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ORIGINAL INVESTIGATION

Gender-dependent consequences of chronic olanzapine in the rat: effects on body weight, inflammatory, metabolic and microbiota parameters

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Abstract
Rationale Atypical antipsychotic drugs (AAPDs) such as olanzapine have a serious side effect profile including weight gain and metabolic dysfunction, and a number of studies have suggested a role for gender in the susceptibility to these effects. In recent times, the gut microbiota has been recognised as a major contributor to the regulation of body weight and metabolism. Thus, we investigated the effects of olanzapine on body weight, behaviour, gut microbiota and inflammatory and metabolic markers in both male and female rats.
Methods Male and female rats received olanzapine (2 or 4 mg/kg/day) or vehicle for 3 weeks. Body weight, food and water intake were monitored daily. The faecal microbial content was assessed by 454 pyrosequencing. Plasma cytokines (tumour necrosis alpha, interleukin 8 (IL-8), interleuin-6 and interleukin 1-beta (IL-1β)) as well as expression of genes including sterol-regulatory element binding protein-1c and CD68 were analysed.
Results Olanzapine induced significant body weight gain in the female rats only. Only female rats treated with olanzapine (2 mg/kg) had elevated plasma levels of IL-8 and IL-1β, while both males and females had olanzapine-induced increases in adiposity and evidence of macrophage infiltration into adipose tissue. Furthermore, an altered microbiota profile was observed following olanzapine treatment in both genders.
Conclusions This study furthers the theory that gender may impact on the nature of, and susceptibility to, certain side effects of antipsychotics. In addition, we demonstrate, what is to our knowledge the first time, an altered microbiota associated with chronic olanzapine treatment.

Keywords Olanzapine · Weight gain · Inflammation · Metabolic syndrome · Gender · Microbiota

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Introduction

Atypical antipsychotics (AAPDs) such as olanzapine represent the mainstay of treatment for schizophrenia and bipolar disorder. AAPDs are a diverse drug class grouped

together based on their lack of extra-pyramidal side effects associated with typical antipsychotics (De Oliveira and Juruena 2006). AAPDs however are associated with their own side effects, most notably weight gain and metabolic dysfunction (Albaugh et al. 2011; Birkenaes et al. 2008; Chintoh et al. 2008; Oriot et al. 2008; Perez-Iglesias et al. 2009). Clinically significant weight gain (> 7%) often occurs in greater than 50% of patients receiving an atypical antipsychotic (Ahmer et al. 2008; Citrome et al. 2011; Patel et al. 2009). This has ramifications for the patient in terms of metabolic and cardiovascular disease co-morbidity as well treatment compliance (Cohen and Correll 2009; Correll et al. 2009; Farwell et al. 2004; Nasrallah 2003; Starrenburg and Bogers 2009).

A number of factors, such as baseline weight (Basson et al. 2001; Gebhardt et al. 2009), therapeutic outcome (Basson et al. 2001; Meltzer et al. 2003) and gender (Aichhorn et al. 2007; Haack et al. 2009), have been proposed to confer susceptibility to the metabolic effects of antipsychotics. In the majority of cases, females have been found to have a higher prevalence of AAPD-induced weight gain (Aichhorn et al. 2007; Haack et al. 2009; Hakko et al. 2006; Verma et al. 2009). However, exceptions exist, where men have increased weight gain (Basson et al. 2001) or no gender bias has been apparent (Lee et al. 2004).

Intriguingly, in rat (and, to a lesser extent, mouse) models of antipsychotic-induced weight gain, there appears to exist gender-dependent effects with female rats showing more robust weight gain following treatment compared to males (Albaugh et al. 2006; Choi et al. 2007). This has led some to challenge the relevance of these models (Pouzet et al. 2003). However, recent studies have demonstrated that male rats do incur a number of detrimental metabolic effects in the absence of weight gain (Albaugh et al. 2011; Minet-Ringuet et al. 2006; Victoriano et al. 2009) and can even incur weight gain with extended protocols (Shobo et al. 2011). Overall, therefore, gender differences in animal models may be more relevant to the clinical setting than previously thought (Weston-Green et al. 2011) and, while current models are not perfect, they are extremely important in tackling the problems associated with AAPDs (Boyda et al. 2010).

It is important to note that AAPDs such as olanzapine can lead to the development of type II diabetes mellitus with or without the presence of overt weight gain (Kim et al. 2010; Newcomer 2004). Therefore, direct and indirect metabolic actions of these drugs are important considerations when assessing their overall metabolic impact. Inflammation has been proposed as an important factor in the development of obesity and the metabolic syndrome (Bastard et al. 2006; Das 2001) and a correlation between increased cytokine production and AAPD-induced weight gain has been observed (Kluge et al. 2009).

The gut microbiota comprises the approximate 10^{13}–10^{14} bacteria which reside within the gastrointestinal tract and exist in a symbiotic relationship with the host. Recently, a role for the gut microbiota in body weight, metabolism and systemic inflammation has begun to be elucidated (Backhed et al. 2004; Backhed et al. 2007; Bailey et al. 2011; Clarke et al. 2010; Ley et al. 2005; Murphy et al. 2010; Turnbaugh et al. 2006). Furthermore, as with many other systems, a definitive gender divide exists in the composition of the gut microbiota in both humans and animals (Fushuku and Fukuda 2008; Mueller et al. 2006). Moreover, the microbiota can have marked effects on the brain–gut axis (Cryan and O'Mahony 2011; Bravo et al. 2011). It is currently unclear whether chronic AAPD treatment can affect microbiota composition in addition to affecting body weight, metabolism and systemic inflammation.

Thus, we investigated the impact of chronic olanzapine treatment on metabolic, inflammatory and microbiome parameters and assessed whether there was a sexually dimorphic response.

Materials and methods

Animals

Male and female Sprague–Dawley rats, initially weighing approximately 200 g, were used (Harlan, UK). The animals were habituated to the animal facility for 1 week. They were housed at four rats per cage (56×38×17 cm), allowed access to standard chow and water ad libitum and kept on a 12-h light–dark cycle with lights on at 7:30 am. All experiments were approved by the Ethical Committee of University College Cork (#2010/013) and carried out in accordance with the Cruelty to Animals Act 1876.

Drug administration

Olanzapine (Discovery Fine Chemicals, UK) was dissolved in a minimal amount of glacial acetic acid (approximately 0.1 ml) and then made to volume with deionised water, with the pH adjusted to approximately 6.0 with 0.1 M NaOH. Vehicle consisted of distilled water acidified with glacial acetic acid and pH was adjusted with 0.1 M NaOH. All solutions were prepared fresh daily. The treatments were administered via intra-peritoneal injection B.I.D. with the first injection between 9:00 am and 10:00 am and the second injection between 4:00 pm and 5:00 pm.

Treatment groups

Rats (n=8) received vehicle, olanzapine 2 mg/kg/day or olanzapine 4 mg/kg/day for 21 days. All groups were

weight-matched prior to commencing treatment. Doses were selected on the basis that they reflect therapeutic concentrations and have been shown to induce weight gain and metabolic side effects previously (Cooper et al. 2005; Fell et al. 2005; Kapur et al. 2003).

