The role of hypothalamic pathways in the metabolic side effects of Olanzapine
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Chapter 3.3

Olanzapine-induced changes in glucose metabolism are independent of the melanin-concentrating hormone system

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ABSTRACT

Atypical antipsychotic drugs such as Olanzapine (Ola) induce weight gain and metabolic changes associated with the development of type 2 diabetes. The mechanisms underlying these undesired side effects are currently unknown. Chagnon et al. showed that the common allele rs7973796 of the prepro-melanin-concentrating hormone (PMCH) gene is associated with a greater body mass index in Ola-treated schizophrenic patients. As PMCH encodes for the orexigenic neuropeptide melanin-concentrating hormone (MCH), it was hypothesized that MCH is involved in Ola-induced weight gain. We have recently reported that the intragastric infusion of Ola results in hyperglycemia and insulin resistance in male rats. In order to test in vivo the possible involvement of the PMCH gene in the pathogenesis of Ola side effects, we administered Ola intragastrically in wild-type (WT) and PMCH knock-out (KO) rats. Our results show that glucose and corticosterone levels, as well as endogenous glucose production, are elevated by the infusion of Ola in both WT and KO animals. Thus, the lack of MCH does not seem to affect the acute effects of Ola on glucose metabolism. On the other hand, these effects might be obliterated by compensatory changes in other hypothalamic systems.
INTRODUCTION

Treatment with atypical antipsychotic drugs is associated with significant weight gain and metabolic disturbances including hyperglycemia and insulin resistance. Olanzapine (Ola) is one of the atypical antipsychotic drugs inducing the most dramatic weight gain (Sacher et al. 2008). The mechanisms underlying the metabolic changes induced by Ola are still far from clear. The susceptibility to body weight changes in psychotic patients due to atypical antipsychotics has been hypothesized to partly be of genetic origin. Chagnon et al. in 2004 used a linkage analysis in 21 families with schizophrenia or bipolar disorders (508 family members) and showed linkage of the chromosomal region 12q24 with the phenotype of obesity under the use of antipsychotics. This region is located at less than 1 centimorgan from the prepro-melanin-concentrating hormone (PMCH) gene, encoding the orexigenic neuropeptide melanin-concentrating hormone (MCH) (Chagnon et al. 2004). Later studies by the same authors in unrelated patients showed that the common allele rs7973796 of PMCH gene is associated with a greater body mass index in Ola-treated patients (Chagnon et al. 2007). Thus it was hypothesised that Ola may stimulate PMCH expression and release, and thereby the development of obesity (Chagnon et al. 2007).

MCH is expressed in the lateral hypothalamus and the zona incerta (Bittencourt et al. 1992; Sita et al. 2007), areas involved in the regulation of eating behaviour and energy homeostasis (Qu et al. 1996). MCH neurons project broadly throughout the central nervous system, suggesting that MCH may function as a neurotransmitter and/or a neuromodulator to regulate behavioural functions (Bittencourt et al. 1992; Skofitsch et al. 1985). Processing of PMCH results in the production of 3 neuropeptides: neuropeptide glycine-glutamic acid (N-GE), neuropeptide glutamic acid-isoleucine (N-EI) and MCH (Saito and Maruyama 2006). When injected centrally in rats, MCH stimulates food intake (Qu et al. 1996). Two receptors are known in humans: MCH receptor-1 (MCHR1) and -2 (Sailer et al. 2001), while only MCHR1 was identified in rodents. Antagonism of this receptor in rodents leads to a decreased food intake and weight gain (Shearman et al. 2003). MCHR1-deficient mice have normal body weight but are hyperphagic and hyperactive (Marsh et al. 2002). PMCH knock-out (KO) mice showed reduced body weight and an increased metabolic rate (Shimada et al. 1998). Conversely mice overexpressing PMCH are obese and insulin resistant (Ludwig et al. 2001).

