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# Hypothalamic histamine H1 receptor-AMPK signaling time-dependently mediates olanzapine-induced hyperphagia and weight gain in female rats

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**Abstract**
Although second-generation antipsychotics induce severe weight gain and obesity, there is a lack of detailed knowledge about the progressive development of antipsychotic-induced obesity. This study examined the hypothalamic histamine H1 receptor and AMP-activated protein kinase (H1R-AMPK) signaling at three distinctive stages of olanzapine-induced weight gain (day 1-12: early acceleration, day 13-28: middle new equilibrium, and day 29-36: late heavy weight maintenance). At the early acceleration stage, the rats were hyperphagic with an underlying mechanism of olanzapine-increased H1R mRNA expression and AMPK phosphorylation (pAMPK), in which pAMPK levels positively correlated with H1R mRNA expression and food intake. At the middle stage, when the rats were no longer hyperphagic, the changes in H1R-AMPK signaling vanished. At the late stage, olanzapine increased H1R mRNA expression but decreased pAMPK which were positively and negatively correlated with weight gain, respectively. These data suggest a time-dependent change of H1R-AMPK signaling, where olanzapine activates AMPK by blocking the H1Rs and causing hyperphagia in the acute phase. The chronic blockade of H1R may contribute to the late stage of olanzapine-induced heavy weight maintenance. However, pAMPK was no longer elevated and actually decreased. This indicates that AMPK acts as an energy sensor and negatively responds to the positive energy balance induced by olanzapine. Furthermore, we showed that an H1R agonist, 2-(3-trifluoromethylphenyl) histamine, can significantly inhibit olanzapine-induced hyperphagia and AMPK activation in the mediobasal hypothalamus in a dose dependent manner. Therefore, lowering H1R-AMPK signaling is an effective treatment for the olanzapine-induced hyperphagia associated with the development of obesity.

**Keywords**
Antipsychotics, olanzapine, histamine H1 receptor, AMP-activated protein kinase, food intake, weight gain

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# Hypothalamic histamine H1 receptor-AMPK signaling time-dependently mediates olanzapine-induced hyperphagia and weight gain in female rats

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**Running title:** H1 receptor and AMP-activated protein kinase signaling in olanzapine-induced obesity

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**Summary**

Although second-generation antipsychotics induce severe weight gain and obesity, there is a lack of detailed knowledge about the progressive development of antipsychotic-induced obesity. This study examined the hypothalamic histamine H1 receptor and AMP-activated protein kinase (H1R-AMPK) signaling at three distinctive stages of olanzapine-induced weight gain (day 1-12: early acceleration, day 13-28: middle new equilibrium, and day 29-36: late heavy weight maintenance). At the early acceleration stage, the rats were hyperphagic with an underlying mechanism of olanzapine-increased H1R mRNA expression and AMPK phosphorylation (pAMPK), in which pAMPK levels positively correlated with H1R mRNA expression and food intake. At the middle stage, when the rats were no longer hyperphagic, the changes in H1R-AMPK signaling vanished. At the late stage, olanzapine increased H1R mRNA expression but decreased pAMPK which were positively and negatively correlated with weight gain, respectively. These data suggest a time-dependent change of H1R-AMPK signaling, where olanzapine activates AMPK by blocking the H1Rs and causing hyperphagia in the acute phase. The chronic blockade of H1R may contribute to the late stage of olanzapine-induced heavy weight maintenance. However, pAMPK was no longer elevated and actually decreased. This indicates that AMPK acts as an energy sensor and negatively responds to the positive energy balance induced by olanzapine. Furthermore, we showed that an H1R agonist, 2-(3-trifluoromethylphenyl) histamine, can significantly inhibit olanzapine-induced hyperphagia and AMPK activation in the mediobasal hypothalamus in a dose dependent manner. Therefore, lowering H1R-AMPK signaling is an effective treatment for the olanzapine-induced hyperphagia associated with the development of obesity.

**Keywords:** antipsychotics; olanzapine; histamine H1 receptor; AMP-activated protein kinase; food intake; weight gain

## 1. Introduction

Antipsychotic-induced obesity is a serious side effect and requires a systematic and in depth understanding of the molecular mechanisms involved in the progressive development of obesity in order to develop appropriate therapeutic strategies. Clinical evidence indicates that atypical antipsychotic-induced obesity (clozapine and olanzapine) occurs in three distinct stages: rapid increases of body weight in the early acceleration stage (first 3 months); a reduced rate of weight gain in the middle new equilibrium stage (3-18 months); and finally, body weight plateaus and is maintained with continuing antipsychotic treatment (> 18 months, late stage) (Allison and Casey, 2001; Pai et al., 2012). In rats, it has been shown that olanzapine-induced obesity has similar patterns of weight gain although each period is shorter (Huang et al., 2006). These findings suggest that the mechanisms may vary during the different stages of olanzapine-induced obesity development.

In the clinic, antipsychotics' affinity for the H1 receptor predicts (Kroeze et al., 2003) and correlates (Matsui-Sakata et al., 2005) with antipsychotic-induced weight gain. In rats, olanzapine treatment alters H1 receptor mRNA expression in the hypothalamus, which is negatively correlated with weight gain (Han et al., 2008). Therefore, the H1 receptor antagonism appears to be a key factor in olanzapine-induced obesity (Deng et al., 2010; He et al., 2013). In fact, betahistine (an H1 receptor agonist/ H3 receptor antagonist) has been used to prevent olanzapine-induced weight gain in humans (Poyurovsky et al., 2005; Poyurovsky et al., 2013) and rats (Deng et al., 2012).

Hypothalamic AMP-activated protein kinase (AMPK) regulates food intake. It has been reported that histamine decreases pAMPK, which can be reversed by clozapine in mouse hypothalamic slices (Kim et al., 2007). Furthermore, clozapine does not elevate pAMPK in H1 receptor knockout mice (Kim et al., 2007). Other investigators have reported that hypothalamic AMPK is activated by olanzapine (Martins et al., 2010; Sejima et al., 2011; Skrede et al., 2013). These data suggest a possibility that H1 receptor agonist may be used to reverse the olanzapine-induced elevation of pAMPK and prevent olanzapine-induced obesity.

Since the mechanisms may vary during the different stages of olanzapine-induced obesity, two key questions are: does hypothalamic H1 receptor-AMPK signaling play a different role in the different stages of olanzapine–induced obesity; and is H1 receptor-AMPK signaling a potential target for treating olanzapine-induced obesity? This study provides evidence for the first time that the time-dependent modulating mechanisms of hypothalamic H1 receptor-AMPK signaling underlie the three developmental stages of olanzapine-induced obesity. We tested the effect of central H1 receptor activation in reducing olanzapine-induced hyperphagia and its relationship with AMPK signaling in the hypothalamus.

## 2. Materials and Methods

**2.1. Animals, drugs and chemicals**. Female Sprague-Dawley (SD) rats (weight 200-225g) were obtained from the Animal Resources Centre (Perth, WA, Australia). Rats were housed at 22°C on a 12h light-dark cycle (lights on 0700 h). Food and water were allowed *ad libitum* throughout the study. All animal procedures were approved by the Animal Ethics Committee, University of Wollongong, and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (2004). Olanzapine (Zyprexa) was purchased from

Eli Lilly, Indianapolis, IN, USA. 2-(3-trifluoromethylphenyl) histamine (FMPH) and pyrilamine were obtained from Sigma-Aldrich (Sigma, NSW, Australia).

### 2.2. Experiment one

To investigate the role of hypothalamic H1 receptor-AMPK signaling at the three stages of olanzapine-induced weight gain, we conducted this experiment to detect changes of the H1 receptor-AMPK signaling under olanzapine treatment for 8, 16 and 36 days. A rat model that mimics olanzapine-induced weight gain has been established in our laboratory (Zhang et al, 2013b). SD rats were randomly divided into three groups and treated with olanzapine for 8, 16 and 36 days to represent early, middle and late stages of olanzapine-induced weight gain (Huang et al., 2006). Olanzapine (1 mg/kg) or vehicle was administered orally three times daily (equivalent to 3 mg/kg/day) at eight-hourly intervals at 0700 h, 1500 h and 2300 h (n=12/group). The rats were fed sweet cookie dough (62% carbohydrate, 22% protein, 6% fibre, 10% vitamins and minerals) mixed with either olanzapine or placebo (Zhang et al, 2013b). Food intake and body weight were recorded every 2 days. The dosage of olanzapine was chosen based on our previous studies (Zhang et al, 2013b). It was calculated towards the clinical relevant dosage of 10mg/day (based on body surface area of different species) (Reagan-Shaw et al., 2008), and mimicked the olanzapine -induced obesity in the clinical setting. Two hours after the last drug treatment, the rats were euthanized using carbon dioxide ($CO_2$) asphyxiation (0900 h to 1100 h). The brains were immediately collected. The hypothalami were quickly dissected on ice, frozen in liquid nitrogen and then stored at -80 °C.

