach, duodenum, and intestines are clearly visible.

110cm) of the intestinal tract in the control group relates to the higher circulating glucose and insulin levels during the first 20 minutes of the IG-GTT. Accordingly, the decreased carmine red concentration in the distal parts of the OLZ 20 group relates to the decreased AUC of glucose between 0-20min and lower circulating glucose level at 15min. The luminal glucose measured in the distal jejunum and ileum of the OLZ groups is surprising because glucose is normally already absorbed in the duodenum and proximal jejunum, where SGLT-1 receptors necessary for active glucose transport are most abundant [25]. Indeed, the increased glucose AUC between 40-60 minutes correlates to the glucose still present in the lumen of the duodenum and jejunum in the OLZ groups.

To study if OLZ's effect on the gastrointestinal transport is caused by changes in peristalsis we set up an *in vitro* study to directly assess whether OLZ influences contractibility of the small intestinal smooth muscles cells by Ach and 5HT. First, we demonstrated that ACh inhibits maximal contractibility to  $86.32 \pm 14.37\%$ , while 5HT inhibits maximal contractibility to  $66.37 \pm 14.21\%$ . These data relate to Nakajima *et al* who demonstrated that gastrointestinal peristalsis is cholinergic regulated and who also showed that 5-HT induced gastric antrum and duodenal contraction can be blocked by atropine, suggesting that the 5-HT pathway is dependent of cholinergic input [26]. We then showed that OLZ is indeed a strong inhibitor of ACh and 5HT-induced smooth muscle contraction.

To investigate the role of the stomach in OLZ's effect, we infused [ $F^{18}$ ]-FDG glucose into the stomach and assessed OLZ's effect on glucose transport towards the gastrointestinal lumen by PET-scan analysis. We used this as a proxy for gastric emptying. The results clearly demonstrate that glucose is increased in the gastric lumen by OLZ, which indeed shows that gastric emptying is delayed by OLZ. The amount of glucose measured in the gastric lumen of the Control animals is already lower compared to the OLZ treated animals, which is the result of the fact that rats were first infused intragastrically with [ $F^{18}$ ]-FDG glucose, and thereafter were put in the PET scanner. Comparative to the first experiment, we also observed an increased expression of [ $F^{18}$ ]-FDG glucose in the duodenum of the OLZ treated animals compared to control treated animals.

One explanation underlying the diminished gastric emptying and ileal transport might be a possible effect of OLZ on the release of incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP, also known as: gastric inhibitory peptide) causing the so-called "ileal brake"[27]. Although Lykkegaard *et al* demonstrated that the GLP-1 analogue liraglutide is capable of reversing OLZs adverse metabolic effects in rats [28], Van der Zwaal *et al.*, however,



did not show any change in post meal circulating GLP-1 concentrations 75 minutes after s.c. OLZ (1mg/kg) administration [4]. Moreover, Vidarsdottir *et al* also did not find any differences in GLP-1 levels in OLZ treated human subjects [29]. This seems to dismiss a role for GLP-1 in the effects of OLZ to induce abovementioned gastrointestinal effects. Ogawa *et al* demonstrated that exogenous GIP inhibits intestinal glucose absorption by reducing gastrointestinal motility [30]. These data are homologous to the OLZ-induced gastrointestinal changes in the study described here. Ogawa *et al* also show that this effect of GIP is not mediated via GLP-1, but via a somatostatin pathway. In addition, Sohndi *et al* show a 3-fold increase of GIP mRNA in the rat small intestine after 28 days of 20 mg/kg Clozapine treatment [31]. However, the only relation between OLZ and GIP we were able to find in the literature is described by Ono *et al*, who show that schizophrenic patients carrying a polymorphism on the GIPR A allele developed hyperinsulineamia more easily after an oral glucose load [32]. The exact mechanisms via which OLZ may act on GIP or GLP-1 secreting intestinal cells is beyond the scope of this study, but deserves specific attention.

Further research is necessary to unravel the mechanisms underlying OLZ's effects on gastrointestinal functioning. Particularly because decreased peristalsis is related to delayed but increased nutrient absorption changing fuel utilization [27,33], and may contribute to the increased weight gain and insulin resistance associated with OLZ treatment.

In summary, we showed *ex vivo* that OLZ inhibits duodenal and jejunal smooth muscle contraction directly via antagonizing serotonin and acetylcholine. Additionally, we demonstrated *in vivo* that OLZ delays glucose absorption from, and transport through, the gastrointestinal tract, resulting in lower circulating insulin levels because of reduced amounts of glucose entering the circulation. Not surprisingly, a major percentage of SGA users are co-treated with laxatives to stimulate bowel movement and avoid constipation [9]. The enteric nervous system is predominantly regulated via serotonergic and cholinergic pathways, and therefore it should be realized that some of the negative metabolic effects seen in patients using SGAs might already originate at the level of the gut.

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