We have shown earlier that intragastric Ola induces hyperglycemia and insulin resistance (Girault et al. 2012). In the present study, we determined whether the metabolic side effects of Ola persist in rats in the absence of the MCH system using the PMCH KO rat model (Smits et al. 2006). We acutely administered Ola intragastrically to PMCH KO and wild-type...
(WT) rats to investigate the role of MCH in the pathogenesis of the metabolic side effects of Ola.

MATERIALS AND METHODS

Ethic statement
All experiments were approved by the animal care committee of the Royal Netherlands Academy of Arts and Sciences.

Animals
The PMCH KO rat (Pmch$^{Hubr}$) was generated by target-selected ENU-driven mutagenesis (Smits et al. 2006). Briefly, high-throughput re-sequencing of genomic target sequences in progeny from mutagenized rats revealed an ENU-induced premature stop codon in exon 1 (K50X) of PMCH gene in a rat (Wistar/Crl background). The heterozygous mutant animal was outcrossed to WT Hsd Wistar background for six generations to eliminate confounding effects from background mutations induced by ENU. Further details regarding the selection of the animals can be found in Mul et al. (Mul et al. 2010). WT littermates (with similar genetic backgrounds) were used as controls. PMCH KO rats were viable into adulthood and fertile and appeared phenotypically normal despite their lower body weight. Two rats were housed together until the surgery, under controlled experimental conditions (12:12-h light-dark cycle, light period 06:00–18:00, 21 ± 1°C, 60% relative humidity). The standard fed diet (semi-high-protein chow: RM3, 26.9% crude protein, 11.5% fat, and 61.6% carbohydrates; 3.33 kcal/g AFE; SDS, Witham, UK) was provided ad libitum together with water. Only male rats were used in the present study.

Genotyping
Genotyping was done using the KASPar SNP Genotyping System (KBiosciences, Hoddesdon, UK; as described in (van et al. 2008) using gene-specific primers (forward common: TTAAT ACATT CAGGA TGGGG AAAGC CTTT; reverse wild type: GAAGG TGACC AAGTT CATGCT CGATC TTTCT GCGGT ATCTT CCTT; and reverse homozygous: GAAGG TCGGA GTCAA CGGAT TCGAT CTTTC TGCGG TATCT TCCTA). All pups were genotyped at 3 weeks of age. Genotypes were reconfirmed after experimental procedures.

Drugs
The dose of Ola chosen in the present study was identical to the effective dose used in our
previous experiments (Girault et al. 2012) and selected to parallel the clinical setting based on 70% dopamine D\textsubscript{2} receptor occupancy, which represents a threshold in humans associated with optimal clinical response (Kapur et al. 2003). The route of administration was chosen such that a continuous infusion of freshly made solution was possible in freely moving, undisturbed animals. Using a surgically implanted intragastric (IG) catheter, animals were treated with a primed 36 mg/kg/h infusion during 5 minutes followed by a continuous 3 mg/kg/h infusion for 160 minutes (i.e., in total 3.66 mg/rat) of Ola (ChemPacific Corporation, Maryland) dissolved in acidified MilliQ water (pH=6). Ola solution was prepared in MilliQ water acidified with HCl (1M) and then brought back to pH 6 using NaOH (1M).

Surgical procedures
Animals were anesthetized by an intramuscular injection of 0.6 ml/kg Hypnorm (Janssen, High Wycombe, Buckinghamshire, UK) and a subcutaneous injection of 0.15 ml/kg Dormicum (Roche, Almere, The Netherlands). Silicon catheters were placed into the right jugular vein and the left carotid artery for intravenous infusions and blood sampling. The vascular lines were closed using a mix of polyvinylpyruvidon (PVP; Sigma-Aldrich Corp., St. Louis, MO), heparin (LEO Pharma, Ballerup, DK) and amoxicillin (Centrafarm, Etten-Leur, NL). A silicon cannula was placed in the stomach during the same surgery. IG cannu\text{las} were placed through a 1cm incision on the left side of the abdomen. Catheters and IG cannulas were fixed on the top of the head of the animal using dental cement. These techniques allowed us to perform all our experiments in freely moving animals. Experiments were performed only after recovery of the pre-surgical body weight and with animals in healthy state, i.e. 7 – 10 days post-operative recovery.