### 2.3. Experiment two

To address the question of whether direct activation of central H1 receptors could reduce olanzapine-induced hyperphagia, and whether this effect is linked to hypothalamic AMPK signaling, olanzapine-treated rats were given an intracerebroventricular (icv) injection of an H1 receptor agonist. After acclimatization, each SD rat was surgically implanted with a 24-gauge guide cannula into the lateral ventricle under isoflurane anesthesia (1.0mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.5 mm below the top skull) (George and Charles, 2007). After one week recovery, the rats were orally treated with olanzapine or vehicle (same as in Experiment one) for 4 days. On day 5, the rats received 0, 100 or 200 nmoles (Lecklin et al., 1998) icv injection of the H1 receptor agonist, 2-(3-trifluoromethylphenyl) histamine (FMPH), or saline (n=5-8/group) at a rate of 5 µl/min, and the volume was 5 µl (Group 1, vehicle/saline; Group 2, vehicle/FMPH 200 nmoles; Group 3, olanzapine/saline; Group 4, olanzapine/FMPH 100 nmoles; Group 5, olanzapine/FMPH 200 nmoles). Thirty minutes later, the rats were treated orally with olanzapine at the same dose or with the vehicle. Food intake in all rats was measured at 1, 2, 4, 16 (overnight) and 24h after the olanzapine or vehicle administration. To test whether the suppressive effect of FMPH 200 nmoles on food intake in olanzapine-treated rats could be reversed by H1 receptor antagonism, the rats were given an icv injection of pyrilamine (800 nmoles) before FMPH was given, and then food intake was tested (Group 6, olanzapine/FMPH 200 nmoles/pyrilamine 800 nmoles).To further examine the role of H1 receptor-AMPK signaling in olanzapine-induced hyperphagia, the same treatment was repeated after a three day drug washout period. Rats were then sacrificed using $CO_2$ asphyxiation 1 h after their last treatment (between 1200 h and 1400 h) to detect H1 receptor-AMPK signaling in the hypothalamus. The hypothalamic nuclei were identified and dissected according to the standard rat brain atlas (George and Charles, 2007). Briefly, rat brains were snap-frozen, cut at 500 µM coronal sections from Bregma -2.16 mm to

-3.60 mm, -1.44 mm to -2.04 mm, and -2.16 mm to -3.60 mm, for arcuate nucleus (Arc), paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) respectively. The temperature was set at -18°C. The nuclei were dissected using micro-punches (#57401, Stoelting Co, Wood Dale, IL, USA) (Zhang et al, 2013b). The Arc was dissected in an overlapping pattern over the third ventricle. Since the Arc is small, the punched tissue primarily contained Arc, but the inclusion of adjacent brain areas cannot be ruled out, therefore the punched tissue containing Arc was named as the mediobasal hypothalamus (MBH).

### 2.4. Real-time quantitative PCR

Real-time quantitative PCR was used to detect the mRNA expression of the H1 receptors, AMPKα, acetyl-CoA carboxylase α (ACCα, a downstream target of AMPK), corticotrophin-releasing hormone (CRH), leptin receptor and orexin-A. Dissected hypothalamus was homogenized and RNA was isolated using Purlink$^{TM}$ RNA Mini Kit (Life Technologies, NSW, Australia) following the manufacturer's instructions. RNA was converted to cDNA using Superscript® VILO$^{TM}$ cDNA Synthesis Kit (Life Technologies). RT-PCR was performed using LightCycler® 480 Real-Time PCR instrument (Roche Applied Science, NSW, Australia) with the TaqMan® Gene Expression Assays (Life Technologies): H1 receptor (assay no. Rn00566691_s1); AMPKα2 (assay no. Rn00576935_m1); ACCα (assay no. Rn00573474_m1); CRH (assay no. Rn01462137_m1); leptin receptor (assay no. Rn01433205_m1), neuropeptide Y (NPY) (Rn00821417_m1), agouti-related peptide (AgRP) (Rn01431703_g1) and orexin-A (assay no. Rn00565995_m1). The β-actin (assay no. Rn00667869_m1) was used as an endogenous control. The amplification was run for 40 cycles of denaturation at 95°C followed by annealing/extending at 60°C. The $2^{-\Delta\Delta CT}$ method was used to calculate the results (Schmittgen and Livak, 2008).

### 2.5. Western blot

Western blot was performed following the procedure previously described (du Bois et al., 2012). Tissues were homogenized in NP40 cell lysis buffer (Life Technologies) containing a protease inhibitor cocktail, beta-glycerophoshate and phenylmethanesulfonyl fluoride (Sigma, NSW, Australia). Protein concentration was detected by DC protein assay (Bio-Rad Laboratories, Gladesville, Australia). Proteins were loaded onto 4-12% Bis-Tris gels (Bio-Rad). Following electrophoresis for 50 min at 200V, the proteins were transferred to polyvinylidene difluoride (PDVF) membranes (100V for 1h) (Bio-Rad). Membranes were then blocked for 1h in 5% bovine serum albumin (BSA) in tris buffered saline with 0.1% Tween 20 (TBST) and incubated with the primary antibodies in 1% BSA at 1:1000 overnight at 4°C for AMPKα, ACC, and phospho-AMPKα (pAMPKα) (Cell Signaling Technology, Danvers, MA, USA) and phospho-ACCα (pACCα) (Millipore, Billerica, MA, USA). Following washing in TBST (3×5min), membranes were incubated with goat anti-rabbit (1:5000 Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and horseradish peroxidase conjugated secondary antibody for 1h at 25°C. Immunoractive proteins were detected using the enhanced chemiluminescence (ECL) kit (GE Healthcare, Rydalmere, NSW, Australia). The results were quantified using Bio-Rad Quantity One software. The quantification was normalized to β-actin, according to previous studies (Ferno et al., 2011).

### 2.6. Statistical analysis

The statistics were performed by the SPSS 19.0 program (Chicago, IL, USA). In Experiment one, two-way analysis of variance (ANOVA) (olanzapine × time as repeated measure) followed by an independent unpaired student's *t*-test (two-tailed) were used to analyze the

statistical differences of food intake and weight gain between olanzapine and control groups in every 2 days. The differences in H1 receptor, AMPKα and ACCα mRNA expression, and pAMPKα and pACCα protein expression were analyzed using an independent unpaired student *t*-test (two-tailed). In Experiment two, Kruskal–Wallis H test followed by Mann–Whitney U tests were used to compare the difference in food intake, the hypothalamic leptin receptor, orexin-A, corticotrophin-releasing hormone (CRH) and NPY and AgRP mRNA expression following the icv injection. One-way ANOVA followed by *post hoc* Tukey's multiple comparison were used to analyze the statistical differences of pAMPKα and pACCα expression in different hypothalamic regions. Correlations were identified using Pearson's correlation. All data were presented as mean ± SEM. Statistical significance was defined as $p \leqslant 0.05$.

