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Olanzapine-induced endoplasmic reticulum stress and inflammation in the hypothalamus were inhibited by an ER stress inhibitor 4-phenylbutyrate

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ABSTRACT

Antipsychotics are the most important treatment for schizophrenia. However, antipsychotics, particularly olanzapine and clozapine, are associated with severe weight gain/obesity side-effects. Although numerous studies have been carried out to identify the exact mechanisms of antipsychotic-induced weight gain, it is still important to consider other pathways. Endoplasmic reticulum (ER) stress signaling and its associated inflammation pathway is one of the most important pathways involved in regulation of energy balance. In the present study, we examined the role of hypothalamic protein kinase R like endoplasmic reticulum kinase- eukaryotic initiation factor 2α (PERK-eIF2α) signaling and the inflammatory IkappaB kinase β- nuclear factor kappa B (IKKβ-NFκB) signaling pathway in olanzapine-induced weight gain in female rats. In this study, we found that olanzapine significantly activated PERK-eIF2α and IKKβ-NFκB signaling in SH-SY5Y cells in a dose-dependent manner. Olanzapine treatment for 8 days in rats was associated with activated PERK-eIF2α signaling and IKKβ-NFκB signaling in the hypothalamus, accompanied by increased food intake and weight gain. Co-treatment with an ER stress inhibitor, 4-phenylbutyrate (4-PBA), decreased olanzapine-induced food intake and weight gain in a dose- and time-dependent manner. Moreover, 4-PBA dose-dependently inhibited olanzapine-induced activated PERK-eIF2α and IKKβ-NFκB signaling in the hypothalamus. These results suggested that hypothalamic ER stress may play an important role in antipsychotic-induced weight gain.

1. Introduction

Antipsychotics (APs) are widely used in the treatment of schizophrenia. However, APs particularly olanzapine and clozapine, are associated with weight gain and other metabolic side-effects. Previous studies have suggested that multiple factors are involved in AP-induced weight gain, including the histamine H1 receptor and its associated 5′ AMP-activated protein kinase (AMPK) signaling (He et al., 2014), H3 receptor (Lian et al., 2014; Poyurovsky et al., 2013, 2005), serotonin receptor 2C (Lord et al., 2017), ghrelin-mediated growth hormone secretagog receptor (GHS-R) signaling (Tagami et al., 2016; Zhang et al., 2014b), and neuropeptides (Huang et al., 2006; Zhang et al., 2014b). It has long been established that the cannabinoid receptor 1 (CB1 receptor) is involved in the metabolic mechanisms of food intake (Lazzari et al., 2011; Manca et al., 2013; Mastinu et al., 2012, 2013). Recently, a study in female rats reported that olanzapine-induced food intake and weight gain was markedly inhibited by the CB1 receptor inverse agonists rimonabant or NESS06SM (Lazzari et al., 2017), suggesting the importance of the CB1 receptor in AP-induced weight gain. Moreover, interesting research demonstrated that the microbial fermentation of prebiotics, B-GOS® attenuated olanzapine-induced weight gain in female rats (Kao et al., 2018). However, in the clinic, there is still a lack of medication for preventing and treating AP-induced weight gain. To find out the interaction of APs with other signaling pathways that mediate energy balance is very important for treating AP-induced weight gain and for development of novel non-obesogenic APs.

The endoplasmic reticulum (ER) is a continuous membrane system in the cells of eukaryotic organisms. ER stress refers to a pathophysiological process in which ER function is disordered under conditions of hypoxia, oxidative stress, undernutrition or imbalance of calcium ion

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homeostasis (Cnop et al., 2012). ER stress is mediated by three proteins: protein kinase R-like ER kinase (PERK), inositol requiring enzyme 1(IRE1) and activating transcription factor 6 (ATF6) (Cnop et al., 2012). Under non-stress conditions, the three proteins are inactive in combination with glucose-regulated protein 78 (GRP78). GRP78 is also referred to as the immunoglobulin heavy chain-binding protein (BiP) (Lee, 2005). When ER stress occurs, PERK, IRE1, and ATF6 dissociate from GRP78, and PERK is then activated by phosphorylation. pPERK thereby phosphorylates the downstream eukaryotic initiation factor 2α (eIF2α) to inhibit the translation and synthesis of transcription factor 4 (ATF4). ATF4 up-regulates GRP78/BiP expression (PERK-eIF2α signaling), and this effect contributes to the restoration of endoplasmic reticulum homeostasis (Cnop et al., 2012). Therefore, up-regulation of GRP78/BiP expression is a widely used marker of ER stress (Cnop et al., 2012). It is noteworthy that activation of hypothalamic ER stress PERK-eIF2α signaling leads to obesity (Cnop et al., 2012; Ozcan et al., 2009; Ramirez and Claret, 2015; Zhang et al., 2008). In high-fat diet (HFD) induced obese rodents (both males and females), the expression of pPERK, peIF2α and GRP78/BiP in the hypothalamus was significantly increased (Cakir et al., 2013; Melo et al., 2014). In male mice model, activation of hypothalamic ER stress leads to hyperphagia and inhibition of intra-capsular brown adipose tissue (IBAT) decomposition and heat production, resulting in severe obesity (Ozcan et al., 2009; Qiang et al., 2017). Conversely, inhibition of the hypothalamic ER stress by 4-phenylbutyrate (4-PBA) and tauroursodeoxycholic acid (TUDCA) reduces food intake and increases heat production in IBAT in male obese mice, thereby inhibiting obesity (Melo et al., 2014; Ozcan et al., 2009; Ramirez and Claret, 2015)

In the pathological process of obesity, hypothalamic ER stress is related to inflammation (Zhang et al., 2008). It has been demonstrated that activation of hypothalamic PERK- eIF2α signaling activates nuclear factor kappa B (NFκB), one of the most important transcriptional regulators in inflammatory response (Zhang et al., 2008). HFD feeding significantly activated hypothalamic ER stress and IKKβ-NFκB signaling, whereas intraventricular injection of an ER stress inhibitor TUDCA markedly inhibited hypothalamic IKKβ-NFκB signaling, accompanied by decreased food intake and weight gain in mice (gender not shown) (Zhang et al., 2008). Activation of hypothalamic IKKβ-NFκB signaling by constitutively active IKKβ cloned lentiviral vector induced increased food intake and weight gain in mice. On the contrary, inhibition of hypothalamic IKKβ-NFκB signaling by dominant-negative IKKβ cloned lentiviral vector significantly protected mice from obesity (Zhang et al., 2008). These results suggested that overnutrition activates hypothalamic IKKβ-NFκB signaling at least partly via elevating hypothalamic ER stress. Moreover, it has been found in male mice that NFκB activation increased the transcriptional activity of pro-opiomelanocortin (POMC) in the hypothalamus, suggesting involvement of NFκB in feeding control (Jang et al., 2010). Furthermore, the involvement of NFκB in a male murine model of neurodevelopment disorder has been reported recently (Bonini et al., 2016).

