Citation: Transl Psychiatry (2013) 3, e208; doi:10.1038/tp.2012.138
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www.nature.com/tp

# Haloperidol and olanzapine mediate metabolic abnormalities through different molecular pathways

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**The pathogenesis of antipsychotic-induced disturbances of glucose homeostasis is still unclear. Increased visceral adiposity has been suggested to be a possible mediating mechanism. The aim of this study was to investigate, in an animal model, the differential effects of olanzapine and haloperidol on visceral fat deposition (using magnetic resonance imaging(MRI)) and on critical nodes of the insulin signaling pathway (liver-protein levels of IRS2 (insulin receptor substrate 2), GSK3α (glycogen synthase kinase-3α), GSK3β, GSK3α-Ser21, GSK3β-Ser9). To this end, we studied male Sprague–Dawley rats treated with vehicle ($n = 8$), haloperidol (2 mg kg$^{-1}$ per day, $n = 8$), or olanzapine (10 mg kg$^{-1}$per day, $n = 8$), using osmotic minipumps, for 8 weeks. The haloperidol group showed a higher percentage of visceral fat than both the olanzapine group and the vehicle group, whereas there was no difference between the olanzapine and the vehicle group. In terms of insulin signaling pathway, the olanzapine group showed significantly reduced IRS2 levels, reduced phosphorylation of GSK3α and increased phosphorylation of GSK3β, whereas there was no difference between the haloperidol and the vehicle group. Our data suggest that different molecular pathways mediate the disturbances of glucose homeostasis induced by haloperidol and olanzapine with a direct effect of olanzapine on the insulin molecular pathway, possibly partly explaining the stronger propensity of olanzapine for adverse effects on glucose regulation when compared with haloperidol in clinical settings.**
*Translational Psychiatry* (2013) **3,** e208; doi:10.1038/tp.2012.138; published online 15 January 2013

## Introduction

The mortality rate of individuals with serious mental illnesses is two to three times higher than in the general population,[1] and up to 60% of the excess mortality rate in patients with schizophrenia is attributable to physical illnesses.[2] Indeed, it is widely acknowledged that patients with schizophrenia suffer from higher rates of metabolic syndrome and obesity-related illnesses, such as type 2 diabetes mellitus, dyslipidaemia, hypertension and cardiovascular disease.[3] Reasons for the increased prevalence of physical illnesses in schizophrenia are complex, but perhaps the most important is the fact that antipsychotic medications increases body weight and predisposes to metabolic abnormalities.[4] The molecular mechanisms underlying these metabolic effects of antipsychotics are, however, yet unclear. Moreover, there is some evidence that olanzapine and clozapine are associated with an increased risk for diabetes compared with other antipsychotics.[5,6] Among the other antipsychotics, for example, haloperidol has been less consistently associated with weight gain and abnormal glucose regulation,[4] but again, whether olanzapine and haloperidol have different metabolic effects at a molecular level it is not well understood yet.

One possible pathophysiological mechanism for olanzapine-induced hyperglycemia is the reported weight gain and/or change in body fat distribution with a predominant visceral fat type.[7] However, previous studies in humans and rodents have suggested that, at least for olanzapine, the effects on glucose homeostasis and insulin sensitivity precede weight gain and changes in visceral adipose-tissue deposition.[7–10] Therefore, alternative mechanisms may occur through a direct effect of olanzapine on humoral and/or cellular insulin pathways, leading to impairment of insulin action, and ultimately leading to insulin resistance.[11,12]

In light of this literature, we focussed our attention on the insulin pathway. The insulin receptor, in presence of insulin, phosphorylates insulin receptor substrate (IRS) proteins, which are the key mediators of insulin activity. The IRS proteins are linked to the activation of the phosphatidylinositol 3-kinase-AKT/protein kinase B pathway, which is responsible for most of the metabolic actions of insulin, including glucose uptake, gluconeogenesis and glycogen synthesis. Of note is that glycogen synthase kinase-3 (GSK3), the key enzyme involved in the regulation of glycogen synthesis, has two isoforms, GSK3α and GSK3β. Both are constitutively active, and their activities are primarily regulated by the phosphorylation of an N-terminal serine, Ser21 of GSK3α and Ser9 of GSK3β.[13] This N-terminal phosphorylation of GSK3 results in inhibition of its activity and a subsequent activation of the glycogen synthase, and thereby in glycogen synthesis.[13]