## 3. Results

### 3.1. Hyperphagia contributed to rapid weight gain in the early stage of olanzapine-induced obesity

Olanzapine treatment induced a significant elevation in food intake during the first 12 days when it was measured every 48h (Figure 1A; two-way analysis of variance (olanzapine × time as repeated measure) followed by an independent student's *t* test for each time point, all $p < 0.05$, n=12/group) but not during day 14-36 (all $p > 0.05$, Student's *t* test). Rats treated with olanzapine had a higher body weight throughout the whole treatment period (all $p < 0.05$, Student's *t* test), during which olanzapine rapidly increased body weight in the first 12 days (early stage), slowed down from day 13 to 28 (middle stage), and then reached a plateau from day 29 to 36 (late stage) (Figure 1B). Pearson's correlations revealed that the cumulative food intake was significantly correlated with body weight gain in the early stage of

olanzapine-induced obesity but not in the middle and late stages (Figure 1C, D, E). To confirm the relationship between food intake and body weight in the different stages of weight gain, food efficiency (grams of weight gained/grams of food consumed) was also calculated. At the early stage, the food efficiency significantly increased in the olanzapine-treated group compared with the control group (control vs. olanzapine: 0.034 ± 0.004 vs. 0.049 ± 0.002, $p$ = 0.007, Student's $t$ test). In the middle and late stages, the food efficiency of the olanzapine-treated rats was not significantly different to that of the control group (middle stage: control vs. olanzapine: 0.021 ± 0.001 vs. 0.018 ± 0.004, $p$ = 0.480; late stage: 0.013 ± 0.003 vs. 0.018 ± 0.007, $p$ = 0.552, Student's $t$ test).

[Insert Figure 1 around here]

### 3.2. Olanzapine induced time-dependent changes of hypothalamic H1 receptor-AMPK signaling differing in the three stages of obesity development

In order to investigate the role of hypothalamic H1 receptor-AMPK signaling in olanzapine-induced obesity, rats were treated with olanzapine for 8, 16 and 36 days to represent the three stages of olanzapine-induced weight gain. In the early stage, olanzapine treatment induced a significantly elevated hypothalamic H1 receptor mRNA expression (125 ± 7%, $p$ = 0.015 vs. control, n=5/group, Student's $t$ test) but an unchanged AMPKα2 and acetyl-CoA carboxylase α (ACCα, a downstream target of AMPK) mRNA expression compared to the control rats (Figure 2A). The hypothalamic pAMPKα expression (activated AMPK) but not pACCα expression was significantly increased by olanzapine (Figure 2D, E; 124 ± 8%, $p$ = 0.021; 119 ± 12%, $p$ = 0.354, respectively, n=5-6/group, Student's $t$ test). Importantly, Pearson's correlation revealed that the pAMPKα and pACCα expression were positively correlated with H1 receptor mRNA expression as well as the last 48h food intake (Table 1). These data suggest that olanzapine-induced hyperphagia and weight gain are

associated with the activation of AMPK via a blockade of H1 receptor by olanzapine. In the middle stage, when the rats were no longer hyperphagic, both the changes in H1 receptor mRNA expression and pAMPKα and pACCα protein expression vanished (Figure 2B, D, F). In the late stage, olanzapine significantly increased hypothalamic H1 receptor mRNA expression (130% ± 5, $p = 0.046$, Student's *t* test) compared to the control rats (Figure C). The H1 receptor mRNA expression was significantly positively correlated with weight gain ($r = 0.712$, $p = 0.016$). However, both the ACCα mRNA expression (Figure 2C; 80 ± 6%, $p = 0.030$, Student's *t* test) and the pAMPKα and pACCα protein expression were significantly decreased compared with the control rats (Figure 2D, G; 84 ± 1%, $p = 0.005$; 79 ± 5%, $p = 0.023$, respectively, Student's *t* test). Notably, the pAMPKα expression was negatively correlated with H1 receptor mRNA expression and weight gain (Table 1). These data suggest that as olanzapine treatment was prolonged, AMPK activity declined and was less effective in down-regulating food intake.

[Insert Figure 2 around here]

Since hypothalamic AMPK can also be inhibited by leptin and insulin (Minokoshi et al., 2004), we measured the plasma leptin and insulin levels of rats with late stage olanzapine-induced obesity. These rats had significantly increased plasma leptin than the control group (control vs. olanzapine: 8.4 ± 1.8 vs. 15.0 ± 1.5 ng/ml; $p = 0.027$, n=6/group, Student's *t* test). Their insulin level was not significantly different (control vs. olanzapine: 1.2 ± 0.4 vs. 1.5 ± 0.3 ng/ml; $p = 0.158$, n=6/group, Student's *t* test). These results suggest that leptin may be involved in inhibiting AMPK signaling at the late stage of olanzapine-induced obesity.

[Insert table 1 around here]

### 3.3. Histamine H1 receptor agonist, 2-(3-trifluoromethylphenyl) histamine, attenuated olanzapine-induced hyperphagia

Having observed the importance of the central H1 receptor-AMPK signaling in olanzapine-induced weight gain, we further investigated whether the direct activation of the central H1 receptors could reduce olanzapine-induced hyperphagia and weight gain. The H1 receptor agonist, 2-(3-trifluoromethylphenyl) histamine (FMPH), was injected into the lateral brain ventricle of rats treated with either olanzapine or vehicle for five days. Olanzapine treatment significantly increased food intake and cumulative body weight gain from day 2 (Figure 3A and B). Rats on olanzapine treatment followed by an icv injection of saline (controls) showed a significant increase in food intake in 1, 2, 4 and 16 h at 85%, 112%, 61%, 19%, respectively (Figure 3C; all $p < 0.05$ vs. vehicle/saline, Mann-Whitney U test). This effect was significantly attenuated by central FMPH treatment from 1 to 16 h time points (Figure 3C; Kruskal-Wallis H test for each time points: 1 h: $H(5) = 13.251$, $p = 0.021$; 2 h: $H(5) = 11.121$, $p = 0.049$; 4 h: $H(5) = 15.954$, $p = 0.007$; 16 h: $H(5) = 10.957$, $p = 0.052$; 24 h: $H(5) = 8.139$, $p = 0.149$, n = 5-8/group). Olanzapine-induced hyperphagia was reduced by 64%, 51%, 44%, 21%, respectively as measured at 1, 2, 4 and 16 h by FMPH 200 nmoles (all $p < 0.05$ vs. olanzapine/saline, Mann-Whitney U test). The inhibitory effect of FMPH 100 nmoles was also evident, with the most significant changes found in 4 and 16 h at 42% and 28%, respectively ( $p = 0.003$, $p = 0.073$ vs. olanzapine/saline, Mann-Whitney U test). However, the FMPH 200 nmoles injection did not significantly reduce food intake at any time points tested compared with the controls (all $p > 0.05$ vs. vehicle/saline, Mann-Whitney U test). We also investigated whether blocking the H1 receptor could reverse the effect of FMPH on food intake in olanzapine-induced hyperphagia, by treating rats with pyrilamine 800 nmoles (an H1 receptor antagonist) before FMPH was given. However, pyrilamine 800 nmoles was not able to reverse the effect of FMPH on food intake at all-time points (all $p > 0.05$ vs. olanzapine/FMPH

200nmols, Mann-Whitney U test). These data suggest that the activation of the central H1 receptor is effective for treating olanzapine-induced weight gain.

[Insert Figure 3 around here]

### 3.4. FMPH treatment inhibited olanzapine-induced AMPK activation in the hypothalamic mediobasal hypothalamus (MBH)

To understand the mechanisms by which FMPH attenuated olanzapine-induced hyperphagia, we tested the dose dependent effect of FMPH on AMPK signaling in olanzapine-treated rats. We examined the effect of olanzapine and FMPH on AMPK signaling in specific hypothalamic regions including the MBH and paraventricular nucleus (PVN) as these regions are significantly involved in H1 receptor (Sakata et al., 1988; Fukagawa et al., 1989; Ookuma et al., 1989; Umehara et al., 2010) and AMPK (Lage et al., 2008; Lopez et al., 2008) regulation of food intake. In the MBH, we found that olanzapine treatment significantly increased pAMPKα (127 ± 8% vs. vehicle/saline) and pACCα (134 ± 9% vs. vehicle/saline) expression compared with the vehicle-treated rats (Figure 4A, B; ANOVA, $F_{(5,28)} = 2.867$, $p = 0.033$; $F_{(5,31)} = 4.312$, $p = 0.004$, respectively, n=5-7/group, Tukey post hoc tests). Injecting 200 nmoles FMPH significantly decreased pAMPKα and pACCα expression compared with saline in the olanzapine-treated rats (101 ± 3%, $p = 0.040$; 99% ± 6%, $p = 0.046$, respectively, Tukey post hoc tests). FMPH 100 nmoles did not attenuate the olanzapine-induced activation of pAMPKα (110 ± 5%) and pACCα (127 ± 8%) in the MBH ($p > 0.05$, Tukey post hoc tests). Consistent with food intake data, the pyrilamine treatment did not significantly reverse the suppressive effect of FMPH on hypothalamic pAMPKα (107 ± 6%) and pACCα (101 ± 9%) in olanzapine-treated rats ($p > 0.05$, Tukey post hoc tests). It was noted that the pAMPKα expression positively correlated with food intake at 1h (Table 2). These data suggest that the central activation of the H1 receptors reduced the olanzapine-induced food intake, probably via

the inhibition of MBH AMPK signaling. However, in the PVN, olanzapine treatment did not significantly increase the pAMPKα and pACCα expression compared with vehicle (Figure 4C).