Daily measurements

Body weight, food intake and water intake were measured each morning to the nearest 0.01 g using an electronic balance. This was carried out prior to the first injection.

Locomotor activity

On day 22, animals were allowed 1 h to habituate to the testing room (13:00–14:00) before being placed into the centre of a rectangular plastic box (60×50×40 cm). The behaviour was recorded via an overhead camera for 30 min (14:00–14:30) and locomotor activity was analysed using a tracking software system (Ethovision, Noldus, The Netherlands).

Sample collection

Animals were sacrificed by decapitation and trunk blood was collected in EDTA-coated tubes and centrifuged for 15 min at 6,000 rpm. Aliquots were derived from the plasma supernatant and stored on dry ice. The brain was quickly excised and dissected and each brain region was initially stored in RNALater for 24 h. The gonadal, mesenteric and subcutaneous fat deposits were carefully excised and weighed to the nearest 0.0001 g. The gonadal and mesenteric deposits were added together as a measure of visceral fat. The frontal lobe of the liver was snap-frozen in isopentane and stored on dry ice. All samples were frozen at −80°C for later analysis. Faecal pellets were collected directly from the animals on day 22 on dry ice and quickly stored at −80°C.

Plasma analysis

Concentrations of the cytokine tumour necrosis factor alpha (TNF-α), interleukin-8, (IL-8), interleukin-6 (IL-6) and interleukin-1 beta (IL-1β) were analysed using a commercially available electrochemiluminescence multiplex system (MSD, Gaithersburg, MD, USA). The highly sensitive assay has a range of 9.8–40,000 pg/ml. The plates were analysed on a SECTOR Imager 2400 from Mesoscale Discovery. A total of 25 μl of plasma was used for each well and all samples were analysed in duplicate.

Plasma leptin was analysed using a commercially available enzyme-linked immunosorbent assay kit (Millipore, Billerica, MA, USA). The assay range of this assay is 0.2 to 30 ng/ml. A total of 10 μl of plasma was used for each well and all samples were analysed in duplicate.

Plasma ghrelin was measured using a mouse/rat total ghrelin multi-array assay from Mesoscale discovery (MSD, Gaithersburg, MD, USA). The assay has a sensitivity of 11–5,000 pg/ml. All samples were measured in duplicate and analysed on a SECTOR Imager 2400 from Mesoscale Discovery.

Gene analysis

RNA from brain and liver samples was extracted for gene analysis using a commercially available kit (Agilent Technologies, CA, USA). Qiagen RNeasy Lipid Mini Kit was used for adipose tissue (QIAGEN, Valencia, CA, USA). mRNA was reverse-transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems) in a G-storm thermocycler (G-storm, Surrey, UK). Gene expression was analysed using TaqMan Gene Expression Assays and the AB7300 system (Applied Biosystems). The expression value of each gene was normalised to that of β-actin. All samples were analysed in triplicate.

Microbial community composition: pyrosequencing

For analysis of the microbial community composition, total DNA was extracted from the faecal pellets of two rats per cage using the QIAamp DNA stool mini-kit according to the manufacturer's instructions (Qiagen, West Sussex, UK) coupled with an initial bead-beating step. Universal 16S rRNA primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region, i.e. the forward primer F1 (5′-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5′-TACCRGGGTHTC TAATCC), R2 (5′-TACCAGAGTATCTAATTC), R3 (5′-CTACDSRGGTMTCTAATC) and R4 (5′-TACNVGGG TATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp), were used for Taq-based PCR amplification. Sequencing was performed on a Roche 454 GS-FLX using titanium chemistry by the Teagasc454SequencingPlatform. Resulting raw sequences reads were quality-trimmed as previously described (Claesson et al. 2009). Trimmed FASTA sequences were then BLASTed (Altschul et al. 1997) against a previously published 16 s rRNA-specific database (Urich et al. 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson et al. 2007a; Huson et al. 2007b). MEGAN assigns reads to NCBI taxonomies by employing the lowest common ancestor algorithm. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarization. A bit score of 86 was selected as previously used for 16S ribosomal sequence data (Urich et al. 2008). Phylum and family counts for each subject were extracted from MEGAN. Clustering and alpha diversities were generated with the MOTHUR software package (Schloss et al. 2009).

Statistical analysis

Two-way repeated measures analysis of variance (ANOVA) was used to analyse body weight change, food and water intake and locomotion, with gender and treatment as factors. Two-way ANOVA was used for gene and cytokine analysis with gender and treatment as factors. Further analysis was carried out using Tukey's post-hoc test. $p<0.05$ was considered as statistically significant.

Results

Body weight gain

The effect of olanzapine on body weight gain was significantly affected by gender (F (1, 42)=26.906, $p<0.001$) and time (F (2.85, 119.56)=271.878, $p<0.001$). There was a significant interaction between gender and treatment (F (2, 42)=4.883, $p=0.01$). There was also a significant interaction between gender and time (F (2.85, 119.56)=34.425, $p<0.001$) and a gender×treatment×time (F (5.69, 119.56)=15.101, $p<0.001$). The increase in the weight in the female rats was evident within the first days of treatment, with significant increases observed in animals given olanzapine (2 mg/kg) on days 5 to 15 and days 19–23 inclusive ($p<0.05$ to $p<0.001$). Females treated with olanzapine (4 mg/kg) displayed significant weight gain on days 3 to 15 inclusive ($p<0.05$ to $p<0.001$) but subsequently showed a reduction in body weight returning to normal levels such that the animals receiving olanzapine (2 mg/kg) were significantly increased compared to those receiving olanzapine (4 mg/kg) on days 21–23 inclusive ($p<0.05$) (Fig. 1a). In the male rats, no difference between treatment groups was observed (Fig. 1b).

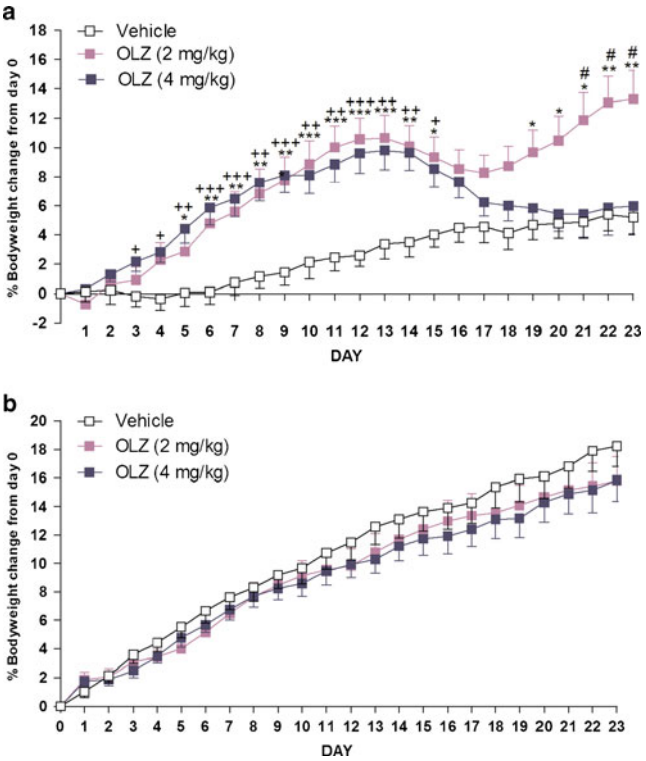

Fig. 1 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on percentage body weight gain in **a** female rats and **b** male rats treated for 21 days B.I.D. First injection on day 1. Data shown represent mean±SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ OLZ (2 mg/kg) significantly different versus vehicle group. $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ 4 mg/kg significant versus vehicle group. $^{\#}p<0.05$, OLZ (2 mg/kg) significant versus OLZ (4 mg/kg) group