There is evidence of antipsychotics influencing the insulin signaling pathway in both *in vivo* and *in vitro* models, although most of these studies have been conducted on

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**Keywords:** adipose tissue; antipsychotic; GSK3; insulin; IRS2; metabolic syndrome
Received 22 October 2012; accepted 10 November 2012

brain tissue.[14–16] To our knowledge, only three previous studies have investigated the effect of antipsychotics on the insulin signaling pathway in peripheral tissue. The first study reported an attenuation of the insulin signaling pathway with decreased protein levels of IRS2 in liver tissue of type 2 diabetic rats, following administration of chlorpromazine.[17] A more recent study showed an increased phosphorylation of AKT in tibialis muscle of clozapine-treated mice.[14] The third study showed that the acute administration of olanzapine to cultures of L6-rat skeletal muscle cells leads to an impairment of the insulin signaling and glycogen synthesis, diminishing insulin-stimulated AKT and GSK3α/β phosphorylation.[18] Interestingly, olanzapine has been recently found to readily fit within the binding pocket of GSK3β, suggesting a direct effect of olanzapine on the activity of this kinase.[19] When looking at possible mechanisms mediating insulin resistance, the study of hepatic tissue is particularly relevant, as the liver is one of the main organs involved in the regulation of glucose homeostasis, maintaining the normal concentration of blood glucose through its ability to store glucose as glycogen, and produces glucose from glycogen breakdown.[20]

To date, no study has investigated the effect of olanzapine and haloperidol on visceral adipose-tissue deposition and insulin signaling pathway in peripheral tissue in the same animals. The aim of the present study was to investigate molecular pathways through which antipsychotics may lead to the development of diabetes/insulin resistance, studying the effects of haloperidol and olanzapine in male rats on visceral fat deposition (using magnetic resonance imaging (MRI)), and on critical nodes of the insulin signaling pathway in the liver (assessing liver protein levels of IRS2, GSK3α, GSK3β, GSK3α-Ser21, GSK3β-Ser9).

## Materials and methods

**Animals.** Twenty-four male Sprague–Dawley rats (Charles River UK,, Kent, UK), initial body weight 240–250 g (9 weeks of age) were housed four per cage under a 12 h light/dark cycle (7:00 AM lights on) with food and water available *ad libitum*, as previously described.[21] Room temperature was maintained at $21 \pm 2\,^{\circ}$C and relative humidity at $55 \pm 10\%$. Animal experiments were carried out with local ethical approval and in accordance with the Home Office Animals (Scientific Procedures) Act, UK.

**Experimental design.** Eight animals were treated with vehicle, eight with haloperidol and eight with olanzapine. Vehicle (β-hydroxypropylcyclodextrin, 20% wt/vol, acidified by ascorbic acid to pH 6), haloperidol ($2\,\mathrm{mg\,kg^{-1}}$ per day; Sigma-Aldrich, Gillingham, Dorset, UK) and olanzapine ($10\,\mathrm{mg\,kg^{-1}}$ per day; Biophore Pharmaceuticals, Hyderabad, Andra Pradesh, India) were administered to the animals using osmotic minipumps for 8 weeks ($\sim$5 human years, considering 11.8 rat days equals 1 human year).[22] The doses of each antipsychotic were chosen based on previous D2 receptor occupancy studies in our laboratory;[23] serum-plasma levels achieved following chronic administration in this study reflect D2 occupancy in the range of 75–90%,[23] similar to clinical exposure. The osmotic pump delivers at a steady rate in comparison to daily injections where drug levels fall to undetectable levels within 24 h (half-life $<$2.5 h in rats for most antipsychotics). The mean drug plasma levels in our animals were as following: haloperidol $20.58 \pm 1.99\,\mathrm{ng\,ml^{-1}}$, olanzapine $60.13 \pm 20.17\,\mathrm{ng\,ml^{-1}}$. The MRI-safe osmotic minipumps (Alzet Model 2ML4, 28 days; Alzet, Cupertino, CA, USA) filled with drug or vehicle solutions were inserted subcutaneously on the back flank under isoflurane anesthesia (5% induction, 1.5% maintenance) and replaced once after 28 days. *In vivo* abdominal MRI scans were acquired after 8 weeks of antipsychotic treatment. Animals were then killed under terminal anesthesia (sodium pentobarbital, $60\,\mathrm{mg\,kg^{-1}}$ intraperitoneal). Liver tissues were rapidly dissected, snap frozen in isopentane and stored at $-80\,^{\circ}$C until further analysis could be performed. Body weight was measured biweekly, starting before minipump implantation until termination.