[Insert Figure 4 around here]

### 3.5. FMPH increased the mRNA expression of CRH in the PVN in olanzapine-treated rats

The histaminergic system is associated with CRH, leptin and orexin-A in body weight regulation (Gotoh et al., 2005; Jorgensen et al., 2005). Therefore we examined the effect of olanzapine and FMPH on hypothalamic CRH, leptin receptor and orexin-A expression 1 h after the last drug treatment. A previous study showed that olanzapine reduces CRH release under the condition of K(+)-stimulation (Tringali et al., 2009). In our study (Figure 5A), olanzapine did not significantly decrease PVN CRH mRNA expression (60 ± 10% vs. vehicle/saline group, $p > 0.05$, n=4-6/group, Kruskal-Wallis H test followed by Mann-Whitney U test). However, CRH mRNA expression was significantly increased by both FMPH 200 nmoles and FMPH 100 nmoles compared with the olanzapine/saline group (172 ± 37%, $p = 0.033$; 88 ± 6%, $p = 0.043$, respectively, Mann-Whitney U test). Compared with the vehicle/saline group, a single injection of 200 nmoles FMPH did not significantly increase CRH mRNA expression (147 ± 28%, $p = 0.251$, Mann-Whitney U test). In addition, olanzapine decreased the expression of the leptin receptor in the MBH (86 ± 3% vs. vehicle/saline, $p = 0.028$, n=4-6/group, Kruskal-Wallis H test followed by Mann-Whitney U test) compared with the vehicle, and this decrease could not be reversed by 200 nmoles or 100 nmoles FMPH (Figure 5B). The histaminergic system innervates the orexin-A system associated with food intake and body weight regulation (Gotoh et al., 2005; Jorgensen et al., 2005). However, we found that

orexin-A mRNA expression was not changed by either the 5 day olanzapine treatment or the central acute administration of FMPH (Figure 5C).

Neuropeptides in the hypothalamus including neuropeptide Y (NPY) and agouti-related peptide (AgRP) are also important parameters in causing olanzapine-induced weight gain (Ferno et al., 2011; Zhang et al., 2014). Therefore, we examined the effect of olanzapine and FMPH on the expression of MBH NPY and AgRP 1 h after the last drug treatment. Olanzapine treatment for 5 days significantly increased MBH NPY and AgRP mRNA expression (Figure 5D, E; 154 ± 14%, $p = 0.011$; 137 ± 20%, $p = 0.050$, respectively, Mann-Whitney U test). However, both 200 and 100 nmoles FMPH did not significantly attenuate the increased NPY (Figure 5D; 122 ± 14%, $p = 0.221$; 137 ± 13% $p = 0.142$, Mann-Whitney U test) or AgRP (Figure 5E; 112 ± 4%, $p = 0.213$; 119 ± 23%, $p = 0.670$, Mann-Whitney U test) mRNA expression induced by olanzapine.

[Insert Figure 5 around here]

## 4. Discussion

The present data suggest that olanzapine-induced weight gain can be divided into three phases, in which different modulating mechanisms are involved. Excessive food intake may be the main reason for the rapid increase in body weight in short-term olanzapine treatment (early stage). We demonstrated different roles of the hypothalamic H1 receptors and AMPK signaling in the three different stages of olanzapine-induced obesity development. In the early stage, in accordance with olanzapine-induced hyperphagia, we found increased hypothalamic H1 receptor mRNA expression and activated AMPK signaling. We found positive correlations between the pAMPK level and H1 receptor mRNA, food intake, and weight gain. This suggests that olanzapine activates AMPK by blocking the H1 receptors in olanzapine-induced

hyperphagia and body weight gain. This was supported by our further test that a single icv injection of an H1 receptor agonist significantly attenuated olanzapine-induced hyperphagia and AMPK activation. Coinciding with our findings, previous studies also reported the activation of hypothalamic AMPK by olanzapine treatment (< 2 weeks) (Sejima et al., 2011; Skrede et al., 2013). Although one study showed that short-term olanzapine treatment inhibited hypothalamic AMPK activity (Ferno et al., 2011), their measurement of AMPK was done 20 h after the last drug treatment, and thus the inhibition of pAMPK may be due to the negative feedback mechanism caused by high body weight rather than being a direct effect of olanzapine.

[Insert table 2 around here]

As treatment was prolonged, we found that alterations of hypothalamic H1 receptor-AMPK signaling vanished (middle stage), followed by increased H1 receptor mRNA expression but decreased pAMPK protein expression (late stage). The fact that AMPK phosphorylation returned to normal and was then inhibited suggests that AMPK acts as an energy sensor by negatively responding to positive energy balance. Hypothalamic AMPK activity is also inhibited by insulin and leptin (Minokoshi et al., 2004; Lopez et al., 2008). In the present study, we observed increased plasma level of leptin in the late stage of olanzapine-induced obesity. This may contribute to the down-regulation of hypothalamic AMPK signaling. In fact, it has been suggested that the combined effects of hyperinsulinemia and increased leptin secretion may lead to the inhibition of hypothalamic AMPK activity in mice with diet-induced obesity (Martin et al., 2006; Viollet et al., 2010).

During chronic olanzapine treatment, the continuous weight gain without hyperphagia indicated that other factors besides AMPK may also be involved in olanzapine-induced weight

gain. Previous evidence in clinical and animal models has demonstrated that olanzapine can decrease energy expenditure, which leads to weight gain (Stefanidis et al., 2008; Cuerda et al., 2011). Possibly, the decreased energy expenditure may largely account for the increase in weight gain especially at the later stages. However, in the case of late stage olanzapine-induced obesity, both hypothalamic pAMPK and its directly downstream target pACC expression were decreased. This evidence indicates that hypothalamic AMPK may drive a negative energy balance which is not strong enough to reduce the weight gained after chronic olanzapine treatment, although the exact mechanism for the maintenance of heavy weight in the late stage still requires further study. In fact, other studies have shown that AMPK signaling is decreased in diet-induced obesity in rodents, including the whole mouse and rat hypothalamus (Fei et al., 2012; Whittle et al., 2012) and the mouse hypothalamic PVN (Martin et al., 2006). Similarly, the current study showed decreased hypothalamic AMPK signaling in obese rats induced by chronic olanzapine treatment. In addition, data from our group and others have reported that long term olanzapine treatment is associated with decreased BAT temperature, BAT uncoupling protein 1 expression (Stefanidis et al., 2008; Zhang et al., 2013) and peroxisome proliferator activated coactivator 1α (Zhang et al., 2013). These results could be a direct effect of olanzapine on BAT via lowering sympathetic activity in the late stage. This can occur independently of its effect on hypothalamic AMPK signaling. The hypothalamic H1 receptors are involved in the regulation of energy expenditure, partly by regulating the sympathetic outflow to BAT (Masaki et al., 2004; Yasuda et al., 2004; Hong et al., 2006; Masaki and Yoshimatsu, 2006; Lundius et al., 2010). A recent review by our group has suggested that second generation antipsychotics (including olanzapine) may also decrease energy expenditure by blocking the H1 receptors in the central nervous system (He et al., 2013). In the present study, at the late stage of olanzapine-induced obesity, H1 receptor mRNA was increased, and

the H1 receptor mRNA expression was significantly positively correlated with weight gain. Therefore, it is possible that during long-term olanzapine treatment, the antagonism of the hypothalamic H1 receptors may contribute to an olanzapine-induced decrease in energy expenditure and the maintenance of high body weight. Additionally, future studies on the effect of olanzapine on browning markers on white adipose tissue would be helpful (Fisher et al., 2012). Since chronic olanzapine treatment also increases subcutaneous and visceral white adipose tissue (Weston-Green et al., 2011), it is desirable to examine whether olanzapine treatment affects the expression of fat storage enzymes such as lipoprotein lipase and fatty acid synthesis in inguinal subcutaneous and visceral white adipose tissue.