Food and water intake

Olanzapine treatment significantly affected food intake (F (2, 90)=21.212, $p<0.001$). There was also a significant effect of gender (F (1, 90)=530.485, $p<0.001$) and a significant time×gender×treatment interaction (F (3.34, 150.66)=4.28, $p<0.01$). Hyperphagia was observed in the female rats in both treatment groups compared to vehicle in week 1 (2 mg/kg, $p<0.05$; 4 mg/kg, $p<0.001$) and week 2 (2 mg/kg, $p<0.05$; 4 mg/kg, $p<0.01$). The observed increases in the rats treated with olanzapine (4 mg/kg) were significantly greater than in those receiving olanzapine (2 mg/kg) group in week 1 ($p<0.05$). In week 3, hyperphagia persisted in the animals treated with olanzapine (2 mg/kg) ($p<0.05$) but was significantly reduced in those receiving olanzapine (4 mg/kg) compared to vehicle-treated rats ($p<0.01$) and the olanzapine (2 mg/kg) group ($p<0.01$). In the male rats, the olanzapine (4 mg/kg) group displayed a significant increase in food intake compared to the vehicle group in week 1 ($p<0.05$) but not in the subsequent weeks (Fig. 2a).

Olanzapine treatment also induced significant increases in water intake (F (2, 90)=6.573, $p<0.01$) with a further significant effect of gender (F (1, 90)=51.397, $p<0.001$) and time (F (2, 180)=14.637, $p<0.001$). There was also a significant gender×treatment interaction (F (2, 90)=4.895, $p<0.01$). The water intake followed a similar pattern to food intake with increases seen in the female rats treated with olanzapine (2 and 4 mg/kg) compared to vehicle-treated rats in week 1 ($p<0.05$, $p<0.01$), respectively, and week 2 ($p<0.001$, $p<0.05$). In week 3, only the female rats receiving olanzapine (2 mg/kg) displayed significant increases versus vehicle-treated animals ($p<0.05$). The male rats did not show differences in any of the weeks (Fig. 2b).

Locomotor activity

In the locomotor activity test, there was a significant effect of time (F (3.45, 144.68)=221.217, $p<0.001$) and treatment (F (2, 42)=7.264, $p<0.01$). Post-hoc analysis showed that

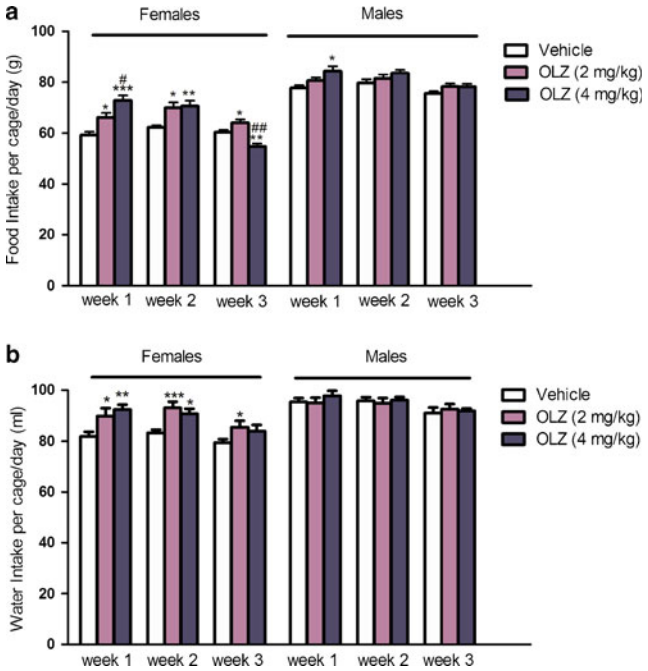

Fig. 2 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on **a** food intake and **b** water intake in female and male rats treated for 21 days B.I.D. Data shown represent mean ± SEM. Food and water intake shown as amount consumed per cage per day. *$p<0.05$, **$p<0.01$, ***$p<0.001$ significantly different versus vehicle group of the same gender. $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus OLZ (2 mg/kg) group of the same gender

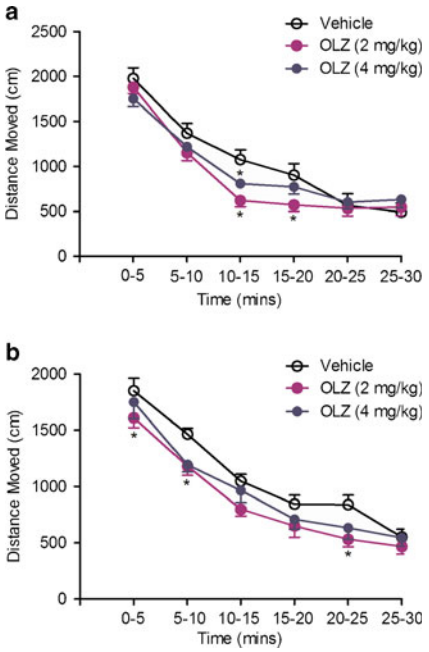

Fig. 3 Effect of olanzapine (*OLZ*) (2 mg/kg and 4 mg/kg) on locomotor activity in **a** female and **b** male rats treated for 21 days B.I.D. Locomotion measured as distance moved. Data shown represent mean ± SEM. *$p<0.05$ versus vehicle

there was a reduction in locomotion in female rats treated with olanzapine (2 mg/kg) between 15 and 20 min ($p<0.001$) and between 20 and 25 min ($p<0.05$). The female rats treated with olanzapine (4 mg/kg) only displayed decreased activity between 15 and 20 min ($p<0.05$). The male rats treated with olanzapine (2 and 4 mg/kg) exhibited reduced locomotor activity between the 5- and 10-min time points ($p<0.05$ and $p<0.01$, respectively). The male rats treated with olanzapine (2 mg/kg) further showed reduced movement between 15 and 20 min and between 20 and 25 min ($p<0.05$) (Fig. 3).

