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Hypothalamic ghrelin signalling mediates olanzapine-induced hyperphagia and weight gain in female rats

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Abstract

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Disciplines

Medicine and Health Sciences

Authors

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Abstract

Excessive weight gain is a major metabolic side effect of second-generation antipsychotics (SGAs) in the treatment of schizophrenia. Ghrelin is an orexigenic hormone secreted mainly from the stomach, which can induce weight gain and hyperphagia through regulating neuropeptides at the hypothalamus. Accumulating evidence implicates a relationship between ghrelin signalling and SGA-induced hyperphagia and weight gain. We report that olanzapine (a SGA with high weight gain liability) potently and time-dependently up-regulate ghrelin and ghrelin signalling, leading to hyperphagia and weight gain in female Sprague-Dawley rats, an action reversed by i.c.v. injection of a ghrelin receptor (GHS-R1a) antagonist. These findings indicate a crucial role of ghrelin signalling in hyperphagia induced by olanzapine, supporting the notion that GHS-R1a antagonist may be useful for pharmacological treatment of SGA-induced weight gain resulted from hyperphagia.

Introduction

Schizophrenia affects up to one-and-a-half per cent of the general population worldwide (American Psychiatric Association, 2000). Olanzapine is a second-generation antipsychotic (SGA) widely used for treating schizophrenia (Leucht et al., 2009; Komossa et al., 2010). However, the metabolic side effects including weight gain and hyperphagia have led to drug withdrawals, symptom relapse and reduced drug compliance for some patients (Lieberman et al., 2005; Correll et al., 2011).

Ghrelin, an orexigenic hormone secreted primarily from the stomach, is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a; also called 'ghrelin receptor') (Kojima et al., 1999). Ghrelin stimulates food intake and body weight gain in both humans and rodents (Wren et al., 2001; Druce et al., 2005; Adachi et al., 2010), by up-regulating neuropeptide Y (NPY) and agouti-related peptide (AgRP) expressions (Nakazato et al., 2001), activating NPY/AgRP neurons (Cowley et al., 2003), and inhibiting proopiomelanocortin (POMC) neurons (Cowley et al.,

2003) in the arcuate nucleus (Arc) of the hypothalamus, where GHS-R1a is highly expressed (Guan et al., 1997; Zigman et al., 2006; Harrold et al., 2008).

Clinical studies have demonstrated that circulating levels of ghrelin is altered by olanzapine, possibly in a time-dependent manner (Togo et al., 2004; Murashita et al., 2005, 2007; Palik et al., 2005; Hosojima et al., 2006; Esen-Danaci et al., 2008; Kim et al., 2008; Perez-Iglesias et al., 2008; Roerig et al., 2008; Tanaka et al., 2008; Basoglu et al., 2010; Vidarsdottir et al., 2010; Chen et al., 2011; see Review by Zhang et al., 2013). Further, mRNA expressions of the GHS-R1a receptor at the hypothalamus have been reported to be up-regulated by olanzapine in rats (Davey et al., 2012). Additionally, the up-regulating effect on Arc NPY and AgRP, and the down-regulating effect on POMC mRNA of olanzapine have also been reported (Fernø et al., 2011; Weston-Green et al., 2012a). These studies suggest that ghrelin signalling could play an important role in olanzapine-induced hyperphagia and weight gain. In the current study, we used a GHS-R1a antagonist, D-Lys3-GHRP-6, injected i.c.v. into the brain of an olanzapine-induced hyperphagic rat model, to determine the acute effect of olanzapine on rats with intact or blocked ghrelin signalling pathway. In addition, according to a recent review of clinical and animal studies, the effects of SGAs (including olanzapine) on circulating ghrelin levels are tri-phasic (Zhang et al., 2013). Therefore, we tested the time-dependent effect

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of olanzapine on circulating ghrelin and ghrelin signalling in three cohorts of rats treated with olanzapine or control for different durations. Furthermore, we conducted a pair-feeding experiment to determine whether the effects on ghrelin and ghrelin signalling were due to a direct effect of olanzapine treatment or a secondary effect of hyperphagia/weight gain induced by olanzapine. Finally, the transcriptional factors forkhead box O1 (FOXO1), phosphorylated cyclic AMP response element binding protein (pCREB) and brain specific homeobox (BSX) have been reported as important mediators for the expressions of hypothalamic NPY, AgRP and POMC in the central ghrelin-signalling pathway (Kim et al., 2006; Kitamura et al., 2006; Sakkou et al., 2007; Nogueiras et al., 2008; Lage et al., 2010). In the current studies, hypothalamic expressions of these upstream ghrelin-signalling markers were also measured.

Method

Animals

Female Sprague-Dawley rats (201–225 g) were obtained from the Animal Resource Centre (Australia). Rats were individually housed at 22 °C, 12-h light-dark cycle with lights on at 07:00 h. All animals had *ad libitum* access to water and a standard laboratory chow diet (3.9 kcal/g; 10% fat, 74% carbohydrate and 16% protein). After one week of acclimatization, animals were trained to self-administer the placebo sweet cookie-dough. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Australian Government National Health and ⏐Medical Research Council, 2004).

Oral drug treatment

A cookie-dough (62% carbohydrate, 22% protein, 6% fibre, 10% vitamins and minerals) method was employed as previously reported (Han et al., 2008; Weston-Green et al., 2011; Deng et al., 2012). Briefly, a mixture of corn starch (30.9%), sucrose (30.9%), gelatine (6.3%), casein (15.5%), fibre (6.4%), minerals (8.4%) and vitamins (1.6%) was produced. Three times per day (at 7.00 h, 15.00 h and 23.00 h), cookie-dough mixed with either olanzapine (1 mg/kg BW) (Eli Lilly, USA) or placebo was served to the corresponding animals. The dosage of oral olanzapine administration (1 mg/kg, t.i.d.) was chosen based on our previous dose-dependent experiments (Weston-Green et al., 2011), which have been confirmed to be able to consistently induce hyperphagia and weight gain in our animal model (Deng et al., 2012; Weston-Green et al., 2012a,b; Lian et al., 2013). This dosage is clinically relevant based on D2 receptor occupancy (Kapur and Mamo, 2003), and is equivalent to a human dosage of approximately 10 mg/day (for a 60 kg person),

according to dosage translation between species based on body surface area, following an FDA guideline for clinical trials (Centre for Drug Evaluation and Research FDA, 2005; Reagan-Shaw et al., 2008). Animals were observed during the administration period to ensure complete consumption of the pellets.