**MRI acquisition and analysis.** Animals were scanned after 8 weeks of treatment, and regional adiposity was determined by calculating the cross-sectional area of fat measured on an image slice centered at the level of the kidneys, as previously described.[24] Imaging was performed on a 7.0T horizontal small bore magnet (Varian, Palo Alto, CA, USA) connected to a console running VnmrJ acquisition software (v2.3; Varian). A quadrature birdcage radiofrequency coil with 72 mm internal diameter (Varian) was used for signal transmission and reception. The optimal imaging protocol consisted of coronal $T_2$-weighted (TR 1250 ms; TE 12 ms) spin echo images using a $256 \times 256$ matrix size, 16 averages per phase encoding step, 10 contiguous 1-mm-thick slices, with an in-plane spatial resolution of 625 $\mu$m.

The visceral fat of the animals was measured using the semi-automated region of interest tool in VnmrJ (Varian). At a slice level through the middle of both kidneys (Figure 1), all pixels were summed to yield a total body area. The fat content within this abdominal slice was nominally ascribed to all pixels exceeding 1s.d. greater than the signal intensity of the left kidney.[24]

**Western-blot analysis.** To investigate changes in protein expression of IRS2 and GSK3α/β, as well as treatment-induced changes in GSK3α/β phosphorylation, liver tissue was washed three times in ice cold PBS containing protease and phosphatase inhibitors (Pierce, Rockford, IL, USA). The washed tissue was homogenized in standard RIPA buffer with protease and phosphatase inhibitors (Pierce) using a dounce homogenizer, and lysed on ice for 20 min. Lysates were centrifuged at $14\,000 \times g$ for 10 min at $4\,^{\circ}$C and the protein-containing supernatants were used for further analysis.

Protein concentrations were quantified using a bicinchoninic acid colorimetric assay system (Merck, Nottingham, UK). Protein samples were incubated with the kit reaction mixture in a ratio 1:8 for 30 min at $37\,^{\circ}$C, absorbance was measured with a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Brea, USA) at 562 nm. The protein concentration per sample was determined based on a bovine serum albumin standard curve ($0\,\mu\mathrm{g\,ml^{-1}}$,