Previous studies have illustrated that the most obesogenic APs, including olanzapine, clozapine and risperidone, significantly activated ER stress PERK- eIF2α signaling in β-pancreatic cells (Ozasa et al., 2013) and the male mouse liver (Lauressergues et al., 2012), therefore leading to glucose and lipid metabolism dysfunction. A recent study in a female rat model reported that acute clozapine treatment (treated once and rats were sacrificed 1 h after the treatment) increased the protein expression of eIF2α and IRE1 in the liver (Weston-Green et al., 2018). This evidence suggested that AP treatment did activate ER stress PERK-eIF2α signaling. However, the effect of olanzapine on hypothalamic ER stress PERK-eIF2α signaling and IKKβ-NFκB signaling is unclear. The present study aimed to examine the role of hypothalamic ER stress PERK- elF2α signaling and its related IKKβ-NFκB signaling in olanzapine-induced weight gain.

2. Methods

2.1. Cell culture and olanzapine treatment

The human neuroblastoma SH-SY5Y cell line was purchased from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco`s Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Wuhan, Hubei, China, #31,966,021) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher Scientific, #10,270,098 and #15070063, respectively) at 37 °C in a humidified 5% CO_2 incubator. The cells were regularly subcultured to maintain them in the logarithmic growth phase. For olanzapine treatment, cells were seeded in 6-well plates (6×10^5 cells/well). After incubation for 24 h at 37 °C, the cells were divided into three groups (n = 4 samples/group): control, olanzapine high dose (100 μM) and olanzapine low dose (50 μM). The treatment duration for the in vitro experiment was chosen based on a previous study which reported that 100 μM olanzapine treatment for 24 h induced mitochondrial damage in SH-SY5Y cells (Vucicevic et al., 2014). The mitochondrial axis has long been shown to be related to ER stress (Bravo et al., 2012). The olanzapine (Sigma-Aldrich, #O1141) was dissolved in Dimethyl sulfoxide (DMSO) to make a 100 mM stock solution. When used, the corresponding amount of olanzapine stock solution was diluted to the final concentration of 50 μM and 100 μM with DMEM (the final concentration of DMSO was 1/1000 (v/v)). Control cells were treated with DMSO. After 24 h of incubation, the protein was harvested and ready for use.

We have further investigated whether olanzapine-induced activated ER stress was inhibited by an ER stress inhibitor, 4-PBA. In brief, the cells were pre-treated with different doses (2 mM or 1 mM) of 4-PBA for 2 h before 100 μM olanzapine exposure for 24 h. The 4-PBA control group was treated with 4-PBA for 2 h followed by vehicle (DMSO) for 24 h. The olanzapine control group was treated with water for 2 h followed by olanzapine for 24 h. The control group was treated with water for 2 h followed by vehicle for 24 h. The dosage of 4-PBA was based on previous studies (Marwarha et al., 2012; Zamarbide et al., 2013). The 4-PBA (Sigma-Aldrich) (100 mM) stock solutions were prepared by titrating equimolar amounts of 4-PBA with sodium hydroxide to pH = 7.4 and dissolved in DMEM before using.

2.2. Animals and drugs

Female Sprague-Dawley (SD) rats (weight 200–225 g) were obtained from the Animal Resources Centre (SPF (Beijing) Biotechnology Co., Ltd, Beijing, China). The rats were housed under environmentally controlled conditions (22 ± 2 °C on a 12 h light-dark cycle, lights on 0700 h). Rats were allowed *ad libitum* access to standard laboratory chow diet and water throughout the studies. All animal experiments were approved by the Animal Ethics Committee, Wuhan University of Technology. Olanzapine (Zyprexa) was purchased from Eli Lilly, Indianapolis, IN, USA. 4-PBA was purchased from Sigma-Aldrich (# P21005).

2.3. Animal experiment 1

In order to examine the effects of sub-chronic olanzapine treatment on the hypothalamic PERK- eIF2α and IKKβ-NFκB signaling pathway, 24 female SD rats were randomly divided into two groups (n = 12/group) and treated with olanzapine or vehicle for 8 days (He et al., 2014). In animal experiments, our previous studies reported that olanzapine treatment did not increase food intake during the first 24 h treatment (He et al., 2014; Zhang et al., 2014b). Olanzapine treatment for 8 days leads to hyperphagia and rapid weight gain accompanied by increased hypothalamic AMPK expression that is related to ER stress (He et al., 2014; Zhang et al., 2014b). Therefore, 8-day treatment of olanzapine was chosen in this study. Briefly, olanzapine (1 mg kg^{-1}) or