Time-dependent experiment (Experiment 1)

Rats were randomized into either olanzapine (O) or control (C) treatment groups, with three treatment duration cohorts: short-term (8 days), mid-term (16 days) and long-term (36 days) (6 groups; $n = 12$/group). Food intake and body weight were measured every second day. The time frames chosen in the current study were based on evidence observed from both clinical and animal studies, which suggested that along the time course of olanzapine-induced weight gain, there are three typical stages: the initial stage with rapid increase of body weight accompanied with elevated food intake, the middle stage with slow body weight gain and no elevation of food intake, and the late stage with maintenance of the heavy body weight without elevated food intake (Huang et al., 2006; Pai et al., 2012; Deng, 2013).

Short-term pair-feeding experiment (Experiment 2)

Rats were randomised into two groups: pair-fed olanzapine (PO) and pair-fed control (PC) ($n = 12$/group). Rats then received eight days of treatments similar to the short-term cohort in Experiment 1, except that the lab-chow provided was not *ad libitum*, but with restricted food intake for both control and olanzapine groups to 80% of the control group's food intake based on the measurements at the previous time point.

I.C.V. injection experiment (Experiment 3)

After acclimatisation, a 24-guage cannula was surgically implanted under anaesthesia into the lateral ventricle of each rat (1.0 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.5 mm below the top of skull) (Paxions and Watson, 2007). All rats were allowed to recover for one week with close monitoring of post-surgery symptoms. Rats were orally administered with either olanzapine (1 mg/kg BW) (O) or control (C) cookie-dough three times daily for four consecutive days. On the fifth day, 30 min before oral cookie-dough administration, rats were injected with 5 μl of saline (V), low dose (3 nmol) GHS-R1a blockade (D-Lys3-GHRP-6; Tocris Bioscience, USA) (GL), high dose (30 nmol) GHS-R1a blockade (GH), or high dose GHS-R1a blockade followed by rat ghrelin (200 pmol; Tocris Bioscience, USA) (GH/g), via the cannula during 15.00–16.30 h (during the light phase when the normal oral drug administration was delivered). The i.c.v. injection dosages were chosen based on previous studies (Nakazato et al., 2001; Shintani et al., 2001; Asakawa et al., 2003; Kola et al.,

2005). Food was removed from the cages before the injection and reintroduced after cookie-dough administration. Food intake was measured 1, 2, 4, 18 and 24 h post injection. After four days of washout, rats were re-introduced to the original oral treatment regime of olanzapine or control for another four days. On the fifth day, rats were injected with the same drugs as in the first i.c.v. injection, and euthanized 1.5 h post-injection.

Euthanasia and tissue collection

Two hours after the last treatment in Experiment 1 and Experiment 2, and 1.5 h after the last injection in Experiment 3 (between 12:00 h and 14:00 h), rats were euthanized by fast CO_2 infusion (Han et al., 2008; Weston-Green et al., 2011; Deng et al., 2012). Blood samples were collected into EDTA tubes from the left ventricle of the heart; plasma was separated and stored at −80 °C. For ghrelin measurement, blood was treated with 0.8 mM Pefabloc SC (Sigma-Aldrich) and acidified with HCl to a final concentration of 0.05 N. In Experiment 1 and Experiment 2, brains were dissected on an ice plate immediately after euthanasia, snap-frozen in liquid nitrogen and stored in −80 °C. In Experiment 3, whole brains were snap-frozen, cut at 500 μM sections ranging from Bregma −2.16 to −3.66 mm based on a standard rat brain atlas (Paxions and Watson, 2007), using a cryostat (Leica CM 1950; Leica Microsystems, Wetzlar, Germany) with the temperature set at −18 °C. The Arc was dissected using a Stoelting Brain Punch (#57401, Wood Dale, Stoelting Co, USA) in an overlapping pattern over the 3rd ventricle. The punched tissue principally contained arcuate nucleus, but we cannot rule out the inclusion of adjacent brain areas, so the punched tissue was named as the mediobasal hypothalamus (MBH). White adipose tissue (inguinal, mesenteric, peri-renal and peri-ovary) and brown adipose tissue (inter-scapular) were dissected and individually weighed.

Enzyme immunoassay (EIA)

Hypothalamic NPY levels were determined by the NPY EIA kit (Phoenix Pharmaceuticals, USA) using the hypothalamic homogenates collected for Western blot in Experiment 1. Plasma ghrelin levels were detected by the ghrelin (total) EIA kit (Phoenix Pharmaceuticals, USA).

Western blot

The hypothalamus (or MBH) were homogenized in 10vol (v/w) homogenizing buffer (containing NP40, Protease Inhibitor Cocktail, 1 mM PMSF and 0.5 mM β-glycerophosphate). Total protein concentrations were determined by DC-Assay (Bio-Rad, USA), detected by SpectraMax Plus384 absorbance microplate reader (Molecular Devices, USA). Samples were heat-treated in Laemmli buffer at 95 °C, loaded to 8% SDS-PAGE gels

for fractionation, and then transferred onto Immun-BlotTM PVDF membranes (Bio-Rad, USA). The block consisted of 5% BSA in TBST. The membranes were then incubated with POMC, GHS-R1a, pCREB (Santa Cruz Biotechnologies; dilution factor 1:200), or FOXO1 (Cell Signalling Technology; dilution factor 1:1000) antibody in TBST containing 1% BSA overnight at 4 °C. Secondary antibodies were anti-rabbit (for POMC, FOXO1 and pCREB) or anti-goat (for GHS-R1a) IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnologies, USA; dilution factor 1:5000). For visualization, ECL detection reagents were used and films were exposed on the AGFA CP1000 Tabletop Processor (COD Medical, USA). Films were then analysed using the Quantity One software, connected to GS-690 Imaging Densitometer (Bio-Rad, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the hypothalamus (in Experiment 1 and 2) or MBH (in Experiment 3) with PureLink RNA extraction kit (Life Technologies, Australia) according to the manufacturer's protocol. First-strand cDNA was synthesized with VILO cDNA synthesis kit (Life Technologies, Australia) with 20 μl reaction volume. qRT-PCR was carried out in triplicates using TaqMan Gene Expression Assays (Life Technologies, Australia) on LightCycler480[+] (Roche, Germany). The results were normalized to β-actin (cat. no. 4252640E; Life Technologies, Australia), and were expressed as folds different from control. The assay identifications of the target genes were: Npy (Rn01410145_m1), Pomc (Rn00595020_m1), Agrp (Rn01431703_g1), Ghsr (Rn00821417_m1), Foxo1 (Rn01494868_m1); and Bsx (Rn04244809_m1) (Life Technologies, Australia).