The mechanisms by which olanzapine up-regulates H1 receptor mRNA expression remains unknown. It is possible that the increased H1 receptor mRNA expression was due to a negative feedback loop of the H1 receptor antagonism. In addition, the H1 receptors display constitutive activity (Bond and Ijzerman, 2006). Olanzapine, similar to clozapine, may work as an inverse agonist of the H1 receptors which suppress the H1 receptor internalization, which in turn results in H1 receptor up-regulation (Humbert-Claude et al., 2012).

Our study demonstrated that central H1 receptor activation attenuated olanzapine-induced excessive food intake, supporting the statement that central H1 receptor antagonism contributes to olanzapine-induced hyperphagia, and could be a potential target for the treatment of olanzapine-induced obesity. Furthermore, the activation of AMPK in the MBH by olanzapine was attenuated by the H1 receptor agonist. These findings suggest that AMPK acts as a downstream target in H1 receptor-regulated food intake in the MBH, and this nucleus-specific activation of AMPK may be of major importance in contributing to

olanzapine-induced hyperphagia. Further studies that directly inject the H1 receptor agonist into the Arc are required to confirm the role of Arc H1 receptor-AMPK signaling in olanzapine-induced hyperphagia and weight gain. The H1 receptors and AMPK in the PVN are also well-known for their role in regulating food intake (Sakata et al., 1988; Fukagawa et al., 1989; Ookuma et al., 1989; Ookuma et al., 1993). However, the lack of AMPK activation in the PVN in the present study suggests that PVN H1 receptor-AMPK signaling is unlikely to contribute to short-term olanzapine-induced hyperphagia and weight gain.

Histamine/H1 receptor signaling can influence the CRH, leptin and orexin systems in food intake and body weight regulation (Gotoh et al., 2005; Jorgensen et al., 2005). CRH originating from the PVN exerts an anorexic effect and can be activated by histamine (Kjaer et al., 1994). The H1 receptor agonist, 2-thiazolylethylamine (2-TEA), increased CRH expression in the PVN, suggesting that activating H1 receptors may also regulate food intake via CRH (Kjaer et al., 1992; Kjaer et al., 1998). Our study found that the injection of an H1 receptor agonist dose-dependently increased CRH expression compared with saline in olanzapine-treated rats, suggesting that the CRH may also act as a downstream hormone of histamine H1 receptor signaling in olanzapine-induced obesity, however further studies are needed. Since the Arc is responsible for leptin signaling in the regulation of energy balance, the decreased leptin receptor expression in the MBH by olanzapine treatment may induce a resistance to leptin (Panariello et al., 2012). In addition, one study suggested that orexin is involved in antipsychotic-induced weight gain (Fadel et al., 2002). However, our study did not find any change in orexin-A in the LHA under olanzapine or FMPH treatment. Moreover, this study found an increased expression of NPY and AgRP in the MBH. This is consistent with previous studies (Ferno et al., 2011; Zhang et al., 2014) and supports the important role of NPY and

AgRP in olanzapine-induced weight gain. However, the olanzapine-induced increase of NPY and AgRP cannot be completely reversed by a single injection of the H1 receptor agonist. This suggests that the H1 receptor plays a partial role in the olanzapine-induced elevation of NPY and AgRP.

In conclusion, our data have demonstrated that the effect of olanzapine on hypothalamic H1 receptor–AMPK signaling varies during the different stages of obesity development. At the early stage, olanzapine activated hypothalamic AMPK signaling by blocking the H1 receptors leading to hyperphagia. With a prolonged treatment, the rats became obese although the excessive food intake was no longer present, suggesting that other parameters in addition to food intake may also contribute to increased body weight. It is possible that olanzapine affects other pathways related to metabolic regulation besides AMPK (Schmidt et al., 2013). However, we cannot exclude the possibility that desensitization to the drug used, or other unknown feedback mechanisms, may also contribute to the results observed in the present study. In summary, the results of this study may shed light on the future pharmacological development of targeting the H1 receptor–AMPK signaling to assist in minimizing the weight gain side effect of olanzapine treatment.

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**Figure legends**

**Figure 1.** Effects of olanzapine (3 mg/kg/day, tid) on food intake (***A***) and weight gain (***B***) during three stages of olanzapine-induced obesity, and the correlations between cumulative food intake and body weight gain in these three stages (***C, D, E***). Rats were trained to eat cookie dough containing olanzapine or vehicle as indicated for 36 days (see 'Materials and Methods') (n=12/group). All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$ vs. control). FI, food intake; WG, weight gain.

**Figure 2.** Effects of olanzapine on the gene and protein expressions of hypothalamic H1 receptor-AMPK signaling after 8 days (early stage), 16 days (middle stage) and 36 days (late stage) olanzapine treatment (n=5-6/group). ***A***, ***B, C,*** The mRNA expression of the hypothalamic H1 receptor, AMPKα2 and ACCα after 8, 16 and 36 days olanzapine treatment. ***D,*** Western blot of representative samples in the early, middle and late stage of olanzapine-induced obesity, displaying bands of AMPKα, pAMPKα, ACC, pACCα and actin expression in the hypothalamus. ***E, F, G***, Western blot analysis of the protein expression of hypothalamic AMPKα, pAMPKα, ACC and pACCα after 8, 16 and 36 days olanzapine treatment. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$ vs. control).

**Figure 3.** Effects of an acute central injection of the H1 receptor agonist, 2-(3-trifluoromethylphenyl) histamine (FMPH), on olanzapine-induced increased food intake (n=5-8/group). ***A and B***, Food intake and cumulative body weight gain of the rats treated with olanzapine significantly increased from day 2. ***C***, FMPH dose-dependently inhibited olanzapine-induced hyperphagia at different time points. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$). H, high dose; L: low dose; PY: pyrilamine; V/S, vehicle/saline; V/F(H), vehicle/FMPH 200 nmoles; O/S, olanzapine/saline; O/F(L), olanzapine/FMPH 100 nmoles; O/F(H), olanzapine/FMPH 200 nmoles; O/F(H)/PY, olanzapine/FMPH 200 nmoles/pyrilamine 800 nmoles.

**Figure 4.** Effects of an acute central injection of the H1 receptor agonist, 2-(3-trifluoromethylphenyl) histamine (FMPH), on the hypothalamic AMPK signaling in olanzapine or vehicle-treated rats (n=5-7/group). ***A and B,*** Representative western blot and densitometry analysis of pAMPKα and pACCα expression in the mediobasal hypothalamus of the olanzapine or vehicle-treated rats receiving the icv injection of FMPH or saline. ***C,*** Western blot analysis of protein expression of pAMPKα and pACCα in the hypothalamic PVN. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$). V/S, vehicle/saline; V/F(H), vehicle/FMPH 200 nmoles; O/S, olanzapine/saline; O/F(L), olanzapine/FMPH 100 nmoles; O/F(H), olanzapine/FMPH 200 nmoles; O/F(H)/PY, olanzapine/FMPH 200 nmoles/pyrilamine 800 nmoles. MBH, mediobasal hypothalamus; PVN, paraventricular nucleus.

**Figure 5.** Effects of an acute central injection of the H1 receptor agonist, 2-(3-trifluoromethylphenyl) histamine (FMPH), on mRNA expressions of: CRH in the PVN

(***A***), leptin receptor in the MBH (***B***), orexin-A in the LHA (***C***), NPY (***D***), and AgRP (***E***) in the MBH in either olanzapine or vehicle-treated rats (n=4-6/group). All data are presented as mean ± SEM. Statistical significance was defined as $p \leqslant 0.05$ (* $p \leqslant 0.05$). V/S, vehicle/saline; V/F(H), vehicle/FMPH 200 nmoles; O/S, olanzapine/saline; O/F(L), olanzapine/FMPH 100 nmoles; O/F(H), olanzapine/FMPH 200 nmoles; O/F(H)/PY, olanzapine/FMPH 200 nmoles/pyrilamine 800 nmoles. CRH, corticotropin-releasing hormone; LHA, lateral hypothalamic area; MBH, mediobasal hypothalamus; PVN, paraventricular nucleus.