placebo was mixed with sweet cookie dough pellets (62% carbohydrate, 22% protein, 6% fibre, 10% vitamins and minerals). The rats were treated with sweet cookie dough containing olanzapine or placebo three times per day at eight-hourly intervals (0700 h, 1500 h and 2300 h) (equivalent to 3 mg kg^{-1} day^{-1}) (He et al., 2014). Food intake was measured every 24 h, and body weight was measured every 48 h. The dosage of olanzapine was chosen based on our previous studies (He et al., 2014; Liu et al., 2015; Weston-Green et al., 2011a). Based on body surface area of different species, 3 mg kg^{-1} day^{-1} of olanzapine in rats was similar to the clinically relevant dosage of 10 mg day^{-1} (Reagan-Shaw et al., 2008). Two hours after the last treatment, rats were sacrificed by decapitation. The hypothalamic tissues were quickly removed on ice and stored at −80 °C. In the present study, olanzapine formulation (Zyprexa) was used in rats because the olanzapine formulation but not the olanzapine ingredient is commonly used in animal studies to better mirror the human scenario of olanzapine administration (Albaugh et al., 2006; Razavi et al., 2017; Weston-Green et al., 2011b), and this treatment protocol has been proven to mimic the clinical situation of olanzapine-induced weight gain (He et al., 2017; Hu et al., 2014; Lian et al., 2014). Moreover, female rats were used since numerous studies have described the effect of olanzapine on food intake and weight gain in female rats but not in males (Albaugh et al., 2011; Weston-Green et al., 2011b; Zhang et al., 2014c). Similarly, clinical findings showed a high risk of olanzapine-induce obesity in females (Gebhardt et al., 2009; Hakko et al., 2006). According to previous studies in adult female rats (about 200 ± 20 g, 9–10 weeks), the serum level of estradiol is 13–28 pg/mL, the level of progesterone is 31.7 ng/mL, follicle-stimulating hormone level is 15.3 ng/mL and luteinizing hormone level is 2.3 ng/mL (Biegel et al., 1998; Montano et al., 1995; Yan et al., 2009). Olanzapine treatment reduced the serum levels of estradiol and prolactin in female schizophrenia patients (Bergemann et al., 2005) and in female rats (Cooper et al., 2005). It has also been found that olanzapine-induced weight gain is related to the presence of estrogens (Fitzgerald et al., 2003; Skrede et al., 2017). At the end of the experiment, the decapitation procedure was chosen to sacrifice the rats based on previous studies to avoid contamination with other chemicals, such as gases and anaesthetics (Cakir et al., 2013; Castro et al., 2013; van Rijn et al., 2011). Handling and maneuver of sacrificing at the guillotine were undertaken during experiments since habituation to handling prevents the stress of the decapitation procedure (Biggio et al., 1981; Boix et al., 1990). Moreover, the guillotine was well maintained and cleaned between every use to avoid transmission of olfactory clues.

2.4. Animal experiment 2

To investigate whether the changes in the hypothalamic ER stress signaling were a secondary effect of overfeeding in olanzapine-induced weight gain, we conducted an acute (one-day olanzapine treatment) study to examine the effect of olanzapine on the hypothalamic ER stress signaling before the onset of hyperphagia, since overfeeding started at about day 6 of oral olanzapine treatment but not day 1–4 (3 mg kg^{-1} day^{-1}) as described in Animal Experiment 1. Food intake was measured for 24 h. The rats (control and olanzapine group; n = 6/group) were treated with olanzapine or vehicle for one day and were sacrificed 2 h after the last olanzapine treatment and the hypothalamic tissues were collected and ready for use.

2.5. Animal experiment 3

To further investigate whether hypothalamic ER stress PERK- eIF2α signaling is linked to olanzapine-induced hyperphagia, the rats receiving olanzapine treatment were co-treated with an ER stress inhibitor, 4-PBA. Briefly, after acclimatization for 1 week, SD rats were randomly divided into 5 groups (n = 8/group) (Group 1, vehicle/vehicle (Con); Group 2, olanzapine/vehicle (OLZ); Group 3, olanzapine/4-PBA high dose (400 mg kg^{-1}) (OLZ + 4-PBA H); Group 4, olanzapine/4-PBA low dose (200 mg kg^{-1}) (OLZ + 4-PBA L); Group 5, vehicle/4-PBA high dose (Veh + 4-PBA H). The rats were orally treated with vehicle, olanzapine, olanzapine + 4-PBA, or vehicle + 4-PBA for 8 days, respectively. Consistent with Animal Experiment 1, olanzapine (1 mg kg^{-1}, three times daily (t.i.d), equivalent to 3 mg kg^{-1} day^{-1}) and 4-PBA (high dose: 400 mg kg^{-1}, t.i.d, equivalent to 1.2 g kg^{-1} day^{-1}) were mixed with 62% carbohydrate, 22% protein, 6% fibre, 10% vitamins and minerals to make sweet cookie dough pellets. The dosages of 4-PBA were chosen based on a previous study (Kawasaki et al., 2012). Food intake was measured every 24 h, and body weight was measured every 48 h. At the end of the experiment, rats were sacrificed by decapitation. The hypothalamic tissues were collected and stored at −80 °C.

2.6. Western blot procedures

Western blot was conducted based on the procedure in (He et al., 2014). The SH-SY5Y cells and hypothalamic tissues were homogenized in RIPA cell lysis buffer containing a protease inhibitor cocktail and phenylmethanesulfonyl fluoride (Beyotime, Hubei, China). The protein concentration was detected by BCA protein assay (Beyotime, Hubei, China, Cat. No. P0010). 10–40 μg proteins were loaded onto 5–8% gels (Bio-Rad Laboratories, Wuhan Hubei, China) and run for 1.5 h at 120 V. The proteins were then transferred to polyvinylidene difluoride (Merck Millipore, Billerica, MA, USA, #IPVH00010) membranes at 100 V for 1 h. The membranes were blocked in 5% non-fat milk for 2 h in tris buffered saline with 0.1% Tween 20 (TBST) at room temperature. The membranes were then incubated with primary antibodies in 1% non-fat milk at 4 °C overnight (1:1,000, primary antibodies are: phospho-PERK (pPERK) (Thr 980), peIF2α (Ser 51), ATF4, and pNFκB (Ser 536) (Cell Signaling Technology, Danvers, MA, USA, #3179, #3597, #11,815 and #3033 respectively), pIKKβ (phospho Y199) and GRP78/BiP (Abcam, #ab21685 and #ab59195, respectively), and TNF-α, IL-6 and IL-1β (Proteintech, Hubei, China, 60291-1-ig, 21865-1-ap; Bioss, Beijing, China Bs-0812 r, respectively). The membranes were washed in TBST (5 × 5 min), and incubated with goat anti-rabbit and goat anti-mouse (1: 5000 BOSTER Biological Technology Ltd, Wuhan, Hubei, China, #BA1051 and #BA1054), and horseradish peroxidase conjugated secondary antibody at 37 °C for 2 h. After washing with TBST (5 × 5 min), the membranes were detected with the enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific). The results were quantified by Bandscan software. The quantification of protein was normalized to those of β-actin. Based on the literature, the optic density values of olanzapine-treated samples were commonly normalized to β-actin (Kao et al., 2018; Zhang et al., 2014b) and total protein (Ozasa et al., 2013). We have previously confirmed that olanzapine did not change β-actin expression in the hypothalamus during different periods of olanzapine treatment (He et al., 2014; Zhang et al., 2014b). In terms of the total protein levels, it has been reported that olanzapine did not change the total protein levels in the hamster pancreatic β cell line (Ozasa et al., 2013). However, we did not find evidence of whether olanzapine would change total protein levels in the rat hypothalamus. Therefore, in the present study, β-actin was used as an internal control the same as previous studies (He et al., 2014; Zhang et al., 2014b).