Experimental procedures
During the experiment, animals were permanently connected to blood-sampling and infusion lines, which were attached to a metal collar and kept out of reach from the rats by means of a counterbalanced arm. This allowed all manipulations to be performed outside the cages without handling the animals. The metal collars were attached the day before the experiment. Before the day of the experiment, food was restricted to 20 g overnight. Two hours before the experiment, rats were handled to connect them to the blood sampling and infusion lines and all remaining food was removed.

1. Basal endogenous glucose production
To assess endogenous glucose production (EGP), [6,6\textsuperscript{2}H\textsubscript{2}]glucose was used as a tracer.
The method has been reported before in Girault et al. (2012). In brief, blood samples were taken at t=-5 min for background enrichment (t=0 was at 11.00 a.m.), at t=90, t=95 and t=100 min to determine enrichment during the equilibrium state and every 20 min from t=120 till t=260 min to determine enrichment during the experimental state.

Vehicle (Veh; MilliQ water at pH=6 to mimic the pH of the Ola solution) for the IG infusion (1 ml/h) started together with a continuous [6,6-$^2$H$_2$]glucose via the jugular vein at t=0. After the t=100 min blood sample (at about 12.40 a.m.), Veh IG infusion was changed to Ola or Veh solution (36 mg/kg/h during 5 minutes and 3 mg/kg/h until the end of the experiment). At the end of the experiment, animals were sacrificed by a lethal intravenous injection of pentobarbital. The experimental procedure is summarized in Figure 1.

![Figure 1: Experimental protocol](image)

**Figure 1: Experimental protocol.** Experimental protocol showing the time line of the infusions (IV and IG) and the timing of blood sampling.

**Laboratory methods/analysis**

Glucose concentrations were determined using a glucometer (Abbott). Blood samples were collected in tubes containing heparin on ice and centrifuged. Plasma was stored at -20°C until further analysis.

Plasma insulin and corticosterone concentrations were measured using radioimmunoassay kits (Millipore, Billerica, USA and MP Biomedicals, Orangeburg, USA, respectively). Plasma [6,6-$^2$H$_2$]glucose enrichment was measured by gas chromatography-mass spectrometry (GCMS) (Ackermans et al. 2001), and EGP was calculated by the methods of Steele (Steele 1959).

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS version 17.0. A p<0.05 was considered statistically significant. A 2-
way ANOVA with repeated measures was performed to compare glucose levels, endogenous glucose production, corticosterone and insulin levels. If appropriate, post-hoc analysis was performed using One-Way ANOVA.

RESULTS

On the surgery day, body weight of WT animals was 388.6±12.7g vs. 342.2±5.1g in KO animals. Surgery led to a decrease in body weight for 2 to 3 days and then both groups got back to pre-surgery weight, i.e. 394.5±14.4g for the WT and 332.8±5.4g for the KO animals. Throughout the experiment, body weights of KO animals were significantly lower than that of the WT (Genotype p<0.001, Figure 2).

IG Ola treatment significantly increased blood glucose levels (Time*Treatment p<0.001 and Treatment p<0.001, Figure 3) from 4.33±0.20 and 4.16±0.08 mmol/L during the equilibrium state to 5.73±0.24 and 6.57±0.51 mmol/L at 160 min after the start of Ola for WT-Ola (32% elevation) and KO-Ola (57% elevation), respectively. Although there was a tendency towards an interaction effect of Time*Genotype (p=0.064), plasma glucose values of WT and KO animals did not differ significantly at any of the time points.

Figure 2: Post-surgery body weight of WT and KO animals. Body weight was measured each day from the day of surgery (D0) till the day of the experiment (D11). KO animals showed a significantly lower body weight than the WT animals (Genotype p<0.001) throughout the study. *p<0.001 by post-hoc analysis.
Ola treatment also significantly increased EGP (\textit{Time} × \textit{Treatment} \( p<0.001 \) and \textit{Time} * \textit{Treatment} \( p=0.007 \), Figure 4), with at the end a 26% increase for the WT-Ola and 11% for the KO-Ola. There was no effect of Genotype.