Adipose tissue

Olanzapine treatment significantly increased visceral fat mass (F (2, 42)=16.042, $p<0.001$) and there was a significant gender×treatment interaction (F (2, 42)=6.147, $p<0.01$). Female rats treated with olanzapine (2 and 4 mg/kg) had significantly increased visceral fat mass compared to vehicle-treated animals ($p<0.01$ and $p<0.05$, respectively). The male rats receiving olanzapine (4 mg/kg group) showed significant increases in visceral fat compared to both the male vehicle-treated group ($p<0.001$) and the male olanzapine (2 mg/kg)-treated group ($p<0.05$) (Fig. 4).

Olanzapine treatment also had a significant effect on CD68 expression (F (2, 38)=8.825, $p<0.001$) with an effect of gender (F (1, 38)=16.046, $p<0.001$). Female rats treated with olanzapine (4 mg/kg) displayed significantly increased levels of CD68 mRNA compared to vehicle-treated rats ($p<0.05$). In male rats, olanzapine treatment (2 and 4 mg/kg) resulted in increased levels of CD68 mRNA expression compared to vehicle-treated animals ($p<0.05$) (Fig. 5a).

Interleukin (IL)-6 mRNA expression was significantly affected by gender (F (1, 37)=26.511, $p<0.001$) and there was a significant gender×treatment interaction (F (2, 37)=3.886, $p<0.05$). The female animals receiving olanzapine (4 mg/kg) had increased levels compared to the animals receiving olanzapine (2 mg/kg) ($p<0.05$). The male rats treated with olanzapine did not show significant increases, though those treated with olanzapine (2 mg/kg) had a fourfold increase compared to vehicle-treated rats. The

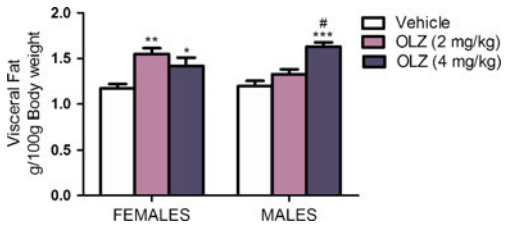

Fig. 4 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on the proportion of visceral fat (gonadal + mesenteric fat deposits) in female and male rats treated for 21 days B.I.D. Data shown represent mean±SEM. **$p<0.01$, ***$p<0.001$ significantly different versus vehicle group of the same gender. $^{\#}p<0.05$ significantly different versus OLZ (2 mg/kg) group of the same gender

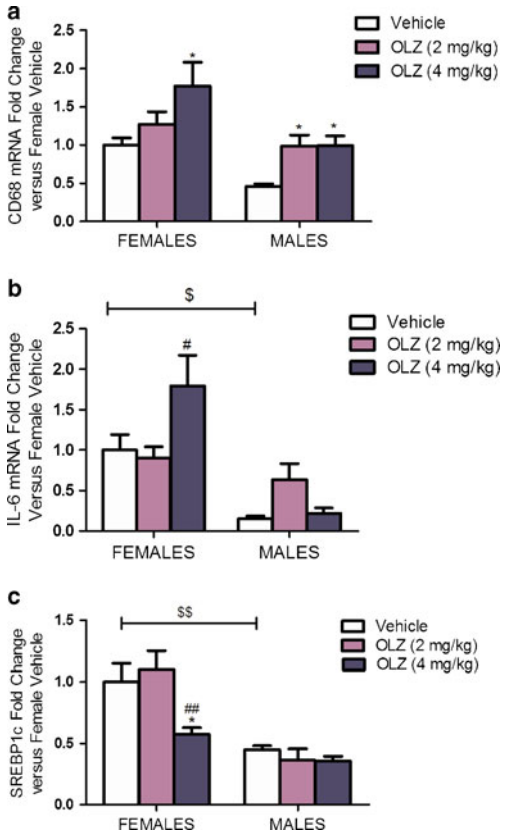

Fig. 5 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on **a** CD68 mRNA expression, **b** IL-6 mRNA expression and **c** SREBP-1c mRNA expression in female and male rats treated for 21 days B.I.D. Data shown represent mean ± SEM. *$p<0.05$ significantly different versus vehicle group of the same gender. $^{\#}p<0.05$, $^{\#\#}p<0.01$ significantly different versus OLZ (2 mg/kg) group of the same gender. $^{\$}p<0.05$, $^{\$}p<0.01$ female vehicle group versus male vehicle group

male vehicle-treated rats had significantly lower expression compared to female vehicle-treated animals ($p<0.05$) (Fig. 5b).

Sterol-regulatory element binding protein 1c (SREBP-1c) expression was significantly affected by olanzapine treatment (F (2, 34)=4.90, $p<0.05$). There was also a significant effect of gender (F (1, 34)=39.189, $p<0.001$) and a significant gender×treatment interaction (F (2, 34)=3.481, $p<0.05$). In female rats, those treated with olanzapine (4 mg/kg) had a significant reduction in the mRNA expression of SREBP-1c compared to both the vehicle ($p<0.05$) and olanzapine (4 mg/kg) groups ($p<0.01$). This reduction was not seen in the male rats, though the male vehicle group had significantly lower levels than the female vehicle-treated animals ($p<0.05$) (Fig. 5c).

Liver

Liver weight as a percentage of body weight was significantly affected by olanzapine treatment (F (2, 42)= 4.512, $p<0.05$) and gender (F (1, 42)=26.466, $p<0.001$). The female rats treated with olanzapine (2 mg/kg) were found to have significantly increased liver weight compared to the vehicle-treated rats ($p<0.05$). No differences were observed in the male rats (Fig. 6).

SREBP-1c mRNA expression in the liver was not affected by treatment but was significantly affected by gender (F (1, 42)=143.439, $p<0.001$). The male and female vehicle-treated groups differed significantly from one another ($p<0.001$) (Table 1).

Carbohydrate regulatory element binding protein (ChREBP) mRNA expression was significantly affected by gender (F (1, 46)=50.085, $p<0.001$) and male vehicle-treated rats were significantly different from female vehicle-treated animals (Table 1).

TNF mRNA in the liver showed no significant differences following olanzapine treatment (Table 1).

Plasma cytokines and leptin

Olanzapine treatment had a significant effect on the circulating plasma levels of IL-8 (F (2, 41)=3.613, $p<0.05$). The female rats treated with olanzapine (2 mg/kg) had increased levels compared to vehicle-treated rats ($p<0.01$) (Fig. 7a).

Plasma levels of TNF-α were significantly affected by gender (F (1, 41)=102.024, $p<0.001$) and there was a significant gender×treatment interaction following olanzapine treatment (F (2, 41)=7.049, $p<0.01$). Male rats treated with olanzapine (4 mg/kg) showed a reduction in circulating levels of TNF-α ($p<0.05$) (Fig. 7b).

Plasma IL-6 levels were significantly affected by treatment (F (1, 41)=4.005, $p<0.05$) and gender (F (1, 41)=7.473, $p<0.01$). There was a significant gender× treatment interaction (F (2, 41)=5.86, $p<0.01$). Male animals treated with olanzapine (2 and 4 mg/kg) had lower levels of IL-6 compared to the male vehicle-treated rats ($p<0.05$ and $p<0.01$, respectively). The vehicle-treated male animals had significantly higher levels than female vehicle-treated rats ($p<0.01$) (Fig. 7c).