Statistics

SPSS (version 15, SPSS, USA) was used. In Experiment 1 and Experiment 2, student's *t*-tests were used on daily food intake, cumulative weight gain, circulating ghrelin and hypothalamic GHS-R1a, NPY, AgRP, POMC, BSX, FOXO1 and pCREB level comparisons. Correlations were identified by Pearson's correlation. In Experiment 3, one-way ANOVAs were used to compare the hourly food intakes, circulating ghrelin levels, and levels of GHS-R1a, NPY, AgRP, POMC, BSX, FOXO1 and pCREB at the MBH, with *post-hoc* Turkey's test for multiple comparisons. Data were expressed as mean±S.E.M, and $p < 0.05$ was considered statistically significant.

Results

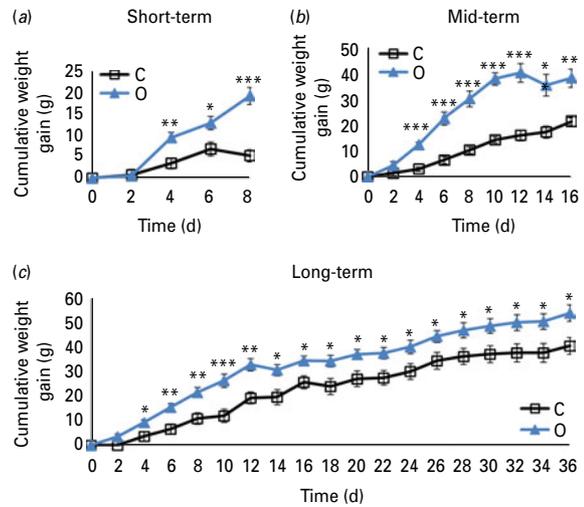### Olanzapine up-regulates circulating ghrelin in the short-term only

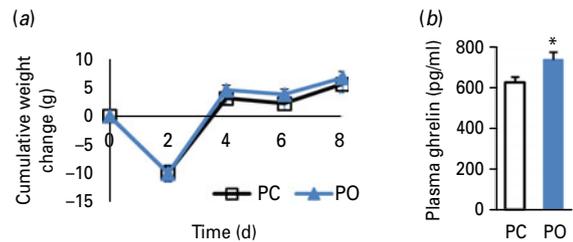To determine the time-dependent effects of olanzapine on circulating ghrelin levels, we performed enzyme
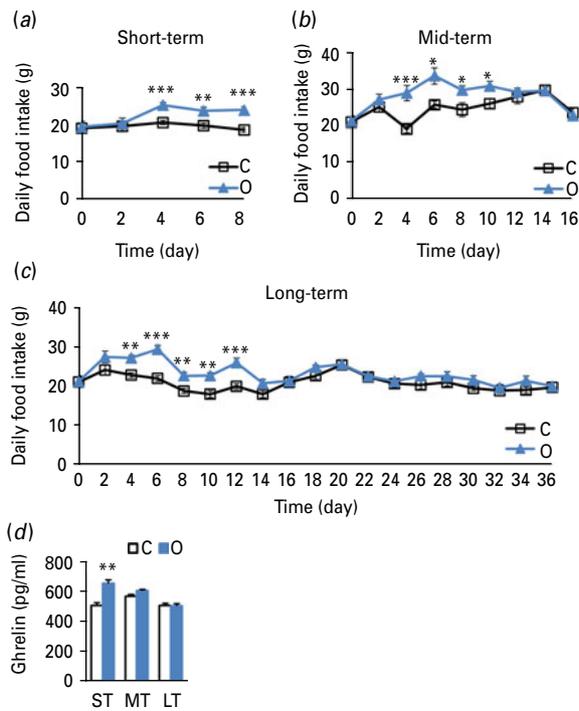
Fig. 1. Olanzapine increases daily food intake and plasma ghrelin levels in a time-dependent manner. In the short-term treatment cohort, daily food intake was increased by olanzapine from day four to day eight (a). In the mid-term treatment cohort, daily food intake was increased by olanzapine from day four to day ten (b). In the long-term cohort, daily food intake was increased by olanzapine from day four to day twelve (c). Olanzapine increased plasma ghrelin in the short-term cohort only (d). *$p < 0.05$ vs. Control; **$p < 0.01$ vs. Control; ***$p < 0.001$ vs. Control; $n = 10$–12 per treatment group.

Fig. 2. Olanzapine increases cumulative body weight gain. Cumulative body weight gain was increased by olanzapine from day four of treatment in the short-term (a), mid-term (b) and long-term (c) cohorts. *$p < 0.05$ vs. Control; **$p < 0.01$ vs. Control; ***$p < 0.001$ vs. Control; $n = 10$–12 per treatment group.
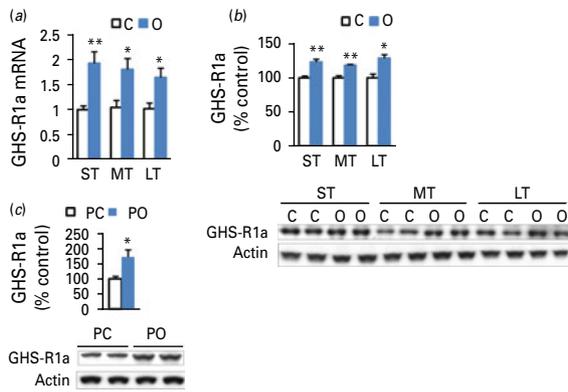

Fig. 3. Effects of pair-fed olanzapine treatments on cumulative weight change and circulating ghrelin levels. Pair-fed olanzapine treated rats had no significant weight change difference compared to the pair-fed control group (a). Pair-fed olanzapine treated rats had elevated plasma ghrelin levels compared to the pair-fed control group (b). *$p < 0.05$ vs. Pair-fed Control; $n = 12$ per treatment group.

immunoassay (EIA) on plasma samples collected from rats of the three treatment cohorts. In line with the effects on food intake (Fig. 1a–c), olanzapine treatment increased plasma ghrelin levels in the short-term (+30%, $p < 0.01$), had a trend of increase in the mid-term (+7%, $p = 0.0515$), but not in the long-term (Fig. 1d). The association between circulating ghrelin and food intake was confirmed by Pearson correlation analysis, which reveals that circulating ghrelin levels were positively correlated with final-day daily food intake ($r = 0.702$, $p < 0.001$; Supplementary Fig. S1a). However, olanzapine increased cumulative body weight gain from day four of treatment, and persisted throughout the whole treatment period (Fig. 2).