2.7. Statistics

The statistical analyses were performed using the SPSS 22.0 program (Chicago, IL). In vitro study, one-way analysis of variance (ANOVA) followed by Dunnett *t*-test were used to analyse the protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, TNF-α, IL-1β and IL-6. In Animal Experiment 1, two-way analysis ANOVA (OLANZAPINE × TIME as repeated measure) followed by an independent unpaired student's *t*-test (two-tailed) were used to analyse the statistical differences in food intake and weight gain between the

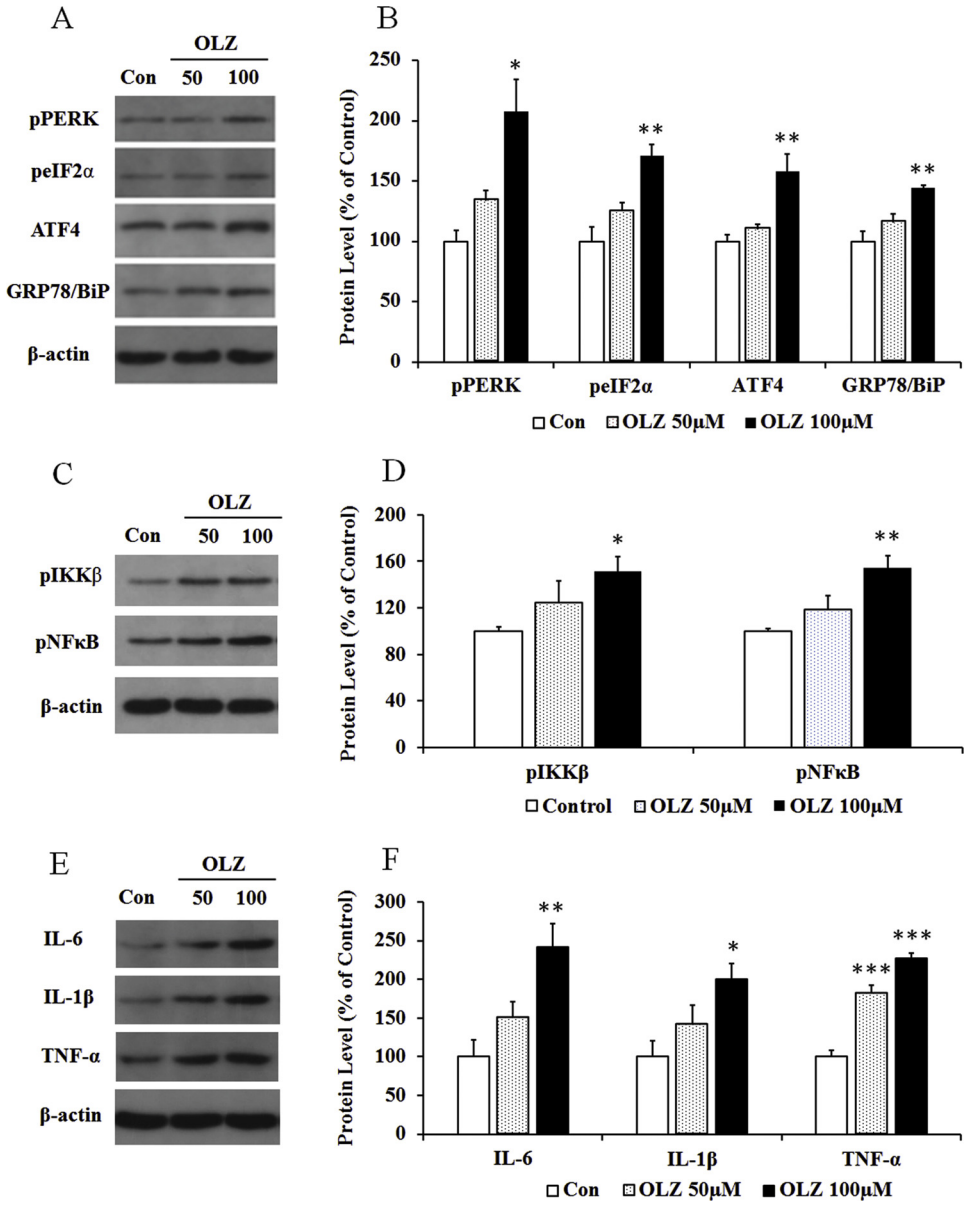

Fig. 1. Effects of olanzapine treatment on the protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α in the human neuroblastoma SH-SY5Y cell line. (***A, C*** and ***E***) Representative Western blot and densitometry analysis of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α. (***B, D*** and ***F***) Western blot analysis of protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (*$p <$ 0.05, **$p <$ 0.01, *** $p <$ 0.001vs. control). Con, control; OLZ, olanzapine.

olanzapine and control groups. The protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, TNF-α, IL-1β and IL-6 were analysed by independent unpaired student's *t*-test (two-tailed). In Animal Experiment 2, the food intake and protein expression of above markers were analysed by an independent unpaired student's *t*-test (two-tailed). In Animal Experiment 3, three-way ANOVAs (OLANZAPINE × 4-PBA × TREATMENT PERIOD) followed by Dunnett *t*-test were used to analyse the difference in food intake and weight gain. The protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ and pNFκB were analysed by one-way ANOVA followed by Dunnett *t*-test. Correlations were carried out by Pearson's correlation. All data were presented as mean ± SEM. Statistical significance was defined as $p <$ 0.05.