\textbf{Figure 3: Blood glucose levels during the experiment.} Ola induces an increase in glucose levels (\textit{Time} × \textit{Treatment} \( p<0.001 \) and \textit{Treatment} \( p<0.001 \)). Post-hoc analysis showed that the effects of \textit{Treatment} and \textit{Time} × \textit{Treatment} were also significant in the separate genotypes. No significant effect of the Genotype or \textit{Time} × Genotype was detected. *\( p<0.05 \) vs. WT-Veh group by post-hoc analysis; #\( p<0.05 \) vs. KO-Veh group by post-hoc analysis.

\textbf{Figure 4: EGP during the experiment.} Ola treatment significantly increased EGP (\textit{Time} × \textit{Treatment} \( p<0.001 \) and \textit{Treatment} \( p=0.007 \)). Post-hoc analysis showed that the effects of \textit{Treatment} and \textit{Time} × \textit{Treatment} were also significant in the separate genotypes. No significant effect of the Genotype was detected. *\( p<0.05 \) from WT-Veh group by post-hoc analysis; #\( p<0.05 \) from KO-Veh group by post-hoc analysis.
Plasma corticosterone levels were increased by the Ola treatment as well (*Time*Treatment p<0.001 and *Treatment p=0.001, Figure 5) from about 10 ng/ml to 261±99 ng/ml for the WT-Ola group and 240±63 ng/ml for the KO-Ola group. The interaction *Time*Genotype*Treatment also showed a significant effect (p=0.024), while no effect of Genotype was noted.

Finally, Ola treatment increased plasma insulin levels (*Time*Treatment p=0.012 and *Treatment p=0.001, Figure 6). Post-hoc analysis showed that this effect was only present in the WT animals (p=0.01), i.e. from 1.75±0.24 to 2.25±0.22 ng/ml, whereas in the KO animals plasma insulin increased (non significantly) from 1.60±0.22 to only 1.70±0.24 ng/ml. The differential effect of Ola in the KO and WT animals is also indicated by the significant effect of the interaction Genotype*Treatment (p=0.046).
DISCUSSION

The present study shows that the acute effects of intragastric Ola administration, such as hyperglycemia, increased EGP and corticosterone levels, are not significantly affected by the absence of hypothalamic MCH expression. Thus, based on the current data in our KO model, the hypothesis by Chagnon et al. (2007) “Ola would stimulate PMCH expression and release, and lead to development of obesity” is not verified since the acute Ola treatment was still able to induce its metabolic side effects in the absence of PMCH gene. But our study only tested the involvement of the PMCH gene in the acute metabolic side-effect of Ola. Thus, the relation between PMCH expression and release and Ola-induced obesity remains to be tested and might still be true. On the other hand, Guesdon et al. showed, that treatment with either Ola (1mg/kg/day per os) or the MCHR1 agonist (30μg/rat/day ICV) for 13 days leads to increased food intake and body weight starting from the 7th day of treatment (Guesdon et al. 2010). Combining the two treatments induced an even more pronounced increase in food intake and body weight. These data thus also support the idea that Ola- and MCH-induced changes in food intake and body weight are mediated by different mechanisms.
Ola treatment in schizophrenic patients caused a biphasic insulin reaction to a hyperglycemic stimulus, in the first weeks a suppression of insulin secretion was observed and subsequently a rebound overcompensation (Chiu et al. 2010). In our rats, administration of Ola led to increased plasma insulin concentrations in the WT rats but not in the PMCH KO rats, pointing towards a role for MCH in the insulin response to Ola. Indeed, MCH receptor mRNA is expressed in rat islets of Langerhans and MCH induces a dose-dependent increase in insulin release from insulin-producing cell lines (Tadayyon et al. 2000). MCH mRNA expression has been found in the whole pancreas (Hervieu and Nahon 1995) and MCHR1 expression was identified in primary human and mouse islets (Pissios et al. 2007). Moreover, Shimada et al. showed that PMCH KO mice display a 27% decrease in insulin level than WT animals (Shimada et al. 1998). Since our PMCH KO animals did not show changes in insulin levels after Ola treatment, we hypothesized that the Ola-induced hyperinsulinemia is mediated via a MCH-dependent mechanism, probably taking place in the pancreas. Moreover, the increased plasma insulin levels in the WT animals might explain the slightly higher glucose levels after Ola treatment in the KO group compared to the WT group.