**Table 1** Correlations within the hypothalamic H1 receptor mRNA, pAMPKα and pACCα protein expression, food intake and weight gain during the three stages of olanzapine-induced obesity.

| | **Early stage** | | **Middle stage** | | **Late stage** | |
|---|---|---|---|---|---|---|
| | **pAMPKα** | **pACCα** | **pAMPKα** | **pACCα** | **pAMPKα** | **pACCα** |
| | **r (P-value)** | **r (P-value)** | **r (P-value)** | **r (P-value)** | **r (P-value)** | **r (P-value)** |
| H1R mRNA | **0.718 (0.029*)** | **0.647 (0.043*)** | -0.057 (0.875) | 0.226 (0.505) | **-0.798 (0.002**)** | **-0.639 (0.025*)** |
| Last 48h FI | **0.747 (0.021*)** | **0.647 (0.031*)** | 0.281 (0.431) | -0.119 (0.713) | -0.225 (0.482) | 0.117 (0.718) |
| Weight gain | **0.717 (0.030*)** | 0.324 (0.332) | -0.432 (0.245) | 0.159 (0.640) | **-0.743 (0.009**)** | -0.230 (0.447) |
| pAMPKα | | **0.691 (0.027*)** | | 0.160 (0.659) | | **0.585 (0.046*)** |

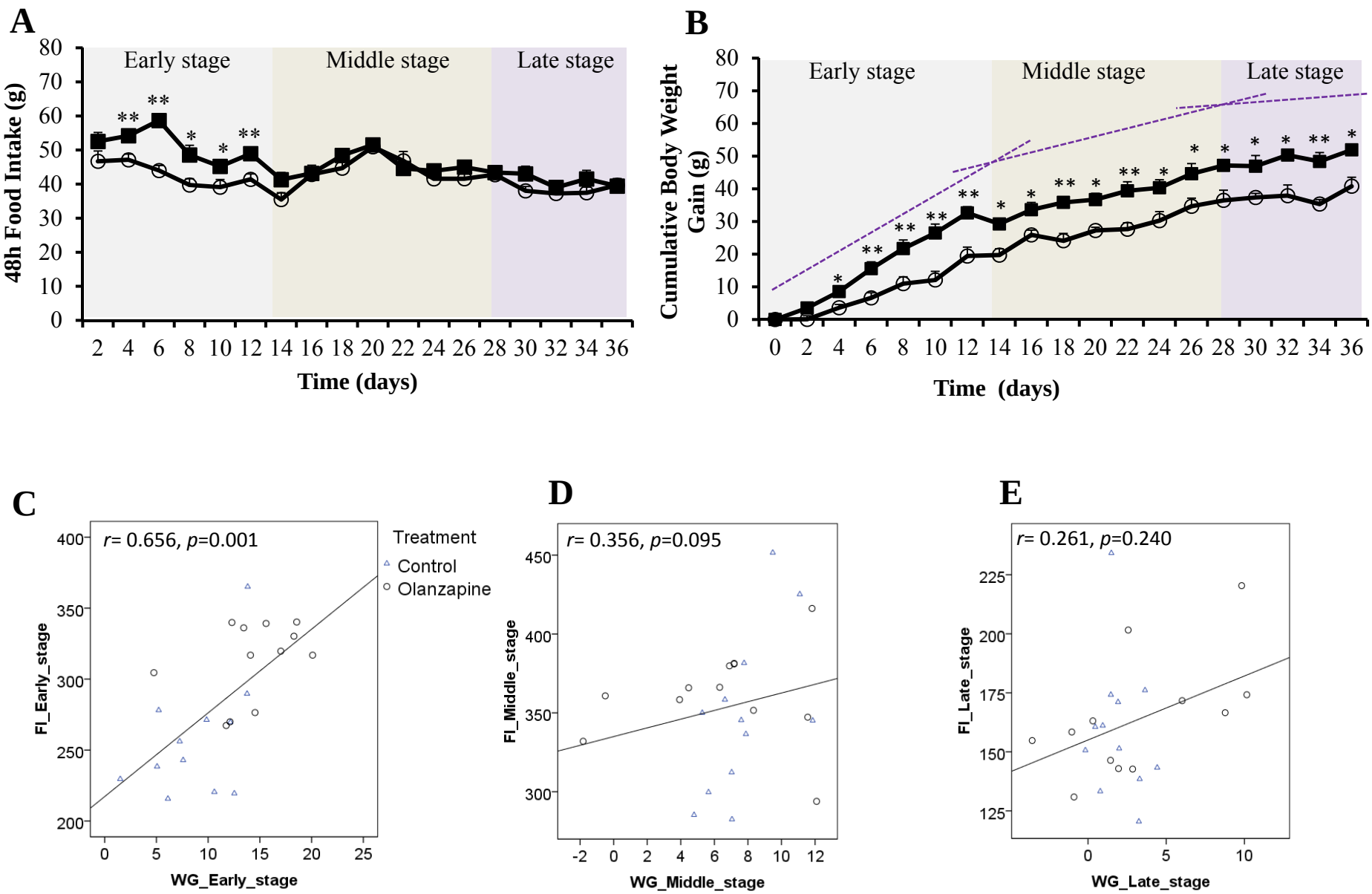
**Abbreviations**: FI: food intake; H1R: histamine H1 receptor; pACCα, acetyl CoA carboxylase phosphorylated Ser79 at ACCα subunits; pAMPKα, AMP-activated protein kinase phosphorylated Thr172 at AMPKα subunits. Statistical significance was defined as $p < 0.05$ (*, $p < 0.05$, **, $p < 0.01$). Significant correlations are indicated in bold.

**Table 2** Correlations within the MBH pAMPKα and pACCα protein expression and food intake during FMPH and olanzapine treatment.

| | | **Food intake (1h)** |
|---|---|---|
| | | r (P-value) |
| **MBH** | pAMPKα | **0.694 (0.000**)** |
| | pACCα | 0.240 (0.179) |

**Abbreviations**: pACCα, acetyl CoA carboxylase phosphorylated at Ser79 at ACCα subunits; pAMPKα, AMP-activated protein kinase phosphorylated Thr172 at AMPKα subunits; MBH, mediobasal hypothalamus. Statistical significance was defined as $p < 0.05$ (**, $p < 0.01$). Significant correlations are indicated in bold.

Figure 1


A
Early stage
Middle stage
Late stage
48h Food Intake (g)
Time (days)
B
Cumulative Body Weight Gain (g)
Time (days)
C
r= 0.656, p=0.001
Treatment
Control
Olanzapine
FI_Early_stage
WG_Early_stage
D
r= 0.356, p=0.095
FI_Middle_stage
WG_Middle_stage
E
r= 0.261, p=0.240
FI_Late_stage
WG_Late_stage