3. Results

3.1. Olanzapine dose-dependently activated PERK- eIF2α and IKKβ-NFκB signaling in human neuroblastoma SH-SY5Y cells

The protein expression of key molecules of PERK- eIF2α and IKKβ-NFκB signaling was examined by Western blot. As shown in Fig. 1A-B, compared with the control group (treated with DMSO), olanzapine 100 μM treated for 24 h markedly increased the protein expression of pPERK (increased by 107.3 ± 26.9%, $p <$ 0.05), peIF2α (by 70.7 ± 9.3%, $p <$ 0.01), ATF4 (by 57.7 ± 14.8%, $p <$ 0.01) and GRP78/BiP (by 44.6 ± 2.0%, $p <$ 0.01). 50 μM olanzapine did not significantly increase the protein expression of pPERK, peIF2α, ATF4 and GRP78/BiP (all $p >$ 0.05). Olanzapine 100 μM largely up-regulated the pIKKβ (increased by 51.5 ± 12.5%, $p <$ 0.05) and pNFκB (by 54.4 ± 10.4%, $p <$ 0.01) protein expression (Fig. 1 C–D). Olanzapine 50 μM treatment did not significantly elevate pIKKβ and pNFκB

expression (all $p > 0.05$). These results suggest that olanzapine activated the PERK-eIF2α and IKKβ-NFκB signaling in a dose-dependent manner in neuroblastoma cells. Moreover, we have investigated the expression of inflammatory cytokines including interleukin 6 (IL-6), IL-1β, and tumor necrosis factor (TNF-α). Olanzapine 100 μM treatment for 24 h increased the protein expression of inflammatory cytokines including IL-6 (by 142.0 ± 30.0%, $p < 0.01$), IL-1β (by 99.6 ± 20.9%, $p < 0.05$) and TNF-α (by 126.6 ± 6.9%, $p = 0.000$) in SH-SY5Y cells. Olanzapine 50 μM did not significantly increase the protein expression of IL-6 and IL-1β ($p > 0.05$), but significantly increased the protein expression of TNF-α (by 82.3 ± 10.5%, $p = 0.000$) (Fig. 1E-F).

3.2. Olanzapine induced activation of PERK- eIF2α and IKKβ-NFκB signaling were inhibited by an ER stress inhibitor 4-PBA in human neuroblastoma SH-SY5Y cells

Consistent with in vitro experiment one, olanzapine significantly increased the protein expression of pPERK (by 91.5 ± 5.2%, $p = 0.000$), peIF2α (by 158.1 ± 4.4%, $p = 0.000$), ATF4 (by 261.6 ± 44.7%, $p = 0.000$), GRP78/BiP (by 258.5 ± 5.5%, $p = 0.000$), pIKKβ (by 170.7 ± 31.5%, $p = 0.000$) and pNFκB (by 126.0 ± 11.3%, $p = 0.000$) compared with those of the control group (Fig. 2A-D). Pre-treatment with 4-PBA high dose significantly reduced olanzapine-induced increased expression of pPERK (reduced by 89.3 ± 4.9%, $p = 0.000$), peIF2α (by 108.6 ± 4.5%, p = 0.000), ATF4 (by 212.4 ± 10.2%, p = 0.000), GRP78/BiP (by 236.4 ± 6.8%, $p = 0.000$), pIKKβ (by 118.2 ± 14.6%, $p < 0.01$) and pNFκB (by 98.8 ± 10.2%, $p = 0.000$) compared with olanzapine. 4-PBA low dose also reduced olanzapine-induced increase in expression of pPERK (reduced by 45.9 ± 9.5%, $p = 0.000$), peIF2α (by 37.8 ± 10.2%, $p < 0.01$), ATF4 (by 151.8 ± 16.3%, $p < 0.01$), GRP78/BiP (by 155.2 ± 26.8%, $p = 0.000$), pIKKβ (by 109.3 ± 14.6%, $p < 0.01$) and pNFκB (by 56.8 ± 15.1%, $p < 0.05$) compared with olanzapine. Moreover, olanzapine evidentially increased the protein expression of IL-6 (by 247.5 ± 6.8%, $p = 0.000$), IL-1β (by 370.2 ± 18.2%, $p = 0.000$) and TNF- α (by 163.2 ± 44.5%, $p < 0.01$). These effects were inhibited by 4-PBA high dose (IL-6, IL-1β and TNF-α: by 175.7 ± 10.3%, 267.1 ± 2.4%, 164.8 ± 17.9%, respectively, all $p < 0.01$) and low dose (IL-6, IL-1β and TNF-α: by 92.8 ± 10.6%, 153.7 ± 34.6%, 131.7 ± 48.4%, respectively, all $p < 0.05$) (Fig. 2E-F).