PMCH expression during early development and puberty is of critical importance for a normal energy balance and the loss of PMCH results in permanently disturbed energy balance during adulthood (Mul et al. 2010). The role of MCH in energy regulation is well established. It should be noted, however, that the entire PMCH gene is inactivated in our KO model which means that the neuropeptides N-GE and N-EI are not expressed either. Thus far, no effects of N-GE on cellular or physiological events have been demonstrated, but N-EI has been implicated in anxiety (Gaston et al. 2011), grooming and locomotor activity (Sanchez et al. 1997). Moreover, the administration of N-EI modifies levels of noradrenalin and dopamine in the brain (Sanchez et al. 2001) and leads to an increased luteinizing hormone release (Attademo et al. 2004). Clearly the lack of those neuropeptides could obscure the effects of Ola on MCH in our experiments.

Previous studies on MCH-KO mice and PMCH KO rats showed that the gene expression of several hypothalamic genes is changed by the KO of this gene (Shimada et al., 1998; Mul et al. 2010). Amongst those changes is a 60% increase of the hypothalamic orexin (Ox) mRNA expression in KO animals at 100 days post-natal (PND100) (Mul et al. 2010). Previously, it has been shown that a peripheral injection of Ola activates neurons in the paraventricular nucleus of the hypothalamus and the lateral hypothalamus/perifornical area (LH/PFA) and that a large part of the LH/PFA neurons are Ox-positive (Stefanidis et al. 2009). Data from
our own group clearly show that ICV Ox administration increases plasma glucose concentrations through an increase in EGP (Yi et al. 2009). Moreover, administration of an Ox-1 receptor antagonist prior the administration of Ola blunts the Ola-induced increased EGP (Girault et al., In press). Since our animals were over PND100, it could well be that an increased Ox expression compensates for the absence of MCH in our study. Especially since MCH and Ox neurons are intermingled in the same region of the LH/PFA and MCH has been shown to increase feeding (Qu et al. 1996). Moreover, there is a reciprocal innervation between Ox and MCH neurons, MCH attenuates the efficacy of the glutamatergic synapses on the Ox neurons (Rao et al. 2008) and part of the effect of Ox may be mediated via stimulation of MCH signalling. The 2 groups of neurons thus appear to fulfil complementary roles in various hypothalamic functions (Tsuneki et al. 2010). Our data on the possible involvement of the Ox and MCH system in the metabolic side effects of Ola are in support of these complementary roles, as an Ox-1 receptor antagonist reduced the hyperglycemic effect of Ola (Girault et al., In press) and MCH-KO tended to increase it (Figure 3).

Chronic treatment with Ola in rats induces fat accumulation (Cooper et al. 2005; Albaugh et al. 2010; Minet-Ringuet et al. 2006; Shobo et al. 2011; van der Zwaal et al. 2010), decreased locomotor activity (Albaugh et al. 2010; Liebig et al. 2010; van der Zwaal et al. 2010), increased meal size (van der Zwaal et al. 2010) but decreased meal frequency (van der Zwaal et al. 2008), and decreased body weight (Smith et al. 2011). In our experiment, the focus was on the acute effects of Ola in a MCH KO model. Therefore, at present we cannot exclude the involvement of the MCH system in more chronic parameters.

In conclusion, our experiment showed that the absence of the MCH system did not significantly affect the hyperglycemia, increased EGP and higher plasma corticosterone levels induced by an acute administration of Ola. However, it is likely that compensatory changes induced by the MCH KO obliterated the effect of an absent MCH system.

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Reference List