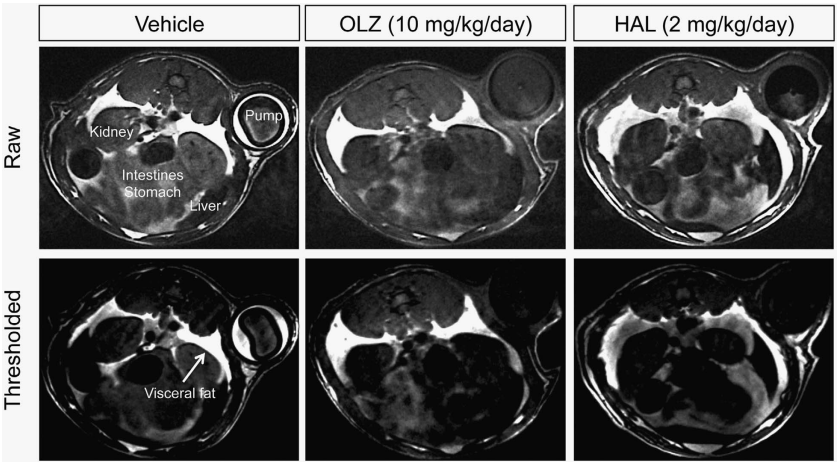


**Figure 1** Visceral fat assessed by $T_2$-weighted MRI in vehicle, olanzapine- and haloperidol-treated rats.

1.25 $\mu$g ml$^{-1}$, 5 $\mu$g ml$^{-1}$, 10 $\mu$g ml$^{-1}$, 25 $\mu$g ml$^{-1}$, 75 $\mu$g ml$^{-1}$, 125 $\mu$g ml$^{-1}$, 250 $\mu$g ml$^{-1}$, 500 $\mu$g ml$^{-1}$, 750 $\mu$g ml$^{-1}$).

Protein samples containing 30 $\mu$g of total protein were boiled for 10 min at 72 °C in 1 × NuPAGE LDS sample buffer (Invitrogen, Paisley, UK) and 1 × NuPAGE sample reducing agent (Invitrogen), and subjected to reducing SDS-PAGE on 10% NuPAGE Bis-Tris gels for 1 h at 200V. Proteins were electrophoretically transferred to Immuno-Blot™ PVDF membranes (Bio-Rad laboratories, Hercules, CA, USA) at 110V for 1.5 h at 4 °C. Transfer efficiency was controlled by Ponceau S staining and by pre-stained protein standards (Invitrogen).

Unspecific binding sites were blocked for 1 h in 5% bovine serum albumin in TBS and immunoprobed with the polyclonal rabbit anti-GSK3α (1:1000), rabbit anti-GSK3$\beta$ (1:1000), rabbit anti-GSK3α-Ser21 (1:1000), rabbit anti-GSK3β-Ser9 (1:1000) (all from Cell Signaling, Danvers, MA, USA), rabbit anti-IRS2 (1:500; abcam, Cambridge, UK) and rabbit anti-beta-actin antibody (1:500; Biolegend, San Diego, CA, USA) in blocking solution at 4 °C over night. Membranes were washed three times for 10 min in TBS containing 0.1% Tween-20 (TBST) and incubated with a HRP-conjugated swine antirabbit secondary antibody (1:2000; DAKO) in 5% non-fat dry milk in TBS for 1 h at room temperature. Membranes were washed three times for 10 min in TBST, and proteins were visualized with enhanced chemiluminescence detection system (GE Healthcare, Chalfont St Giles, UK).

**Statistical analyses.** A one-way ANOVA, followed by LSD post-hoc tests, was used to compare weight and intra-abdominal fat. The western blots analyses for haloperidol and olanzapine group were run at separate times. Therefore, we did not compare directly these two groups within a single statistical analysis, but we used separate Mann–Whitney *U* tests to compare the protein levels between the haloperidol and the vehicle group, and the protein levels between the olanzapine and the vehicle group. Blots for IRS2, GSK3α and GSK3β were normalized to beta-actin. Blots for Ser21-GSK3α and Ser9-GSK3β were normalized to unphosphorylated GSK3α and GSK3β, respectively. Protein levels are presented as fold change compared with controls. Results are presented as mean ± s.e.m.

## Results

**Weight and visceral adipose tissue.** The haloperidol group showed a higher percentage of visceral adipose tissue (%visceral adipose tissue 32.3 ± 1.3) than both the olanzapine group (21.6 ± 2.1; $P = 0.002$) and the vehicle group (23.0 ± 2.6; $P = 0.004$); there was no difference between the olanzapine and the vehicle group ($P = 0.7$, see Figure 1).