In order to determine whether the changes in ghrelin levels were secondary to the elevated food intake induced by olanzapine, we measured the circulating ghrelin levels in a pair-feeding experiment, where food intake of the olanzapine-treated rats was kept the same as the control rats. Our pair-feeding experiment showed that although there was no significant difference in weight gain (Fig. 3a), pair-fed olanzapine rats still exhibited higher

plasma ghrelin level compared to controls (Fig. 3b). These results suggest that the short-term elevation in circulating ghrelin levels under olanzapine treatment is likely to be the cause rather than the consequence of increased food intake.

Olanzapine up-regulates hypothalamic GHS-R1a receptor expressions throughout treatment periods

To determine the time-dependent effects of olanzapine on hypothalamic GHS-R1a receptor expressions, we performed Western blot and quantitative real-time PCR (qRT-PCR) on hypothalamic samples collected from rats of the three treatment cohorts. Olanzapine treated rats showed a small but significant increase in hypothalamic GHS-R1a protein expressions throughout the three treatment cohorts (+23%, $p < 0.01$; +18%, $p < 0.001$; +28%, $p < 0.01$, respectively) (Fig. 4a), which was consistent

Fig. 4. Effects of olanzapine treatments on hypothalamic GHS-R1a mRNA and protein expressions. Olanzapine increased hypothalamic GHS-R1a mRNA levels in the short-, mid- and long-term as detected by qRT-PCR (*a*). Olanzapine increased hypothalamic GHS-R1a protein levels in the short-, mid- and long-term as detected by Western blot (*b*). Pair-fed olanzapine treatment increased hypothalamic GHS-R1a protein expressions (*c*). *$p < 0.05$ *vs.* Control; **$p < 0.01$ *vs.* Control; $n = 5$–6 per treatment group.

with the effect on expressions of GHS-R1a mRNA (+92%, $p < 0.01$; +80%, $p < 0.05$; +64%, $p < 0.05$, respectively) (Fig. 4*b*). This has indicated that the effects of olanzapine on GHS-R1a are independent of those on circulating ghrelin levels. Interestingly, pair-fed olanzapine rats also exhibited elevated GHS-R1a protein expressions at the hypothalamus (Fig. 4*c*), suggesting these alterations were not secondary to the elevated food intake.

Olanzapine increased hypothalamic NPY and AgRP but decreased POMC expressions in the short- to mid-term

Olanzapine has been reported to up-regulate hypothalamic NPY, AgRP, and down-regulate POMC mRNA expressions (Fernø et al., 2011; Weston-Green et al., 2012a). However, the time-dependent effects of olanzapine on these neuropeptides have not been investigated. Therefore, we performed EIA on NPY protein, Western blot on POMC protein, and qRT-PCR on NPY, AgRP, and POMC mRNA levels with hypothalamic tissues from rats of the three treatment cohorts. NPY mRNA was up-regulated in the short-term only (+73%, $p < 0.01$; Fig. 5*a*). AgRP mRNA was up-regulated in the short- and mid-term (+68%, $p < 0.01$; +50%, $p < 0.05$, respectively; Fig. 5*b*), and POMC mRNA was down-regulated in the short- and mid-term by olanzapine treatment compared to control (−54%, $p < 0.01$; −40%, $p < 0.05$, respectively; Fig. 5*c*). Similar results were also observed at the protein/peptide level, where NPY was up-regulated in the short- and mid-term (+35%, $p < 0.001$; +12%, $p < 0.05$; Fig. 5*d*), while POMC was decreased in short-, mid- and long-term, but with different magnitudes of reduction (−36%, $p < 0.01$; −30%, $p < 0.05$; −19%, $p < 0.01$, respectively; Fig. 5*e*). Since the role of hypothalamic NPY and

AgRP is to stimulate food intake, while that of POMC is to inhibit food intake, the alterations on these hypothalamic neuropeptides induced by olanzapine were in line with the time-dependent effects of olanzapine on food intake and circulating ghrelin levels. In fact, circulating ghrelin levels were positively correlated with hypothalamic mRNA levels of NPY and AgRP, while negatively correlated with POMC ($r = 0.785$, $p < 0.001$; $r = 0.740$, $p < 0.001$; $r = -0.766$, $p < 0.001$, respectively; Supplementary Fig. S1*b–d*). Finally, pair-fed olanzapine treated rats also showed similar results in terms of hypothalamic NPY, AgRP and POMC expressions (Fig. 5*f*, g), indicating these alterations are not secondary to increase of food intake.