IL-1β plasma levels were significantly affected by gender (F (1, 41)=5.575, $p<0.05$) and there was a significant gender×treatment interaction (F (2, 41)=4.93,

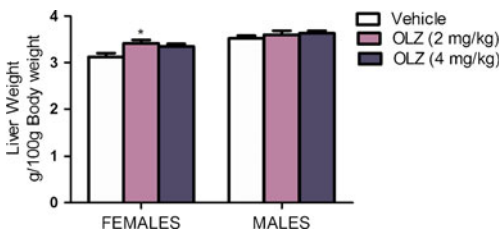

Fig. 6 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on relative liver weight in female and male rats treated for 21 days B.I.D. Data shown represent mean ± SEM. *$p<0.05$ significantly different versus vehicle group of the same gender

Table 1 Effect of chronic olanzapine on gene expression in the liver

Gender	Female			Male		
Olanzapine	Vehicle	2 mg/kg	4 mg/kg	Vehicle	2 mg/kg	4 mg/kg
SREBP-1c	1.0±0.16	0.73±0.07	0.70±0.08	3.26±0.16*	3.57±0.33	3.42±0.44
ChREBP	1.0±0.16	1.09±0.17	0.99±0.17	2.40±0.21*	2.67±0.26	2.70±0.47
TNF	1.0±0.09	0.89±0.14	0.90±0.08	1.82±0.42	0.89±0.20	0.86±0.11

Relative gene expression of SREBP-1c, ChREBP and TNF in liver of olanzapine- and vehicle-treated rats. Values were normalised to female vehicle group

SREBP sterol-regulating element-binding protein, *ChREBP* carbohydrate-regulating element-binding protein, *TNF* tumour necrosis factor

*$p<0.01$ versus female vehicle group

$p<0.05$). In the female rats, those receiving olanzapine (2 mg/kg) had significantly elevated levels compared to vehicle-treated rats ($p<0.05$). The male vehicle-treated rats had significantly higher levels than the female vehicle-treated rats ($p<0.01$) (Fig. 7d).

Plasma leptin levels showed a significant effect of gender (F (1, 42)=12.636, $p<0.001$). The male vehicle-treated animals had higher levels than female vehicle-treated rats ($p<0.05$) (Fig. 8).

Peripheral and central ghrelin

Olanzapine significantly affected the plasma levels of total ghrelin (F (2, 42)=3.143, $p=0.05$). There was also a significant effect of gender (F (1, 42)=9.109, $p<0.01$). The female rats treated with olanzapine (2 mg/kg) had reduced circulating levels of ghrelin ($p<0.05$) and those receiving olanzapine (4 mg/kg) also displayed a trend for reduced levels ($p=0.068$). No significant effects were observed between male groups (Fig. 9).

Hypothalamic expression of the ghrelin 1a receptor mRNA was significantly affected by gender (F (1, 35)= 13.68, $p<0.01$) and there was a significant gender× treatment interaction (F (2, 35)=6.973, $p<0.01$). The male rats treated with olanzapine (4 mg/kg) had significantly higher levels than the vehicle-treated rats ($p<0.05$) (Fig. 10).

Gut microbiota

The effects of chronic olanzapine on the microbial composition of the gut microbiota of the rats was elucidated through high-throughput pyrosequencing (Roche-454 Titanium) of 16S rRNA (V4) amplicons generated from faecal DNA obtained at study termination. Species richness, coverage and diversity estimations were calculated for each

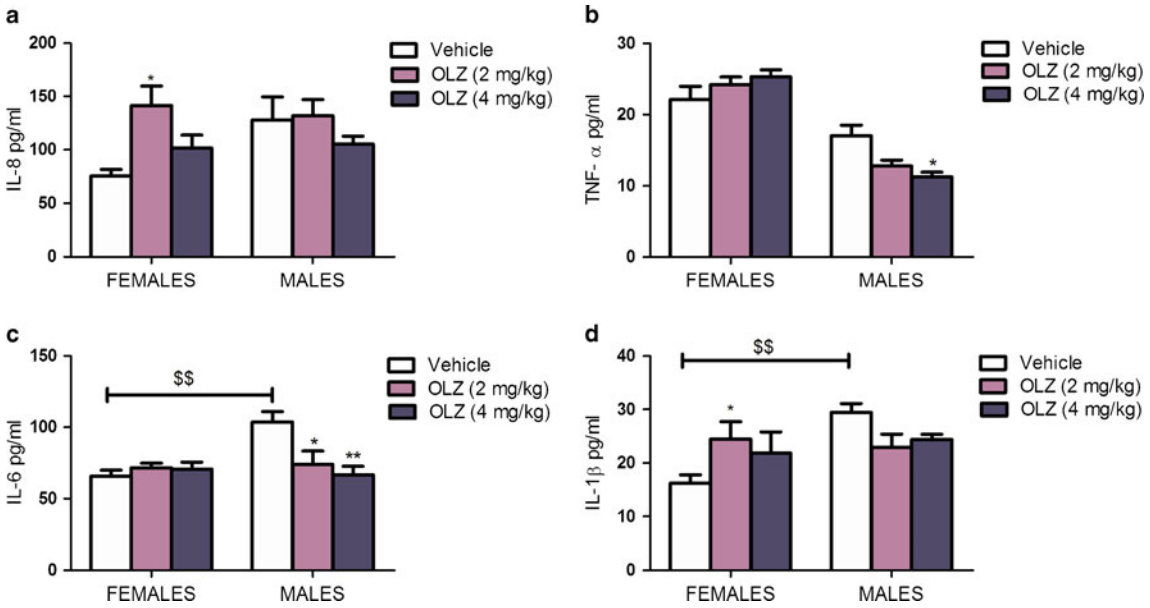

Fig. 7 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on plasma levels of **a** IL-8, **b** TNF-α, **c** IL-6 and **d** IL-1β in female and male rats treated for 21 days B.I.D. Data shown represent mean ± SEM. *$p<0.05$, **$p<0.01$ significantly different versus vehicle group of same gender. $^{\$}p<0.05$, $^{\$\$}p<0.01$, significant difference between female vehicle group and male vehicle group

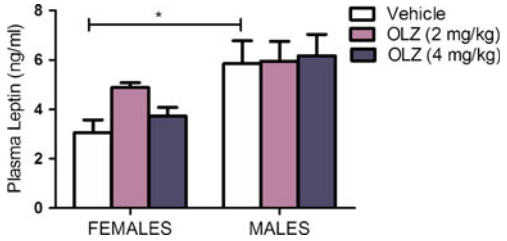

Fig. 8 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on plasma levels of leptin in female and male rats treated for 21 days B.I.D. Data shown represent mean ± SEM. *$p<0.05$ female vehicle group versus male vehicle group

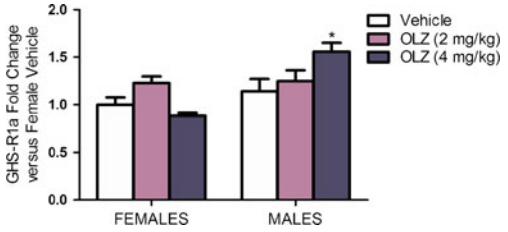

Fig. 10 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on growth hormone secretagogue 1a receptor mRNA expression in the hypothalamus in female and male rats treated for 21 days. Data shown represent mean ± SEM. *$p<0.05$ significantly different versus vehicle group of the same gender

data set (Supplementary Table 1). At the 97% similarity level, the Shannon index, a metric for community diversity, revealed a high level of overall biodiversity within all samples with values exceeding 4.2. The Good's coverage at the 97% similarity level ranged between 84% and 98% for all datasets. The Chao1 richness values indicate good sample richness throughout.