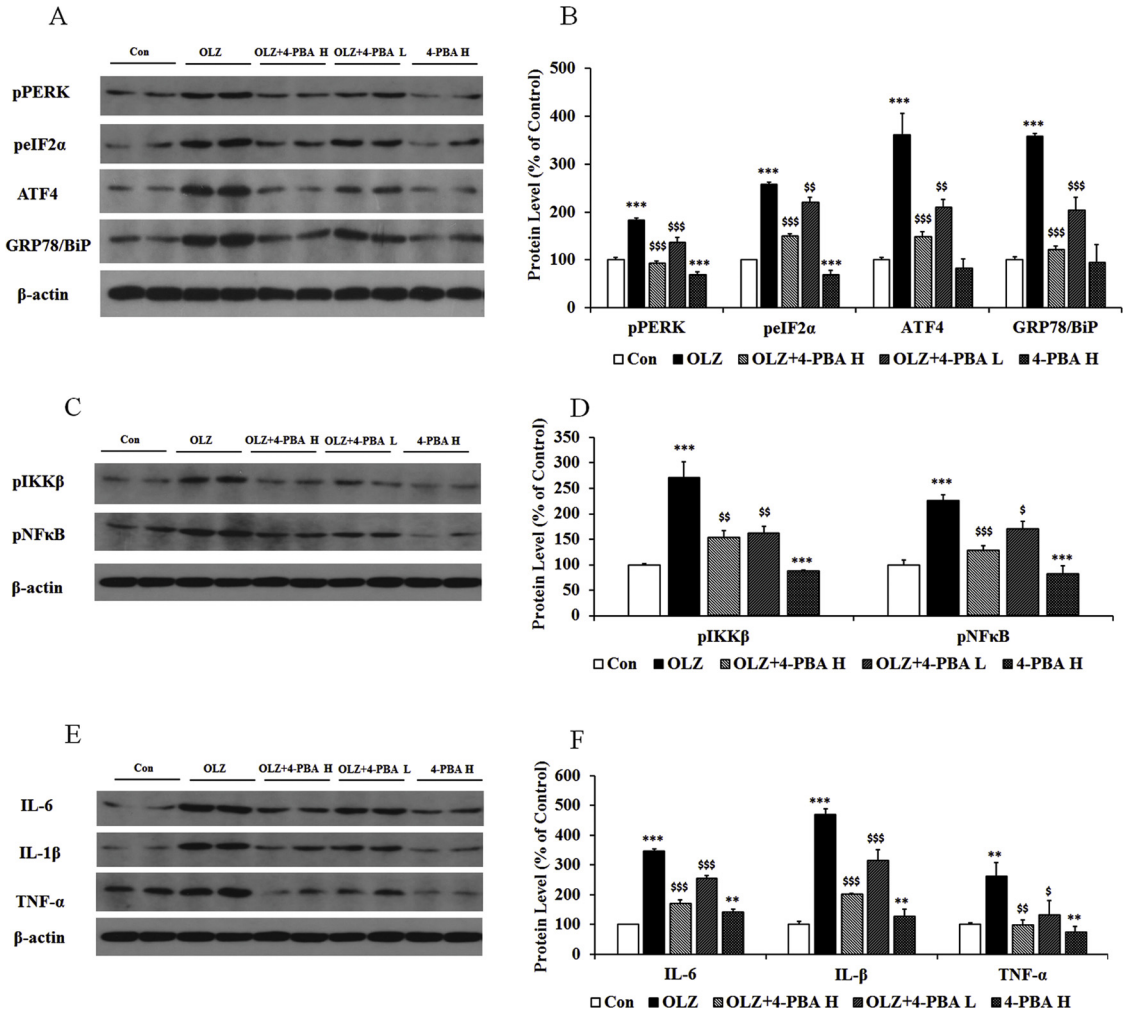

Fig. 2. Effects of olanzapine and 4-PBA treatment on PERK-peIF2α signaling and IKKβ-NFκB signaling in the human neuroblastoma SH-SY5Y cell line. (***A, C*** and ***E***) Representative Western blot and densitometry analysis of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α. (***B, D*** and ***F***) Western blot analysis of protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$ vs. Con; $\$ p < 0.05$, $\$\$ p < 0.01$, $\$\$\$ p < 0.001$ vs. OLZ). Con, control; OLZ, olanzapine; OLZ + 4-PBA H, olanzapine + 4-PBA high dose; OLZ + 4-PBA L, olanzapine + 4-PBA low dose. 4-PBA H, 4-PBA high dose.

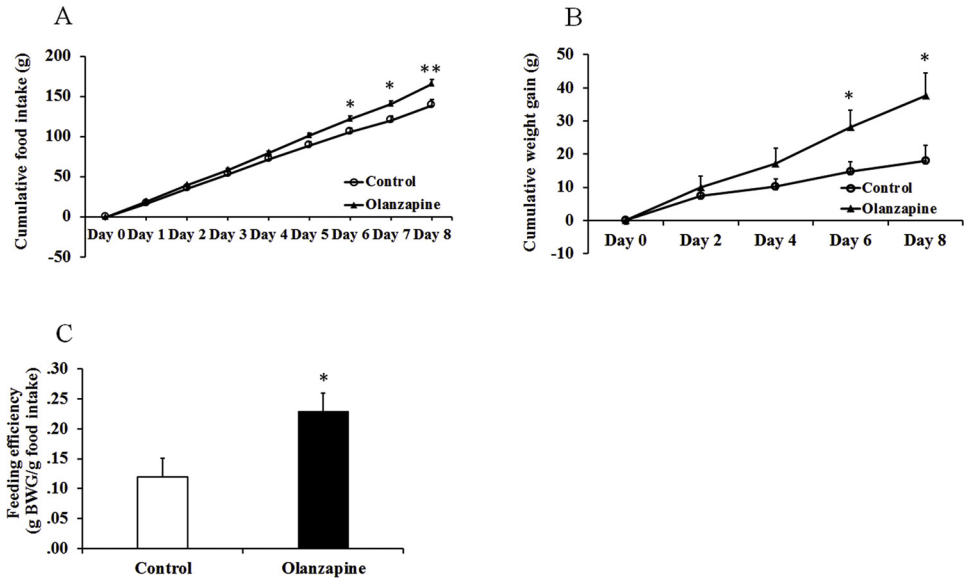

Fig. 3. Food intake (24 h) (***A***), body weight gain (48 h) (***B***) and feeding efficiency (***C***) during 8-day olanzapine treatment. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (*$p < 0.05$, **$p < 0.01$vs. control). Con, control; OLZ, olanzapine.

3.3. Food intake, weight gain and feeding efficiency in rats treated with olanzapine for 8 days

To further understand the role of PERK-eIF2α and IKKβ-NFκB signaling in olanzapine-induced weight gain, an olanzapine-induced obesity rat model has been established. Fig. 3A-B illustrate that olanzapine treatment significantly increased cumulative food intake from day 5 (all $p < 0.05$) and cumulative weight gain (all $p < 0.05$) from day 6 compared to vehicle. Feeding efficiency was increased by olanzapine compared with vehicle ($p < 0.05$, Fig. 3C). Pearson's correlation revealed that cumulative weight gain was significantly correlated with cumulative food intake ($r = 0.886$, $p = 0.000$), the last 24 h food intake ($r = 0.844$, $p = 0.002$) and feeding efficiency ($r = 0.990$, $p = 0.000$).