Olanzapine increased hypothalamic expressions of FOXO1, BSX, and pCREB, the transcriptional factors for NPY and AgRP, in the short- to mid-term

To investigate the time-dependent effects of olanzapine on the hypothalamic transcriptional factors FOXO1, BSX and pCREB, we performed Western blotting on FOXO1 and pCREB protein, and qRT-PCR on FOXO1 and BSX mRNA with hypothalamic tissues from rats of the three treatment cohorts. In line with the time-dependent changes in hypothalamic NPY, AgRP and POMC levels, olanzapine treatment increased hypothalamic FOXO1 protein expressions in the short-term only (+32%, $p < 0.05$; Fig. 6*a*), while pCREB levels were increased in the short- and mid-term (+31%, $p < 0.001$; +14%, $p < 0.05$, respectively; Fig. 6*a*). Similarly, FOXO1 mRNA levels in the hypothalamus were up-regulated by olanzapine treatment in the short-term (+66%, $p < 0.01$) and had a trend of increase in the mid-term (+19%, $p = 0.0503$; Fig. 6*b*); while BSX mRNA levels were elevated in the short-term only (+266%, $p < 0.001$; Fig. 6*c*). These results suggest that olanzapine up-regulates hypothalamic FOXO1, BSX, and pCREB expressions, at least in the early stages of treatments. Pearson's correlation test revealed that there were positive correlations between BSX and NPY ($r = 0.700$, $p < 0.001$), BSX and AgRP ($r = 0.661$, $p < 0.001$), pCREB and NPY ($r = 0.893$, $p < 0.001$), FOXO1 and AgRP ($r = 0.835$, $p < 0.001$), as well as negative correlations between FOXO1 and POMC ($r = -0.865$, $p < 0.001$) (Supplementary Fig. S2*a–e*), indicating that FOXO1, BSX and pCREB are involved in olanzapine-induced alterations in these hypothalamic neuropeptides. Finally, pair-fed olanzapine treated rats also exhibited elevated hypothalamic BSX mRNA levels, as well as pCREB and FOXO1 protein levels (Fig. 6*d*, e), indicating that these up-regulations are not secondary to increase of food intake.

Olanzapine-induced increase in food intake is blocked by GHS-R1a antagonist D-Lys3-GHRP-6

To investigate the cause – effect relationship between the alterations in ghrelin signalling and the increase of food
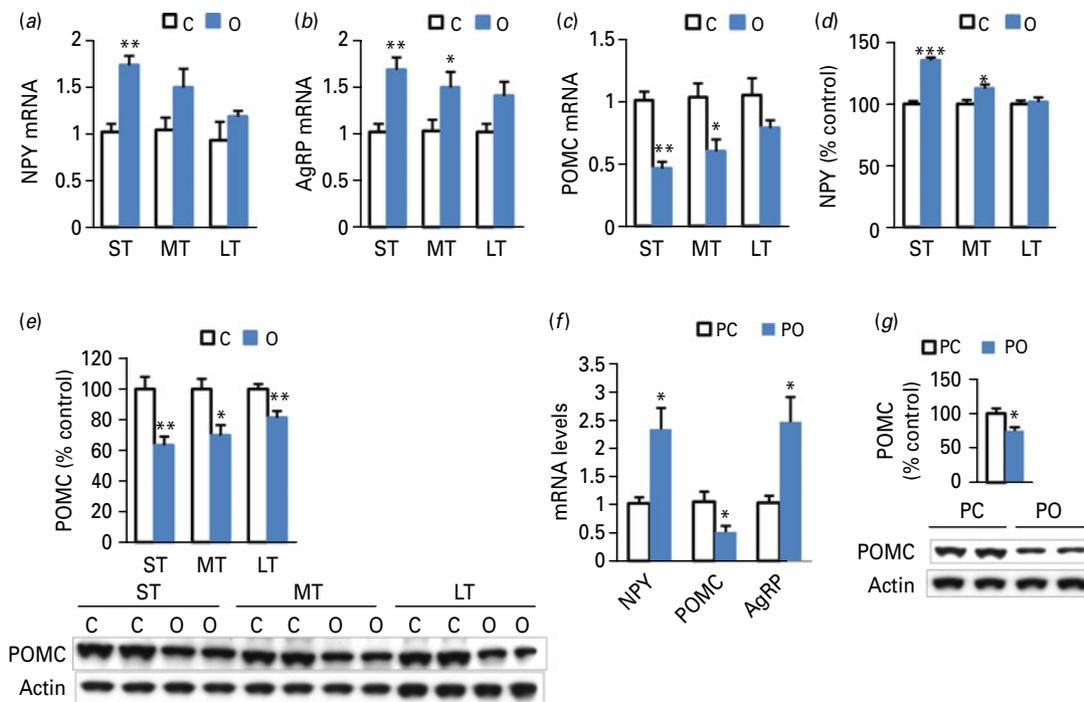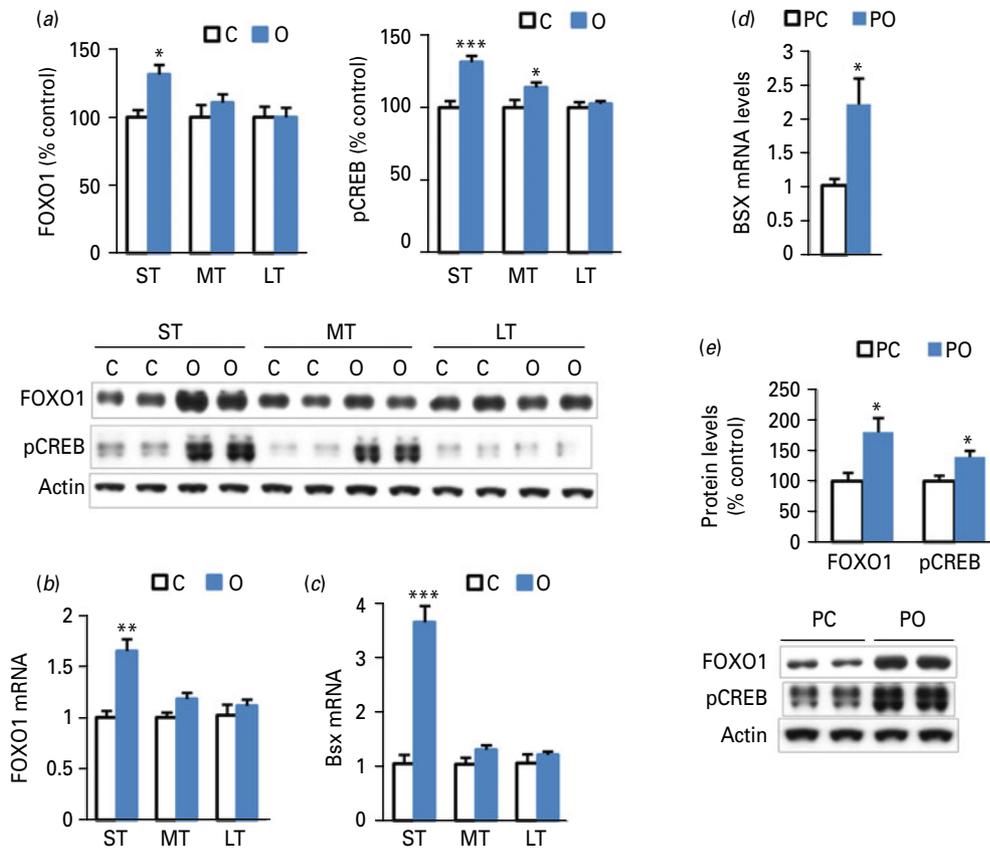
Fig. 5. Effects of olanzapine on hypothalamic expressions of neuropeptides NPY, POMC and AgRP. Olanzapine increased hypothalamic NPY mRNA expressions in the short-term only (*a*). Olanzapine increased hypothalamic AgRP mRNA expressions in the short- and mid- term, but not in the long-term (*b*). Olanzapine reduced hypothalamic POMC mRNA expressions in the short- and mid-term (*c*). Olanzapine increased hypothalamic NPY protein expressions in the short- and mid- term, as detected by EIA (*d*). Olanzapine decreased hypothalamic POMC protein expressions in the short-, mid- and long-term, as detected by Western blot (*e*). Pair-fed olanzapine treatment increased hypothalamic mRNA expressions of NPY and AgRP, but decreased POMC mRNA expressions (*f*). Pair-fed olanzapine treatment down-regulated hypothalamic POMC protein expressions (*g*). *$p<0.05$ *vs.* Control; **$p<0.01$ *vs.* Control; ***$p<0.001$ *vs.* Control; $n=5$–6 per treatment group.