Assessment of the faecal microbiota, in terms of microbial phyla, revealed that olanzapine treatment in the female rats seemed to be associated with increased levels of Firmicutes following olanzapine 2 mg/kg (72.11% versus 84.06%) and olanzapine 4 mg/kg (72.11% versus 88.12%), with increases of 11.95% and 15.99% respectively. Olanzapine treatment of 2 and 4 mg/kg also appeared to reduce diversity compared to vehicle-treated rats evidenced by reductions in the less represented phyla Actinobacteria (3.72% versus 0.34% and 0.15%, respectively) and Proteobacteria (1.60% versus 0.15% and 0.77%, respectively). Animals treated with olanzapine 4 mg/kg also displayed evidence of reduced Bacteriodetes (17.57% versus 10.88%) (Fig. 11a).

In the male rats, olanzapine treatment (2 mg/kg) appeared to impact the microbiota minimally with an apparent reduction in Proteobacteria (3.15% versus 0.94%). Olanzapine treatment of 4 mg/kg, however, seemed to cause an increase in Firmicutes (82.66% versus 91.63%) and a reduction on Bacteriodetes of a similar magnitude (14.08% versus 7.97%) (Fig. 11b).

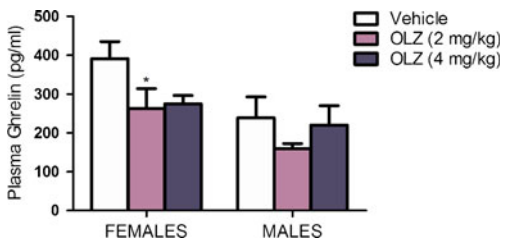

Fig. 9 Effect of olanzapine (*OLZ*) (2 and 4 mg/kg) on plasma levels of total ghrelin in female and male rats treated for 21 days B.I.D. Data shown represent mean ± SEM. *$p<0.05$ significantly different versus vehicle group of the same gender

Correlation analysis

In order to assess the possible relationship between the main physical alterations induced by olanzapine treatment in our model and possible biochemical correlates, we carried out correlation analysis on body weight gain and a number of biochemical plasma markers. For the female rats, a significant correlation was found between body weight gain and plasma leptin (Pearson correlation coefficient= 0.457, r^2 =0 .205, $p<0.05$) (Fig. 12a). A significant correlation was also found for body weight gain and plasma ghrelin (Pearson correlation coefficient=−0.429, r^2=0.185, $p<0.05$) (Fig. 12b). A significant correlation was also observed for body weight gain and plasma IL-8 levels (Pearson correlation coefficient=0.702, r^2=0.493, $p<$ 0.001) (Fig. 12c). Furthermore, a significant correlation was found between visceral fat mass and plasma IL-8 (Pearson correlation coefficient=0.550, r^2=0.303, $p<$ 0.01) (Fig. 12d).

In the male rats, no significant correlation was observed between any of the measured physical and biochemical parameters.

Discussion

Here we show that olanzapine had significant effects on a number of physiological, inflammatory and microbial parameters in the rat and that many, but not all of these, were more pronounced in females compared to males. Olanzapine induced rapid weight gain in female rats and not in male rats, which is consistent with previous reports (Albaugh et al. 2006; Choi et al. 2007). Both male and female rats treated with olanzapine did however exhibit increased visceral fat, though in the males this was the case only at the higher dose. We also show, to our knowledge for the first time, specific alterations to the gut microbiota as a result of antipsychotic treatment, suggesting that microbiota may contribute to AAPD-induced metabolic dysfunction.

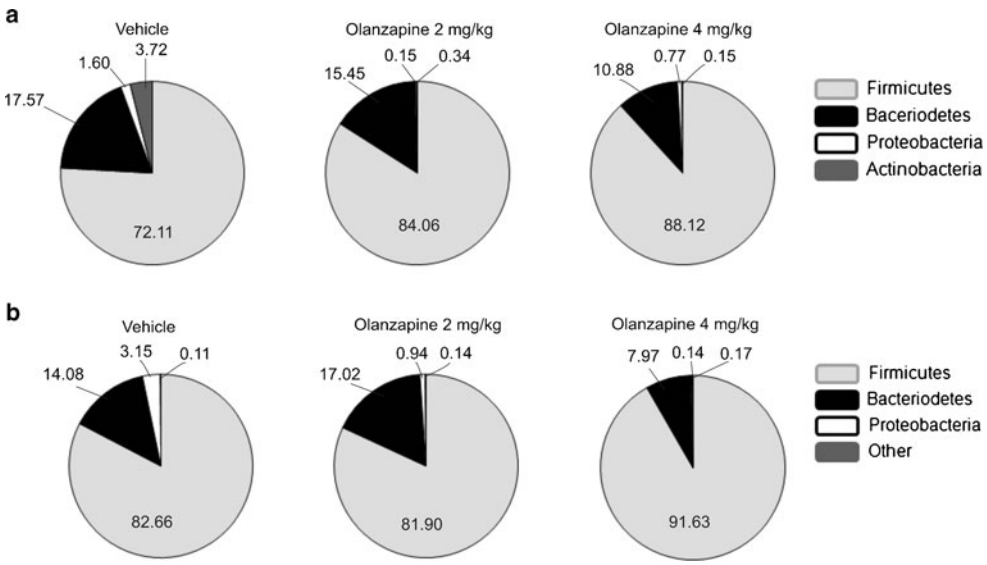

Fig. 11 Proportional composition of the faecal microbiota following 21 days of olanzapine treatment (2 or 4 mg/kg) in **a** females and **b** males. Data represent the cumulative DNA of two pellets per cage for each group

The reason for the gender difference in body weight gain at a pre-clinical level is currently unknown and its significance to the clinical presentation of AAPD-induced metabolic alterations is contentious. One reason for this being that clozapine, an antipsychotic that also causes considerable weight gain in humans, does not appear to do so in rats (Cooper et al. 2008). There is considerable evidence however to suggest that females are more liable to incur antipsychotic-induced weight gain (Aichhorn et al. 2007; Haack et al. 2009; Hakko et al. 2006), although this may reflect gender differences in drug pharmacokinetics (Beierle et al. 1999; Harris et al. 1995). In the present study, we observed a number of gender differences in baseline levels of the plasma cytokines IL-1β and IL-6 as well as local levels of IL-6 in the adipose tissue which may impact on susceptibility to the effects of AAPDs. Gender dimorphism in immune function including cytokine release is well documented and our findings suggest that these may have implications for antipsychotic side effects (Bao et al. 2002; Cannon and Pierre 1997; Yokoyama et al. 2005).