There were no significant differences in body weight at the end of the treatment among the three groups (haloperidol group: 325.9 ± 9.8 g; olanzapine group: 344.3 ± 11.1 g; vehicle group: 360.0 ± 8.7 g, $P = 0.07$), as previously reported.[21] The longitudinal change in body weight over time in the three separate groups has been previously reported, showing weight gain over the 8 weeks in all the three groups, with the antipsychotic-treated animals gaining less weight compared with the control group in the first 2 weeks.[21]

### Insulin signaling pathway

*IRS2.* The olanzapine group showed significantly reduced IRS2 protein levels (fold change: 0.46 ± 0.2; $P = 0.02$) when compared with the vehicle group. No statistical difference was found for IRS2 protein levels between the haloperidol and vehicle groups (fold change: 1.21 ± 0.4; $P > 0.05$; see Figure 2).

*GSK3α and GSK3β.* No significant difference was found in the total protein levels of GSK3α and GSK3β between the haloperidol and the vehicle group (fold change for GSK3α: 1.53 ± 0.3; $P > 0.05$; fold change for GSK3β: 1.34 ± 0.2; $P > 0.05$), or between the olanzapine group and the vehicle group (fold change for GSK3α: 0.96 ± 0.3; $P > 0.05$; fold change for GSK3β: 0.92 ± 0.2; $P > 0.05$; see Figures 3 and 4).

*GSK3α-Ser21 and GSK3β-Ser9.* The olanzapine group showed a significantly reduced phosphorylation of GSK3α-Ser21 (fold change: 0.22 ± 0.1; $P = 0.05$) and an increased phosphorylation of GSK3β-Ser9 (fold change: 1.93 ± 0.3; $P = 0.05$). We found a trend for a reduced phosphorylation of GSK3α-Ser21 (fold change: 0.38 ± 0.2; $P = 0.06$), but no

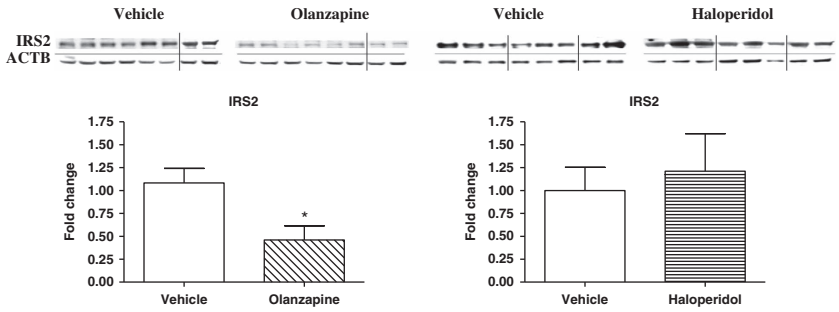


**Figure 2** Hepatic IRS2 protein levels in olanzapine and vehicle group ($P = 0.02$), and in haloperidol and vehicle group ($P > 0.05$). IRS2 blots were normalized to beta-actin.