intake under olanzapine treatment, we administered a ghrelin receptor blockade (D-Lys3-GHRP-6) via i.c.v. injection after the establishment of elevated food intake induced by olanzapine treatment. Our i.c.v. injection experiment showed for the first time that the increase of food intake induced by olanzapine was alleviated by D-Lys3-GHRP-6 (Fig. 7*a*) in the first 4 h post-i.c.v. injection, indicating that the ghrelin-signalling pathway is critical in mediating the acute effect of olanzapine on increasing food intake. In the first four hours post-i.c.v. injection, a high dose of D-Lys3-GHRP-6 completely blocked the elevated food intake induced by olanzapine, while a low dose of D-Lys3-GHRP-6 produced a significant but smaller alleviation effect (Fig. 7*a*), suggesting a dose-dependent effect of D-Lys3-GHRP-6 on reversing the effect of olanzapine on food intake. Interestingly, there was no significant difference between acute food intakes of control treated rats injected with vehicle and those injected with D-Lys3-GHRP-6 (Fig. 7*a*), indicating that the blockage effect of D-Lys3-GHRP-6 on food intake was specific to the olanzapine treated animals, but not an effect on the baseline food intake. Finally, the subsequent ghrelin i.c.v. injection following the high dosage D-Lys3-GHRP-6 injection produced a moderate effect on food intake only in the first hour post-i.c.v. injection

(Fig. 7*a*), suggesting that the ghrelin-signalling pathway was completely blocked by high dose D-Lys3-GHRP-6 after one hour post-injection, confirming the crucial role of ghrelin-signalling in olanzapine-induced elevation of food intake.

GHS-R1a antagonist D-Lys3-GHRP-6 attenuates alterations in hypothalamic NPY, AgRP, POMC, FOXO1, BSX and pCREB expressions induced by olanzapine

To examine the effect of D-Lys3-GHRP-6 on the downstream ghrelin-signalling parameters in the hypothalamus, we performed Western blotting of POMC, FOXO1 and pCREB, and real-time PCR on the mRNA levels of NPY, AgRP BSX and FOXO1, at the MBH of the hypothalamus. Our results revealed that at the MBH, the down-regulation of POMC and up-regulation of FOXO1 and pCREB was blocked by high dose D-Lys3-GHRP-6 injection (Fig. 7*b*). Similarly, the up-regulation of mRNA levels on NPY, AgRP, FOXO1 and BSX by olanzapine was also reversed by high dose D-Lys3-GHRP-6 (Fig. 7*c*). These results suggest that the effects of olanzapine on downstream ghrelin-signalling parameters were attenuated by i.c.v. injection of the ghrelin receptor

Fig. 6. Effects of olanzapine on hypothalamic FOXO1, pCREB, and BSX expressions. Olanzapine increased hypothalamic FOXO1 protein expressions in the short-term only, while increasing hypothalamic pCREB protein expressions in the short- and mid-term (*a*). Olanzapine increased hypothalamic FOXO1 and BSX mRNA expressions in the short-term only (*b; c*). Pair-fed olanzapine treatment increased hypothalamic BSX mRNA expressions (*d*). Pair-fed olanzapine treatment increased hypothalamic FOXO1, and pCREB protein expressions (*e*) *$p < 0.05$ *vs.* Control; **$p < 0.01$ *vs.* Control; ***$p < 0.001$ *vs.* Control; $n = 5$–6 per treatment group.

blockade D-Lys3-GHRP-6. However, EIA analysis revealed that i.c.v/ injection of D-Lys3-GHRP-6 has no effect on circulating ghrelin levels (Fig. 7*d*), suggesting the effect of olanzapine on circulating ghrelin is upstream or independent of the hypothalamic GHS-R1a receptor.

Discussion

A recent review by our group suggested that the effects of second-generation antipsychotics, including olanzapine, on circulating ghrelin levels were tri-phasic: the initial elevation stage at the acute phase, the secondary decrease stage of up to about eight weeks of treatment, and the final re-increase stage thereafter (Zhang et al., 2013). Interestingly, this tri-phasic effect on ghrelin levels has also been substantiated by rodent studies (Zhang et al., 2013). In the current study, we found that eight days of olanzapine treatment increased plasma total ghrelin levels, which is consistent with results in the clinical studies at the first stage of treatment. However, the 16- and 36-d treatment cohorts (representing the second stage of treatment) showed no effect of olanzapine on plasma ghrelin levels, which appears to be in conflict with the