The complex nature of body weight regulation may explain why we did not observe a dose–response relationship in weight gain with olanzapine treatment. This is supported by clinical findings in which lower doses are not

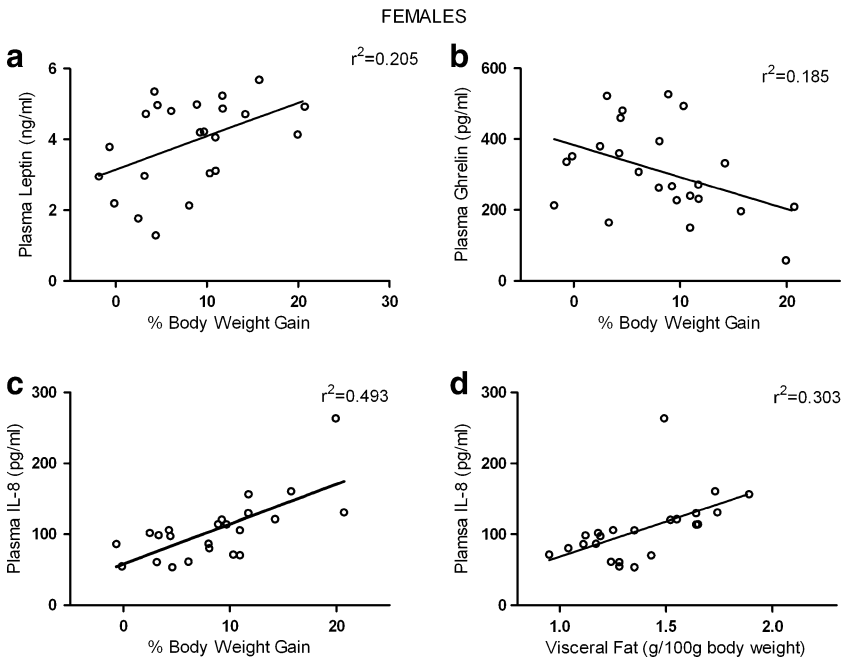

Fig. 12 Correlation analysis between percent body weight gain at day 23 and **a** plasma leptin, **b** plasma ghrelin and **c** plasma IL-8. **d** Correlation analysis between visceral fat and plasma IL-8

necessarily associated with lower weight gain (Citrome et al. 2009). The mechanisms by which olanzapine causes weight gain as in the female rats in this study are unclear but largely attributed to its diverse pharmacological receptor profile (Matsui-Sakata et al. 2005; Newcomer 2005; Reynolds et al. 2006; Roth et al. 2003; Silvestre and Prous 2005). Antagonism of central receptors including serotonin 5-HT_{2C} and histamine H_1 receptors, which play pivotal roles in appetite regulation as well as long-term energy balance (Lam et al. 2008; Masaki et al. 2004; Tsuda et al. 2002), has been particularly implicated in weight gain associated with antipsychotic treatment (Deng et al. 2010; Kroeze et al. 2003; Reynolds et al. 2006; Reynolds et al. 2002). Hyperphagia was observed in the female rats and is believed to drive initial weight gain (Thornton-Jones et al. 2002). This is also seen in clinical studies in which increased appetite is commonly reported by patients initiating olanzapine therapy (Basson et al. 2001; Kluge et al. 2007; Treuer et al. 2009) and in non-psychotic controls (Fountaine et al. 2010).

Male and female animals treated with olanzapine displayed a significant accretion of visceral fat. Interestingly, the female rats treated with olanzapine (4 mg/kg) returned to control body weight but still had increased visceral fat. Furthermore, the male rats treated with olanzapine (4 mg/kg) did not show increases in body weight gain but did however show increased adiposity. This finding supports clinical and pre-clinical studies which found increased adiposity following olanzapine treatment with (Ader et al. 2008; Raskind et al. 2007; Victoriano et al. 2009) and without weight gain (Victoriano et al. 2009). Increased visceral mass is considered a key factor in the development of the metabolic syndrome and in particular the development of insulin resistance (Bjorntorp 1991; Demerath et al. 2008). Thus, these data emphasise that the metabolic threat posed by olanzapine goes beyond merely increases in body weight gain.

Gonadal adipose tissue of female rats treated with olanzapine (4 mg/kg) and male rats treated with olanzapine (2 and 4 mg/kg) displayed increased CD68 expression. CD68 is a glycoprotein which represents a marker of macrophage presence. Macrophage infiltration of adipose tissue is considered a key step in the development of obesity-related inflammation and subsequent insulin resistance (Xu et al. 2003). Interestingly, CD68 expression did not mirror weight gain, even in females. Thus, this suggests that olanzapine can predispose toward a pro-inflammatory state independent of effects on body weight per se. This may have important connotations for patient monitoring following the prescription of AAPDs.

The adipose tissue of female rats treated with olanzapine (4 mg/kg) also displayed inflammation with increased IL-6 gene expression. Though not significant, the male rats receiving olanzapine (2 mg/kg) also displayed the same trend. Like CD68, IL-6 did not follow the pattern of weight gain. However, sorted cell gene analysis of adipose tissue has previously suggested that macrophages and adipocytes secrete roughly equal amounts of IL-6; thus, macrophage infiltration indicated by elevated CD68 expression likely led to elevated IL-6 expression (Wisse 2004). Data on in vitro tests suggest that IL-6 can directly confer insulin resistance (Rotter et al. 2003) and levels are associated with increased risk of type II diabetes (Pradhan et al. 2001). This further suggests therefore that olanzapine can confer risk of such metabolic abnormalities without overt weight gain and that IL-6 may be one mediator of this disguised threat.

The female rats further displayed a pro-inflammatory phenotype with IL-8 and IL-1β being significantly elevated in plasma in the olanzapine (2 mg/kg) group. Increased circulating levels of each of these cytokines have been associated with obesity and implicated in insulin resistance (Kim et al. 2006; Straczkowski et al. 2002). Conversely, male rats treated with olanzapine displayed an anti-inflammatory phenotype with reductions in IL-6 and TNF-α observed. While this is initially surprising, the anti-inflammatory effects of antipsychotics have been recognised for some time (Chedid 1954). Olanzapine has been shown to suppress TNF-α and Il-6 production in mice treated with lipopolysaccharide (Sugino et al. 2009). This discrepancy in circulating cytokines may reflect differences in their primary source of the cytokines (Trayhurn and Wood 2004) which could potentially account for the lack of an observed increase in the males and higher-dose females. Together these findings imply that systemic inflammation associated with olanzapine occurs primarily as a result of body weight gain. Intriguingly, the plasma levels of IL-8 in the female rats showed significant correlation with body weight gain and visceral fat mass, implicating this cytokine in particular as a possible link between inflammation and body weight gain and vice versa and may potentially be a biomarker for recognising the induction of AAPD metabolic side effects. This systemic inflammation may also act to impair metabolism, leading to insulin resistance and increased risk of metabolic syndrome and diabetes as a secondary effect. This emphasises the double-edged risk olanzapine confers on metabolic function with weight gain inducing systemic inflammation and the direct actions of the drug impacting on local inflammatory responses, both of which can converge to induce insulin resistance.