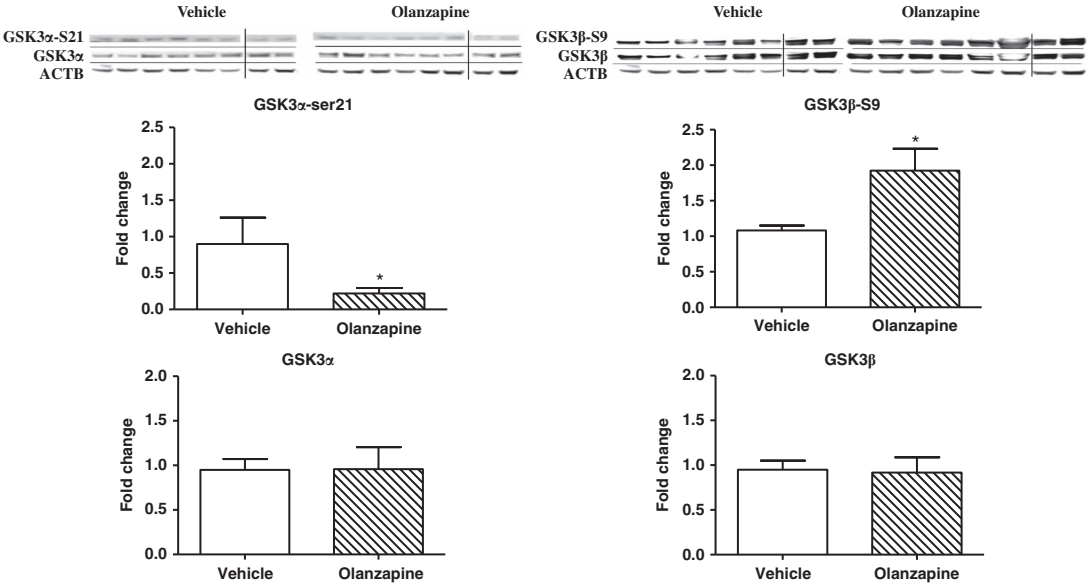


**Figure 3** Hepatic levels of GSK3α, phosphorylated Ser21-GSK3α, GSK3β and phosphorylated Ser9-GSK3β in olanzapine and vehicle group. Olanzapine group showed significantly reduced phosphorylation of GSK3α ($P = 0.05$), and increased phosphorylation of GSK3β ($P = 0.05$) when compared with vehicle group. Blots for GSK3α and GSK3β were normalized to beta-actin. Blots for Ser21-GSK3α and Ser9-GSK3β were normalized to unphosphorylated GSK3α and GSK3β, respectively.

significant difference was found in phosphorylated GSK3β-Ser9 between the haloperidol and the vehicle group (fold change: $1.1 \pm 0.1$; $P > 0.05$; see Figures 3 and 4).

## Discussion

Our findings show different molecular mechanisms underlying the metabolic effects of olanzapine and haloperidol. We find a direct effect of olanzapine on the hepatic insulin signaling pathway, with a reduction in IRS2 levels in the phosphorylation of GSK3α-Ser21, and an increase in the phosphorylation of GSK3β-Ser9, which appears in the absence of an effect on weight or visceral adipose tissue deposition. In contrast, haloperidol increases visceral adipose tissue deposition, but does not significantly affect the expression of proteins involved in the insulin signaling pathway in the liver tissue.

Our study is the first investigating the effects of olanzapine and haloperidol on the hepatic insulin signaling pathway *in vivo*. Previous studies have suggested that IRS2 is a major player in hepatic insulin action.[25] Indeed, knockdown of the hepatic IRS2 expression leads to glucose intolerance, whereas hepatic IRS2 overexpression attenuates gluconeogenesis and reduces fasting-glucose levels.[26,27] In our study, chronic treatment with olanzapine significantly decreases hepatic levels of IRS2, suggesting that olanzapine induces gluco-metabolic abnormalities through an inhibition of the insulin-signaling cascade. Moreover, we find that this effect is not associated with concurrent weight gain or increased visceral fat deposition, supporting previous findings of an effect of olanzapine on glucose homeostasis and insulin sensitivity before weight gain, and/or change in body-fat distribution.[7]

Indeed, and consistently with previous studies, we also did not find an effect of antipsychotic treatment on weight in our male rats.[28–30] However, in contrast with previous findings showing an effect of olanzapine in increasing total body or visceral adiposity,[7,31] we did not find an effect of olanzapine on increasing visceral adipose tissue in our animals. One possible explanation behind these differences is represented by the different methods of drug administration or