reduction effect as shown by the clinical data. In fact, the results from this period of treatment have been inconsistent in both human and rodent studies (Zhang et al., 2013), possibly due to a secondary negative-feedback effect from weight gain induced by antipsychotic treatments. Therefore, the nil effect in plasma ghrelin levels observed in the mid- and long-term cohorts in the current study may be owing to a combined effect of the initial elevation triggered by olanzapine treatment and the secondary reduction by olanzapine-induced weight gain. In addition, consistent with the effect of olanzapine on circulating ghrelin levels, elevation of food intake disappeared from around day 12 of olanzapine treatment in the current study, yet increase of body weight gain persists throughout the long-term treatment period, indicating a non-hyperphagic effect of olanzapine on body weight during the mid- to long-term periods of treatment. In fact, energy expenditure, in particular physical activity and thermogenesis, have been suggested to contribute to the olanzapine's effect on body weight gain (Stefanidis et al., 2009). In light of the non-effect of olanzapine on ghrelin during the mid- to long-term, the role of ghrelin signalling on this long-term effect seems not supported.
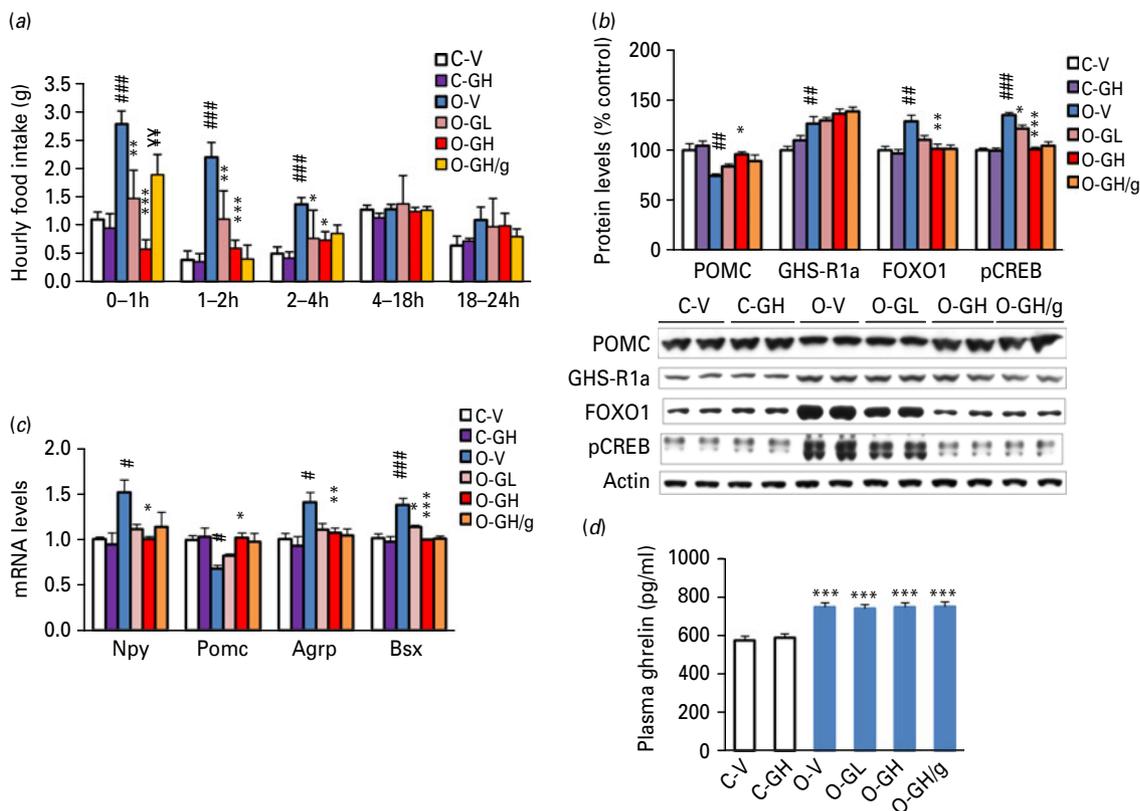
Fig. 7. Acute effects of olanzapine and the ghrelin receptor antagonist D-Lys3-GHRP-6 on food intake, plasma ghrelin levels, protein expressions of POMC, GHS-R1a, FOXO1 and pCREB, and mRNA levels of NPY, POMC, AgRP and BSX at the MBH. Olanzapine treatment produced a significant elevation effect on hourly food intake up to 4 h post-i.c.v. injection of vehicles, which was reduced or eliminated by i.c.v. injection of D-Lys3-GHRP-6 (3 nmol) or D-Lys3-GHRP-6 (30 nmol), respectively (*a*). Olanzapine decreased POMC, and increased FOXO1 and pCREB protein expressions at the MBH, which was eliminated by i.c.v. injection of D-Lys3-GHRP-6 (30 nmol) (*b*). Olanzapine increased GHS-R1a protein expressions at the MBH, which could not be reversed by D-Lys3-GHRP-6 (*b*). Olanzapine increased NPY, AgRP and BSX, but decreased POMC mRNA expressions at the MBH, which were eliminated by i.c.v. injection of D-Lys3-GHRP-6 (30 nmol) ($p < 0.05$; $n = 6$) (*c*). Plasma ghrelin levels were increased by oral olanzapine treatment (d). This effect was not affected by i.c.v. injection of D-Lys3-GHRP-6 (3 nmol) or D-Lys3-GHRP-6 (30 nmol) (*d*) [#]$p < 0.05$ vs. C-V; [##]$p < 0.01$ vs. C-V; [###]$p < 0.001$ vs. C-V;[*]$p < 0.05$ vs. O-V[**]$p < 0.01$ vs. O-V; [***]$p < 0.001$ vs. O-V; [¥¥]$p < 0.01$ vs. O-GH; $n = 5$–6 per treatment group. C-V: the oral control and ICV vehicle group; C-GH: the oral control and i.c.v. high-dose GHS-R1a antagonist group; O-V: the oral olanzapine and ICV vehicle group; O-GL: the oral olanzapine and i.c.v. low-dose GHS-R1a antagonist group; O-GH: the oral olanzapine and high-dose GHS-R1a antagonist group; O-GH/g: the oral olanzapine and i.c.v. high-dose GHS-R1a antagonist with ghrelin co-treatment group.

Additionally, ghrelin has been suggested to play an important role in substrate utilisation (Wortley et al., 2004). Further studies on the long-term effect of olanzapine on energy expenditure parameters are warranted.