Further disruption to normal metabolic functioning was evidenced by reductions in sterol-regulatory binding protein-1c (SREPB-1c) gene expression in the adipose tissue of female rats treated with 4 mg/kg of olanzapine. SREBP-1c is a key regulatory transcription factor which controls a number of genes involved in lipid metabolism (Ferre and Foufelle 2007). Reduced expression of SREBP-

1c in adipose tissue has been observed in obese patients, and subsequent weight loss was associated with increased expression (Kolehmainen et al. 2001). These reductions are likely to be secondary to insulin resistance as insulin is the major regulator of SREBP expression. However, antipsychotics have been shown to activate SREBP in vitro (Ferno et al. 2006; Raeder et al. 2006; Yang et al. 2007) and in a recent in vivo study of risperidone (Lauressergues et al. 2010). However, a recent study also demonstrated down-regulation of SREBP-1c following an initial up-regulation after acute olanzapine treatment (Jassim et al. 2011). A study of clozapine administration was also associated with acute increases in SREBP and associated genes followed by a sustained down-regulation (Ferno et al. 2009). Thus, the long-term effects of antipsychotics on SREBP system are not yet clear but seem to involve feedback mechanisms, and this finding further supports the theory that olanzapine can directly affect lipid handling in the adipose tissue and thus directly contribute to fat deposition and dyslipidemia independent of weight gain (Ferno et al. 2011).

Ghrelin is an orexigenic hormone released from the stomach and is known as the hunger hormone as it is involved in meal initiation (Cummings et al. 2001; Schellekens et al. 2010). We observed reductions in plasma levels of total ghrelin in the female olanzapine-treated rats. The effect of antipsychotics on ghrelin has not been extensively studied though increased levels with prolonged treatment has been found in human patients (Murashita et al. 2005; Sentissi et al. 2008). In our studies, negative feedback may have occurred as a result of hyperphagia driven centrally. Intriguingly, in humans, higher basal plasma levels are associated with females (Greenman et al. 2004). Furthermore, ghrelin levels were found to be inversely correlated with fat mass and body mass index in females but not in human males (Makovey et al. 2007). It must be remembered that ghrelin in vivo exists as acetylated and non-acetylated forms and only the acetylated form can cross the blood–brain barrier and activate central ghrelin receptors. Also, ghrelin displays a circadian rhythm such that the time of day when the animals were sacrificed (morning) may have affected the ghrelin levels. Thus, total plasma ghrelin levels must be interpreted carefully. Hypothalamic ghrelin 1a receptor mRNA was increased in the male olanzapine (4 mg/kg)-treated animals. Central actions of ghrelin are associated with fat deposition (Riley et al. 2005). Thus, these results imply that alterations to the ghrelin system may be one mechanism by which olanzapine increases visceral fat and potentially also appetite and that these effects may be gender sensitive. Moreover, a significant inverse correlation was found between plasma ghrelin and body weight gain.

Leptin is a potent anorexigenic hormone with opposing effects to those of ghrelin. Though not significantly elevated in the treatment groups, a significant correlation was found between body weight gain and plasma leptin. While changes in circulating levels of these hormones likely represent secondary rather than direct actions of olanzapine (Baptista and Beaulieu 2002), they may potentially act as important markers for those at risk for sustained weight gain following commencement of antipsychotic therapy and are important considerations in the assessment of antipsychotics' metabolic impact (Sentissi et al. 2008).

The composition of the gut microbiota appeared to be considerably altered following treatment with olanzapine in the female rats and also in the male rats receiving olanzapine (4 mg/kg). In the female and male rats treated with olanzapine (4 mg/kg), the pooled samples at day 22 show a trend for increased Firmicutes and reduced Bacteriodetes compared to control animals. There was also evidence of reduced diversity at the phylum level in these olanzapine-treated groups with reduced levels of Proteobacteria in both females and males and reduced Actinobacteria in the females. The gut microbiota contributes to metabolism firstly by utilising indigestible complex polysaccharides via fermentation for their own energy and thereby producing short-chain fatty acids which can then be digested and used by the host for energy (Hooper et al. 2002). The microbiota is also involved in cholesterol reduction and the biosynthesis of vitamins that can be used by the host. It is estimated that as much as 10% of our daily energy supply may be provided in this way (Flint et al. 2008).

Furthermore, in their seminal work, Gordon and colleagues showed that germ-free mice (mice devoid of any microbiota) had 40% less body fat than their conventional littermates. Furthermore, colonisation of the germ-free mice with the microbiota of lean control mice led to a significant increase in body fat while colonisation with the microbiota of genetically obese mice (*ob/ob*) led to an even greater level of weight gain (Backhed et al. 2004; Turnbaugh et al. 2006). Furthermore, germ-free mice are resistant to diet-induced obesity (Backhed et al. 2007). This series of experiments also revealed that shifts in the predominant phyla of the microbiota were associated with obesity. An increase in the relative abundance of Firmicutes with a concordant decrease in Bacteriodetes was observed (Ley et al. 2005; Turnbaugh et al. 2008). This shift was also found in a human study of obese versus lean twins and in a study of type II diabetic patients versus non-diabetics, independent of body weight (Larsen et al. 2010; Turnbaugh et al. 2009).

Thus, our findings, while preliminary, are extremely interesting as they are closely in line with the above and other recent studies investigating the role of the microbiota in obesity and energy regulation (Cani et al. 2007; Kalliomaki et al. 2008). However, whether possible alter-

ations to the gut microbiota are a direct result of olanzapine treatment or secondary to other effects is unclear. A direct effect of the drug on intestinal bacteria cannot be ruled out, and a more quantitative analysis of gut flora following olanzapine treatment in the future may provide clarity on this. It is however, tempting to speculate that olanzapine may have influenced the gut microbiota via as yet unknown mechanisms and these changes could well contribute to or exacerbate metabolic dysfunction induced by AAPDs—in particular, fat accumulation. If this is the case, modulation of the gut flora by antibiotic, prebiotic or indeed probiotic therapy may represent a useful adjunctive therapy for olanzapine-induced weight gain in the future.

In this study, systemic inflammation occurred in a gender-dependent fashion and was only observed in female rats and as such likely occurred secondary to weight gain which was also only seen in the females. Importantly, however, both the female and male rats did incur a number of physiologically relevant changes including increased adipose tissue, local inflammation and alterations to the gut microbiota. Thus, this study brings into focus the need to consider the side effects of antipsychotics as a double threat involving not only weight gain but also independent metabolic effects which may include modulation of the gut microbiota. Furthermore, appreciating differences between the sexes may have important clinical implications in not only prescribing the treatment but also monitoring of patients in the future (Seeman 2004).

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Conflicts of interest The authors declare no conflicts of interest.

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