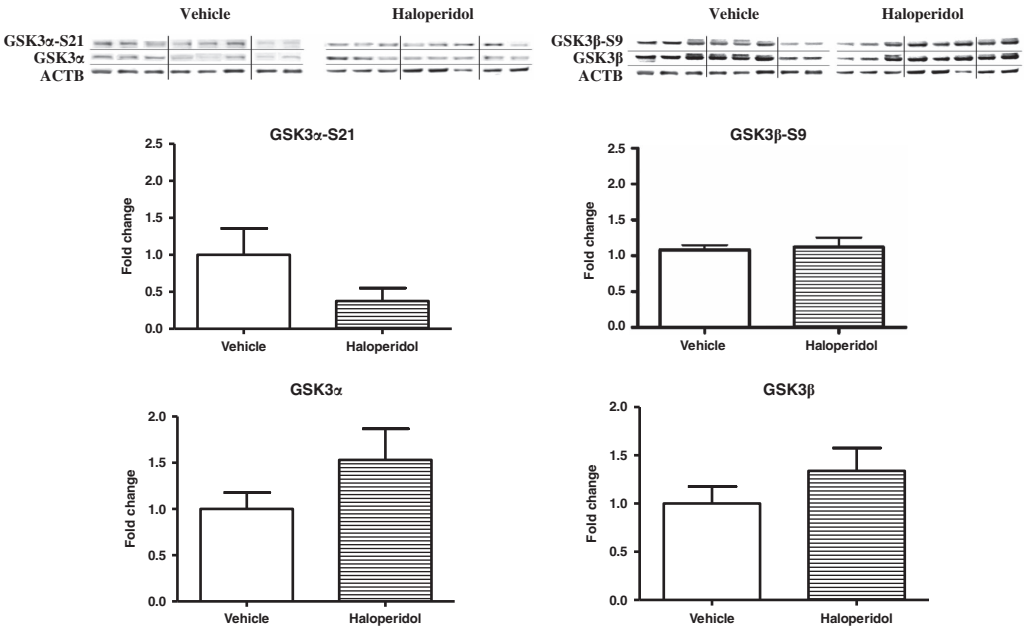


**Figure 4** Hepatic levels of GSK3α, phosphorylated Ser21-GSK3α, GSK3β and phosphorylated Ser9-GSK3β in haloperidol and vehicle group. No significant difference was found in GSK3α, GSK3 β, or in phosphorylated Ser21-GSK3α and Ser9-GSK3β between the haloperidol and the vehicle group ($P > 0.05$). Blots for GSK3α and GSK3β were normalized to beta-actin. Blots for Ser21-GSK3α and Ser9-GSK3β were normalized to unphosphorylated GSK3α and GSK3β, respectively.

antipsychotic concentration across the studies. Indeed, most previous studies looking at antipsychotic metabolic side effects administered antipsychotics by incorporation into the food, and did not report plasma drug concentration.[7,31] Precise regulation of a drug's plasma concentration following administration mixed with food or drinking water is difficult, and therefore exposure to the antipsychotic in these models might not reflect clinical exposure.[23] Similarly to our work, only one previous study used microinfusion pumps to achieve a stable infusion of antipsychotics to look at olanzapine metabolic side effects in male rats.[32] Interestingly, in this study, the authors found an increase in adiposity following olanzapine administration.[32] However, the plasma concentration of olanzapine were at least three-fold higher than those observed usually in humans in clinical settings,[33,34] and than those we had in our animals, possibly explaining the inconsistency with our findings.

In contrast to our findings for olanzapine, we find that haloperidol does increase visceral fat deposition, but does not have any effect on hepatic IRS2 levels. This difference could partly explain the difference between these two antipsychotics in terms of association with diabetes in the clinical setting. Interestingly, previous studies have shown that an excess of visceral adipose tissue can lead to low-grade chronic inflammation, dyslipidemia, insulin resistance, and ultimately, type 2 diabetes and cardiovascular disease, possibly increasing serum levels of free fatty acids, and therefore suggesting a possible alternative pathway to diabetes onset.[35]

Our findings, showing an increased phosphorylation of GSK3β in the liver tissue following treatment with olanzapine, are consistent with previous studies in mouse brain tissue, reporting an increased phosphorylation of GSK3β in the cortex, hippocampus, striatum and cerebellum, upon treatment with olanzapine.[16] Indeed, this is the first study showing this effect on peripheral tissues *in vivo*. Our data appear also to be in line with evidence of a significant elevation of liver glycogen storage in animals treated with higher doses of olanzapine.[19] However, the same findings seem in contrast with the diminished phosphorylation of GSK3β found by Engl *et al.*,[18] following incubation of L6-rat skeletal muscle cells with olanzapine *in vitro*. The difference between these studies could potentially be explained by a difference in the tissues studied. Indeed, olanzapine has been described to acutely change insulin sensitivity in a tissue-specific manner, decreasing insulin sensitivity in muscle tissue, whereas either increasing or not affecting sensitivity in adipose depots.[7] We also did not find an effect of haloperidol on hepatic phosphorylation of GSK3β. These data are consistent with a previous study, showing a lack of acute changes in GSK3β phosphorylation in the cortex, hippocampus, striatum and cerebellum of mice treated with haloperidol,[16] although in contrast with another study reporting increased phosphorylation of both GSK3α and GSK3β in the striatum of rats treated with haloperidol.[15] Indeed, the discrepancies with our study might be again related to the difference in the tissues studied, as our study is the first focussing on the insulin signaling pathway in hepatic tissue.