The expressions of hypothalamic GHS-R1a receptor were elevated by olanzapine through the three treatment cohorts, suggesting that the effects of olanzapine on hypothalamic GHS-R1a levels were independent of those on circulating ghrelin levels. Dimerization between the GHS-R1a receptor and the dopamine D2 receptor at the Arc has been recently reported (Kern et al., 2012), suggesting functional interactions between the D2 receptor and GHS-R1a receptor. However, a recent report has suggested the blockage of D2 receptor may attenuate the effect of ghrelin on food intake (Romero-Pico et al., 2013). Further research is required to elucidate the

relationship between D2 receptor and ghrelinergic signalling in the context of olanzapine-induced hyperphagia and weight gain. Similarly, the serotoninergic 5-HT2c receptor has also been showed to form heterodimers and interact with GHS-R1a receptor (Schellekens et al., 2013).

In the i.c.v. experiment, we showed for the first time that the olanzapine-induced elevation of food intake and alterations of ghrelin-signalling parameters (NPY, AgRP and POMC) were eliminated by acute administration of the GHS-R1a antagonist D-Lys3-GHRP-6 (30 nmol). These findings suggest that ghrelin signalling plays a critical role in mediating the orexigenic effect of olanzapine. Moreover, these findings also suggest a new pharmacological target of body weight management for schizophrenia patients on second-generation antipsychotics.

In fact, D-Lys3-GHRP-6 has been reported to reduce food intake and body weight gain in rodents (Asakawa et al., 2003; Beck et al., 2004). Future studies are required to investigate the chronic effects of co-administration of D-Lys3-GHRP-6 and olanzapine on food intake and the ghrelin-signalling system, as well as the effects of D-Lys3-GHRP-6 administered via routes other than the invasive i.c.v. injection. D-Lys3-GHRP-6 is widely used in animal studies as a potent GHS-R1a blocker (Asakawa et al., 2003; Beck et al., 2004; Zaniolo et al., 2011; Ueno et al., 2012), with similar effects on food intake and alcohol intake as JMV2959, another commonly used GHS-R1a antagonist (Dickson et al., 2011; Moulin et al., 2013). In the current study, as in other rodent i.c.v. injection studies (Asakawa et al., 2003; Beck et al., 2004), D-Lys3-GHRP-6 is well tolerated at the current dosages of i.c.v. injection, with no adverse effects observed. However, future studies using GHS-R1a or ghrelin knockout models are required to confirm the role of the ghrelin-signalling system on SGA-induced hyperphagia and weight gain.

Neuropeptides in the hypothalamus, including NPY, AgRP and POMC are important downstream parameters in the ghrelin-signalling pathways regulating food intake and energy homeostasis, and previous studies have shown that olanzapine can alter their expressions (Fernø et al., 2011; Weston-Green et al., 2012a). Consistent with the effects on ghrelin and food intake, our results showed that hypothalamic expressions of NPY, AgRP and POMC were altered by olanzapine treatment in the short- to mid-term only. We also found for the first time that the hypothalamic expressions of transcription factors for these neuropeptides, including BSX, pCREB and FOXO1, were elevated by short-term olanzapine treatment. BSX has been reported to mediate ghrelin's stimulatory effect on AgRP and NPY gene expressions, while interacting with two other transcription factors FOXO1 and pCREB (Lage et al., 2010). FOXO1 can up-regulate NPY and AgRP and inhibit POMC mRNA expressions at the Arc in rats (Kim et al., 2006). Furthermore, FOXO1 knock-in mice with specific activation at the hypothalamus and pancreas can develop obesity and hyperphagia, with increased AgRP and NPY levels at the hypothalamus (Kim et al., 2012). Finally, central administration of ghrelin can increase BSX mRNA and FOXO1/pCREB protein expressions at the hypothalamus in rodents (Nogueiras et al., 2008). Therefore, the up-regulation of BSX, pCREB and FOXO1 expressions observed under short-term olanzapine treatment in this study are in line with the changes of NPY, AgRP and POMC induced by olanzapine.

Other hypothalamic markers, including p53, Sirtuin 1 (SIRT1), ceramide, mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), carnitine palmitoyltransferase 1 (CPT1), and uncoupling protein 2 (UCP2), have also been indicated as potential upstream targets in the ghrelin-signalling pathway, and may also play a role in SGA-induced hyperphagia (L|ópez et al., 2008; Velasquez et al., 2011; Martins et al., 2012; Ramirez et al., 2013; Skrede et al., 2014), which have been reviewed by our group (Zhang et al., 2013). Further studies are required to access the effect of olanzapine (or other SGAs) on these upstream ghrelin-signalling targets. In fact, the seminal data from our group suggested that the protein levels of pAMPK, pACC, and CPT1c at the hypothalamus were also time-dependently regulated by olanzapine (He et al., 2012) in the same cohorts of rats as in Experiment 1 in the current study, which further substantiates the role of hypothalamic ghrelin-signalling in olanzapine-induced hyperphagia and weight gain.

Finally, in light of the effect of olanzapine in triggering preference to a high fat/high sugar diet over chow diet in rats (Smith et al., 2011), it is possible that the non-effect of olanzapine on food intake during the mid- to long-term treatments was due to the restriction of available foods to lab chows in the current study. However, the effect of olanzapine on food preference is still controversial (van der Zwaal et al., 2010), and remains an interesting topic for future studies.

In summary, we found that the elevation of food intake by olanzapine can be blocked by D-Lys3-GHRP-6, suggesting that the ghrelin-signalling pathway is playing an important role in olanzapine-induced hyperphagia. Further, short-term olanzapine treatment can up-regulate hypothalamic expressions of BSX, pCREB and FOXO1, transcriptional factors for the orexigenic neuropeptides NPY and AgRP, which contributes to olanzapine-induced hyperphagia. Finally, our pair-feeding experiment suggests that the up-regulation of circulating ghrelin and hypothalamic ghrelin-signalling under olanzapine treatment, particularly in the short-term, is not secondary to olanzapine-induced weight gain or elevated food intake. Future research is required to elucidate the detailed molecular pathways involved in olanzapine-induced ghrelin-signalling dysregulations, as well as the interactions between the ghrelin-signalling system and other systems, such as the dopaminergic D2 system and the serotoninergic 5HT2c system, which have been implicated in antipsychotic-induced weight gain.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713001697

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Statement of Interest

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