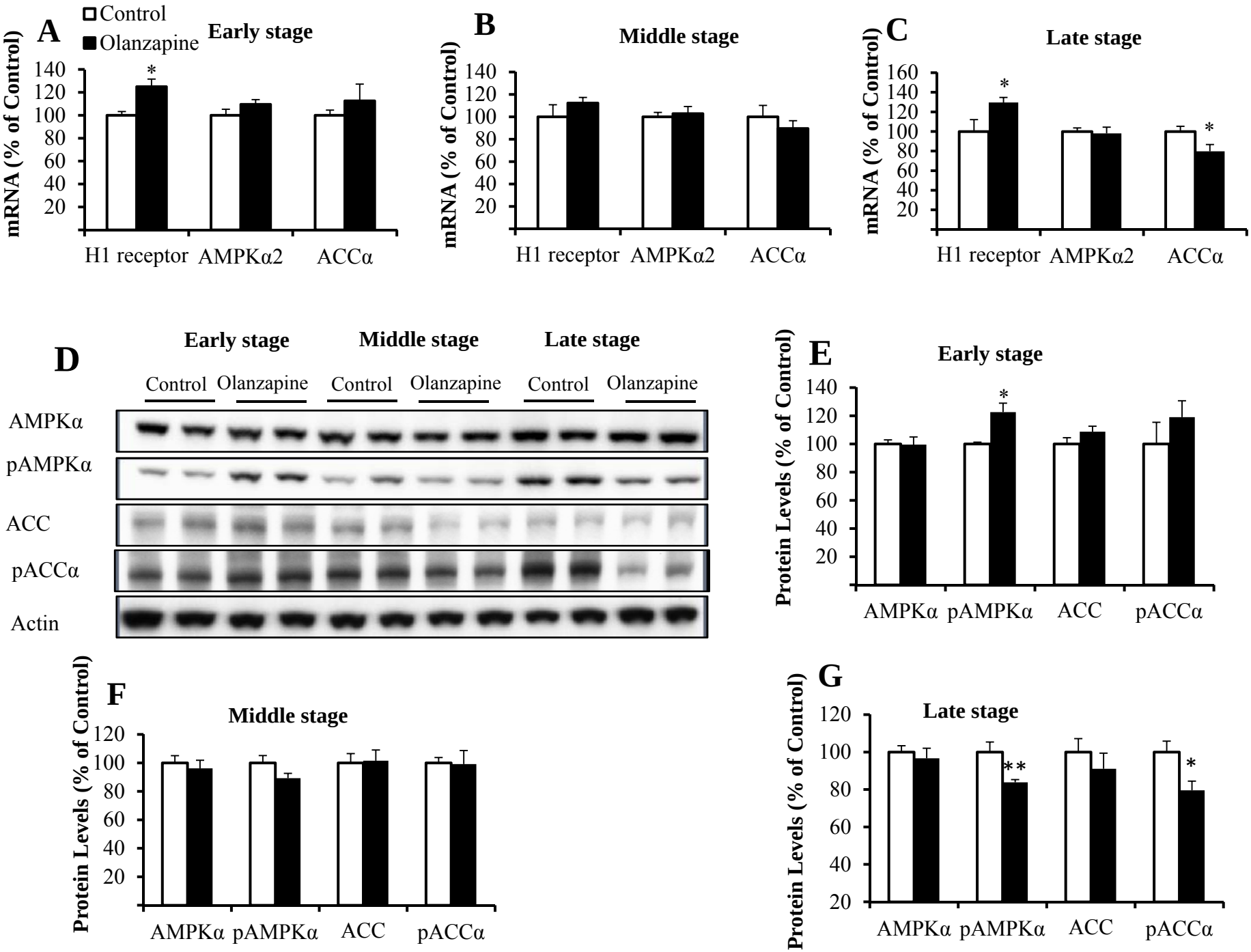

A
Control
Olanzapine
Early stage
mRNA (% of Control)
140
120
100
80
60
40
20
*
H1 receptor
AMPKα2
ACCα
B
Middle stage
mRNA (% of Control)
140
120
100
80
60
40
20
H1 receptor
AMPKα2
ACCα
C
Late stage
mRNA (% of Control)
160
140
120
100
80
60
40
20
*
*
H1 receptor
AMPKα2
ACCα
D
Early stage
Middle stage
Late stage
Control
Olanzapine
Control
Olanzapine
Control
Olanzapine
AMPKα
pAMPKα
ACC
pACCα
Actin
E
Early stage
Protein Levels (% of Control)
140
120
100
80
60
40
20
*
AMPKα
pAMPKα
ACC
pACCα
F
Middle stage
Protein Levels (% of Control)
120
100
80
60
40
20
AMPKα
pAMPKα
ACC
pACCα
G
Late stage
Protein Levels (% of Control)
120
100
80
60
40
20
**
*
AMPKα
pAMPKα
ACC
pACCα

Figure 3

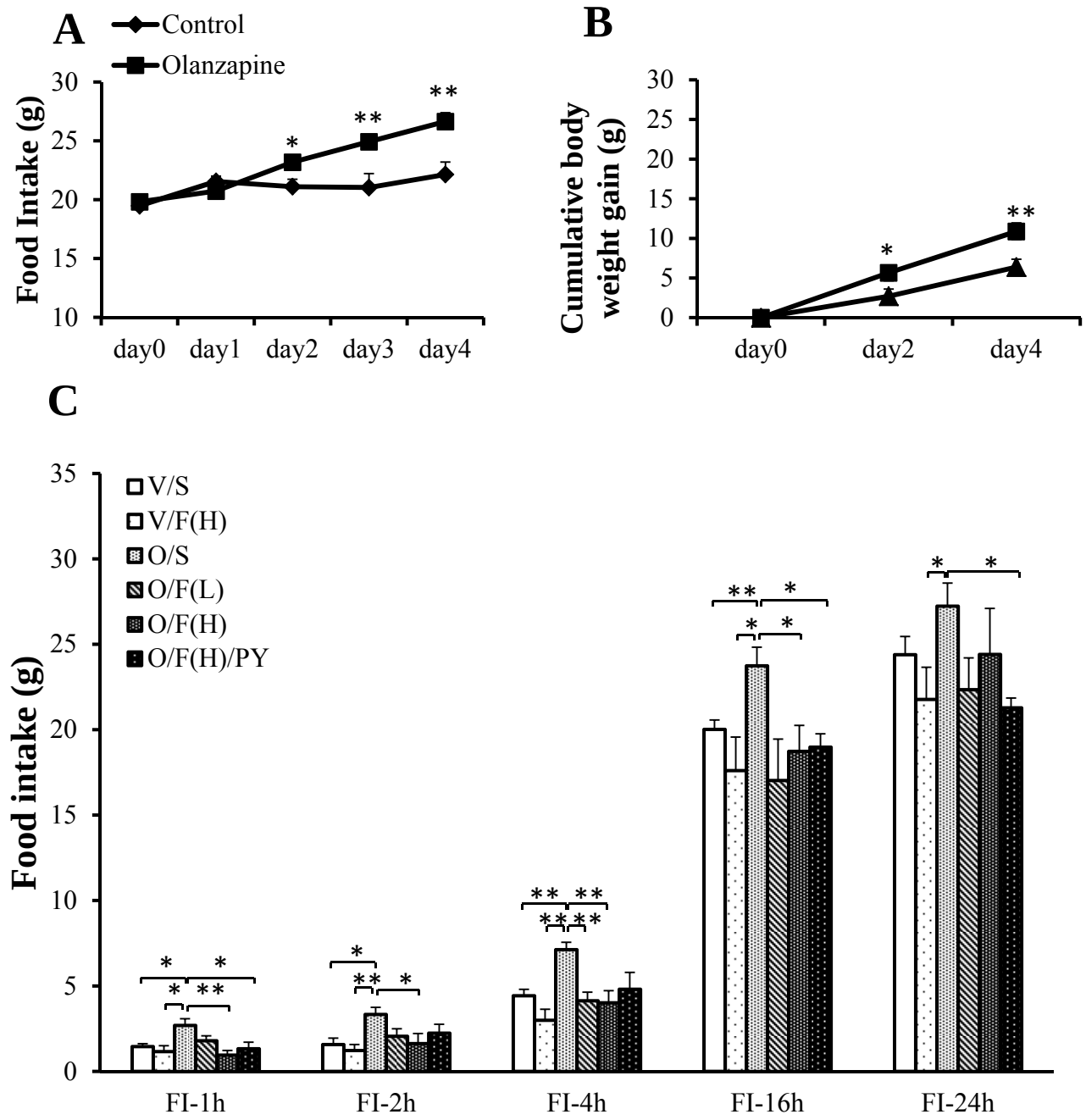
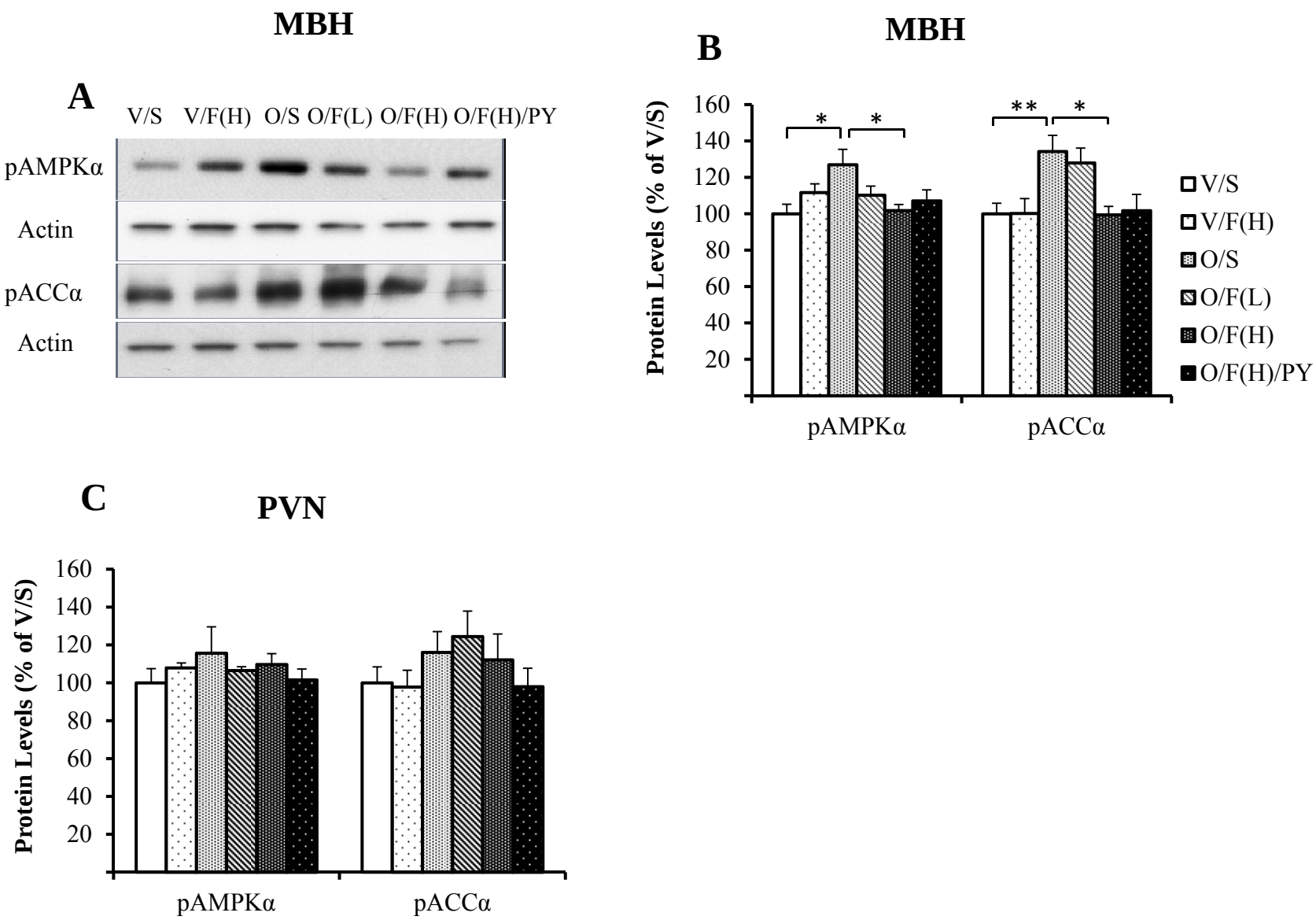