3.4. Olanzapine treatment activated PERK- elF2α and IKKβ-NFκB signaling in the hypothalamus

Western blot was conducted to examine whether olanzapine regulated hypothalamic ER stress PERK- eIF2α signaling and IKKβ-NFκB signaling. We found that olanzapine treatment significantly increased the protein expression of pPERK (increased by 65.5 ± 3.2%, $p < 0.001$), peIF2α (by 60.9 ± 9.0%, $p < 0.01$), ATF4 (by 31.3 ± 7.5%, $p < 0.05$) and GRP78/BiP (by 57.2 ± 11.7%, $p < 0.05$) compared to vehicle (Fig. 4A-B). Moreover, olanzapine treatment increased the protein expression of pIKKβ (by 65.2 ± 11.3%, $p < 0.01$) and pNFκB (by 31.7 ± 11.4%, $p < 0.05$) (Fig. 4C–D). Pearson's correlation revealed that pPERK protein expression was significantly correlated with peIF2α ($r = 0.931$, $p = 0.000$), ATF4 ($r = 0.875$, $p = 0.000$), GRP78/BiP ($r = 0.884$, $p = 0.000$), pIKKβ ($r = 0.837$, $p = 0.001$), pNFκB ($r = 0.720$, $p = 0.012$), cumulative weight gain ($r = 0.722$, $p = 0.018$), and feeding efficiency ($r = 0.639$, $p = 0.036$) and tended to be correlated with the last 24 h food intake ($r = 0.610$, $p = 0.061$). pIKKβ expression was correlated with cumulative weight gain ($r = 0.649$, $p = 0.031$), cumulative food intake ($r = 0.578$, $p = 0.049$), the last 24 h food intake ($r = 0.715$, $p = 0.013$) and feeding efficiency ($r = 0.652$, $p = 0.041$). These results indicated that olanzapine could activate ER stress PERK- eIF2α signaling and IKKβ-NFκB signaling in the hypothalamus, and this effect may be related to olanzapine-induced hyperphagia and weight gain. Moreover, olanzapine treatment increased the protein expression of IL-6 (by 27.9 ± 6.0%, $p < 0.05$), IL-1β (by 30.3 ± 6.4%, $p < 0.01$) and TNF-α (by 54.7 ± 12.5%, $p < 0.05$) (Fig. 4E-F), which are significantly related to IKKβ- NFκB signaling in the hypothalamus in body weight regulation.

3.5. Acute olanzapine treatment did not induce hyperphagia but activated PERK- elF2α and IKKβ-NFκB signaling in the hypothalamus

The data shows that rats treated with olanzapine for 24 h had similar food intake (Fig. 5A, $p > 0.05$, student *t*-test) compared with the control rats. In this experiment, we have found that 24 h olanzapine treatment evidentially increased the protein expression of pPERK (increased by 60.6 ± 10.1%, $p < 0.01$, student *t*-test), peIF2α (by 65.4 ± 9.0%, $p = 0.000$), ATF4 (by 60.5 ± 10.4%, $p < 0.01$), GRP78/BiP (by 33.7 ± 7.2%, $p < 0.01$), pIKKβ (by 96.4 ± 8.9%, $p = 0.000$), and pNFκB (by 66.1 ± 11.3%, $p < 0.01$) compared to vehicle (Fig. 5B-E). Moreover, olanzapine increased the protein expression of IL-6 (by 40.0 ± 5.2%, $p < 0.01$), IL-1β (by 68.0 ± 12.2%, $p < 0.01$) and TNF-α (by 82.1 ± 9.5%, $p = 0.000$) in the hypothalamus (Fig. 5F-G).

3.6. ER stress inhibitor 4-PBA reduced olanzapine-induced food intake and weight gain in a dose- and time- dependent manner

To further investigate whether olanzapine-induced activated ER stress was related to olanzapine-induced obesity, we examined whether an ER stress inhibitor, 4-PBA, could reduce olanzapine-induced food intake and weight gain. In terms of food intake, three-way ANOVA showed that there were significant main effects of treatment (olanzapine) ($F_{[1,266]} = 27.764$, $p = 0.000$), treatment period ($F_{[7,266]} = 957.473$, $p = 0.000$) and 4-PBA ($F_{[2,266]} = 7.745$, $p = 0.001$) as well as significant interactions between treatment and treatment period ($F_{[7,266]} = 2.327$, $p = 0.026$), and interaction between treatment and 4-PBA ($F_{[1,266]} = 3.956$, $p = 0.048$). In terms of weight gain, three-way ANOVA showed that there were significant main effects of treatment ($F_{[1,116]} = 14.563$, $p = 0.000$), treatment period ($F_{[3,116]} = 49.964$, $p = 0.000$) and 4-PBA ($F_{[2,116]} = 5.365$, $p = 0.006$). There was no significant interaction between all the three factors ($p > 0.05$). Post hoc test revealed that the in olanzapine-only group, cumulative food intake was significantly increased from day 5–8 (all $p < 0.05$, Fig. 6A) compared to that of the control group. The 4-PBA high dose + olanzapine co-treated group had a significantly lower