Our findings of increased GSK3β phosphorylation and of decreased GSK3α phosphorylation in the olanzapine group might initially appear conflicting, as we expected the phosphorylation of the two GSK3 isoforms to go in the same direction. Moreover, the increased phosphorylation of GSK3β would suggest an increased activity of glycogen synthase, and therefore, an increased insulin sensitivity, rather than insulin resistance. These apparently conflicting data could be explained by previous studies showing distinct

and tissue-specific biological roles of the two mammalian isoforms of GSK3.[36] In particular, GSK3α has a more critical role than GSK3β in regulating hepatic glucose metabolism and insulin sensitivity,[37] whereas GSK3β is the predominant regulator of glycogen synthase in skeletal muscles.[36] In fact, liver-specific GSK3β-knockout mice display normal metabolic characteristics and insulin signaling.[36] Therefore, we can hypothesize that the increased hepatic phosphorylation of GSK3β following olanzapine treatment might have a secondary role in the hepatic glucose metabolism that could instead be predominantly influenced by the decreased phosphorylation of GSK3α. Indeed, future studies should focus their attention also on proteins upstream and downstream from GSK3 in order to obtain more detailed information about the dysregulation of the insulin signaling cascade, following treatment with olanzapine.

Understanding the mechanisms through which antipsychotic treatment leads to increased metabolic abnormalities and obesity-related illness is essential in order to help reducing mortality rates, and improving quality of life for patients with psychosis. Indeed, our study suggests that different metabolic assessments (measurement of visceral adiposity or glucometabolic assessment) and different intervention approaches might be tailored according to the antipsychotic used.

There are few caveats in the present study. One such limitation is that these findings do not take into account the effect of a potential interaction between psychosis and antipsychotic treatment on the metabolic system. Indeed, metabolic disturbances have also been found in drug-naïve patients with first-episode psychosis, who show higher levels of visceral fat and impaired fasting-glucose tolerance.[38–40] Indeed, psychosis may predispose to metabolic abnormalities also through a sedentary lifestyle,[41] illness-related or genetic factors,[42,43] and childhood trauma.[44] Further studies would need to investigate this interaction in animal models of schizophrenia or directly in individuals with psychosis. A second limitation is represented by the lack of a direct measurement of glucose tolerance and insulin activity in our animals; future studies would need to investigate the role of the abnormalities in the insulin signaling pathway, identified in our study, in the development of insulin resistance. Finally, we did not measure levels of sedation, motor function or food intake following antipsychotic treatment; therefore, we cannot exclude these behavioral effects as possible confounders of our findings.

In conclusion, our data support the presence of different molecular pathways mediating the metabolic side effects of olanzapine and haloperidol, showing a direct effect of olanzapine on the insulin molecular pathway, and possibly partly explaining the stronger propensity of olanzapine for adverse effects on glucose regulation in clinical settings. We propose that diverse assessments and intervention approaches for metabolic abnormalities need to be considered according to the type of antipsychotic used in patients with psychosis.

## Conflict of interest

The authors declare no conflict of interest.

**Acknowledgements**. This research has been supported by the NIHR Biomedical Research Center for Mental Health at the South London and Maudsley NHS Foundation Trust and Institute of Psychiatry, King's College London, and from an ECNP Young Scientist Award, a grant from the University of London Central Research Fund, and a Starter Grant for Clinical Lecturers from the Academy of Medical Sciences, the Wellcome Trust, and the British Heart Foundation to V Mondelli. The data collection was partially supported by MRC Grant G1002198 to S Kapur. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

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