A
Control
Olanzapine
Food Intake (g)
30
25
20
15
10
day0
day1
day2
day3
day4
*
**
**
B
Cumulative body weight gain (g)
30
25
20
15
10
5
0
day0
day2
day4
*
**
C
V/S
V/F(H)
O/S
O/F(L)
O/F(H)
O/F(H)/PY
Food intake (g)
35
30
25
20
15
10
5
0
FI-1h
FI-2h
FI-4h
FI-16h
FI-24h

Figure 4


MBH
A
V/S
V/F(H)
O/S
O/F(L)
O/F(H)
O/F(H)/PY
pAMPKα
Actin
pACCα
Actin
B
MBH
Protein Levels (% of V/S)
160
140
120
100
80
60
40
20
*
*
**
*
pAMPKα
pACCα
V/S
V/F(H)
O/S
O/F(L)
O/F(H)
O/F(H)/PY
C
PVN
Protein Levels (% of V/S)
160
140
120
100
80
60
40
20
pAMPKα
pACCα

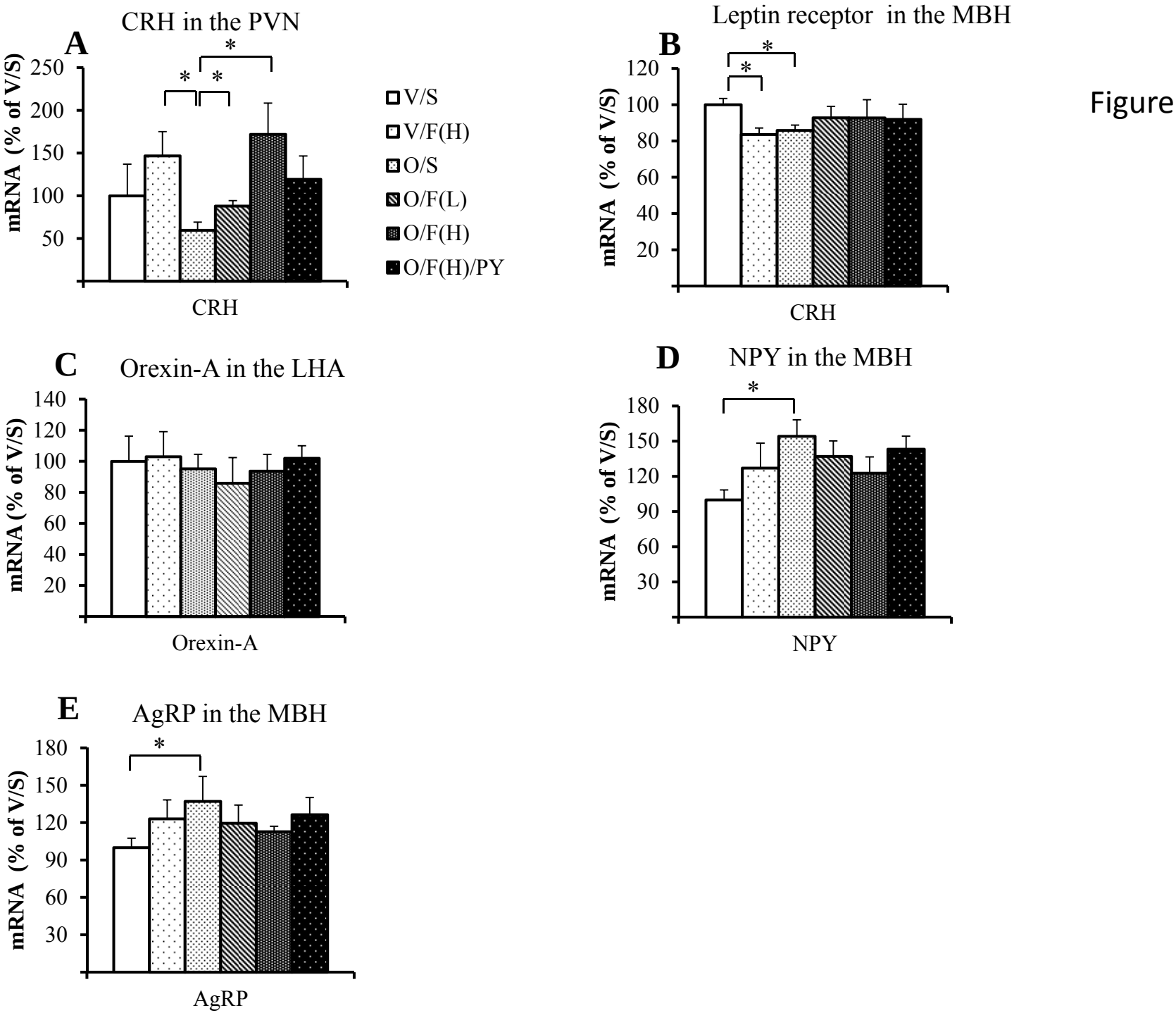

A
CRH in the PVN
mRNA (% of V/S)
250
200
150
100
50
*
*
*
CRH
V/S
V/F(H)
O/S
O/F(L)
O/F(H)
O/F(H)/PY
B
Leptin receptor in the MBH
mRNA (% of V/S)
120
100
80
60
40
20
*
*
CRH
C
Orexin-A in the LHA
mRNA (% of V/S)
140
120
100
80
60
40
20
Orexin-A
D
NPY in the MBH
mRNA (% of V/S)
180
150
120
90
60
30
*
NPY
E
AgRP in the MBH
mRNA (% of V/S)
180
150
120
90
60
30
*
AgRP

Figure 5