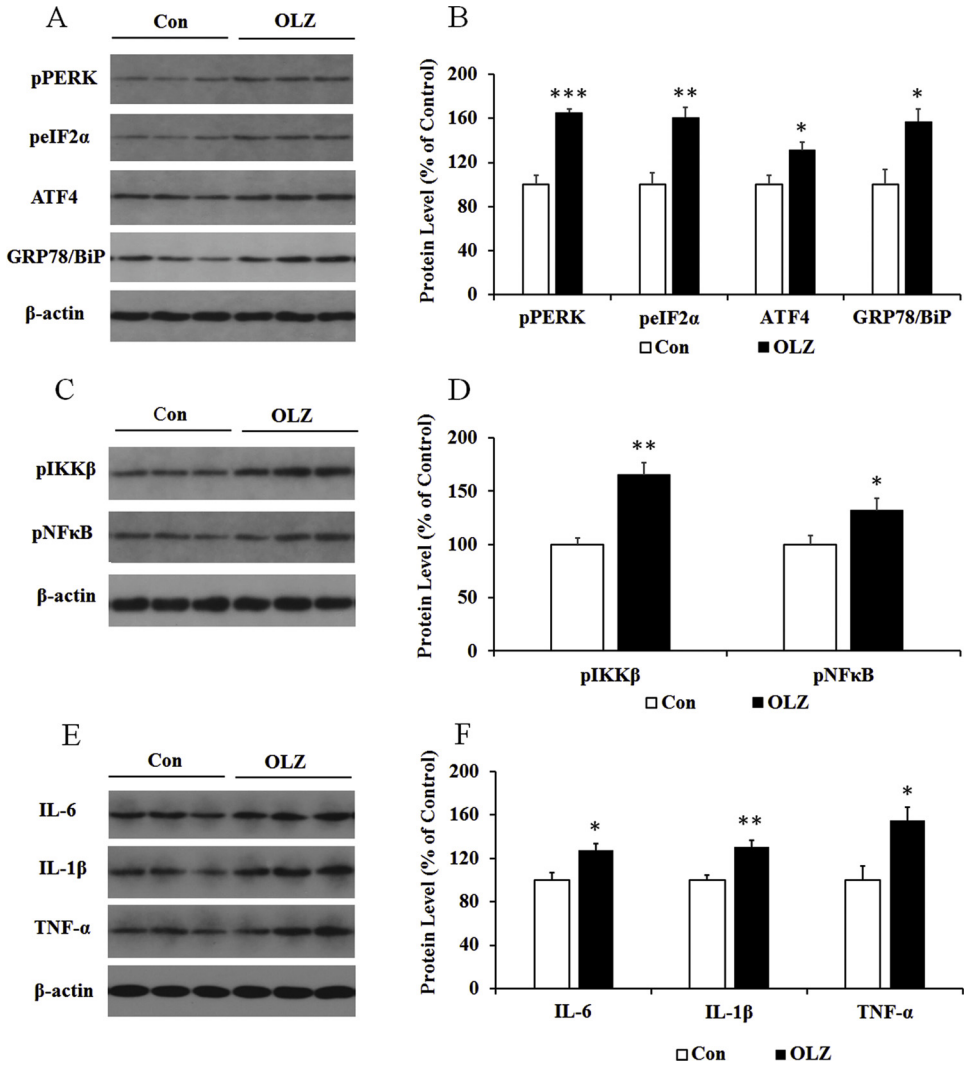

Fig. 4. Effects of 8-day olanzapine treatment on hypothalamic PERK-peIF2α signaling and IKKβ-NFκB signaling. (***A, C*** and ***E***) Representative Western blot and densitometry analysis of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α in the hypothalamus of the olanzapine or vehicle-treated rats. (***B, D*** and ***F***) Western blot analysis of protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α in the hypothalamus. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$vs. control). Con, control; OLZ, olanzapine.

cumulative food intake on day 2–5 (all $p < 0.05$), a trend of lower cumulative food intake on day 6 ($p = 0.052$), but no significantly decreased cumulative food intake on day 7–8 (all $p > 0.05$) compared with the olanzapine-only group. The 4-PBA low dose + olanzapine co-treated group had a significantly reduced cumulative food intake on day 2–3 ($p < 0.05$) but not on day 4–8 (all $p > 0.05$) compared with the olanzapine-only group. On the other hand, olanzapine-only treatment markedly increased weight gain on day 4–8 (all $p < 0.05$) compared with vehicle. 4-PBA high dose treatment significantly reduced olanzapine-induced weight gain on day 2–6 (all $p < 0.05$) and tended to decrease weight gain on day 7–8 ($p = 0.053$) compare with olanzapine-only treatment. 4-PBA low dose treatment significantly decreased olanzapine-induced weight gain only on day 2 ($p < 0.05$) but not on day 3–8 ($p > 0.05$) (Fig. 6B). The olanzapine-only group had a lower feeding efficiency than the control group ($p < 0.05$). 4-PBA high dose treatment tended to reduce olanzapine-induced increase in feeding efficiency compared with the olanzapine-only group ($p = 0.055$). 4-PBA high dose treatment in normal rats had no evident effect on food intake, weight gain and feeding efficiency (all $p > 0.05$) compared with vehicle (Fig. 6C). Pearson's correlation revealed that cumulative weight gain of all rats was significantly correlated with cumulative food intake ($r = 0.803$, $p = 0.000$) and feeding efficiency ($r = 0.967$, $p = 0.000$). These results indicated that 4-PBA treatment could inhibit olanzapine-induced food intake and weight gain in a dose- and time-dependent manner.

3.7. Olanzapine-induced activated hypothalamic PERK-eIF2α and IKKβ-NFκB signaling were inhibited by 4-PBA in a dose-dependent manner

Consistent with Animal Experiment 1, our data revealed a significant effect of olanzapine and 4-PBA treatments on pPERK ($F_{[4,19]} = 26.568$, $p = 0.000$, n = 4/group, Dunnett *post hoc* tests), peIF2α ($F_{[4,19]} = 48.288$, $p = 0.000$), ATF4 (($F_{[4,19]} = 50.912$, $p = 0.000$), GRP78/BiP ($F_{[4,19]} = 29.588$, $p = 0.000$), pIKKβ ($F_{[4,19]} = 17.735$, $p = 0.000$) and pNFκB ($F_{[4,19]} = 59.598$, $p = 0.000$). As shown in Fig. 7A-B, olanzapine markedly increased the expression of pPERK (increased by 132.0 ± 20.2% vs. vehicle (100%), $p = 0.000$), peIF2α (by 204.7 ± 17.6%, $p = 0.000$), ATF4 (by 144.0 ± 7.8%, $p = 0.000$) and GRP78/BiP (by 93.1 ± 11.4%, $p = 0.000$) compared with vehicle. The 4-PBA high dose treatment markedly inhibited olanzapine-induced increase in pPERK (reduced by 97.1 ± 16.2% compared to olanzapine-only group, $p = 0.000$), peIF2α (by 141.6 ± 11.6%, $p = 0.000$), ATF4 (by 113.7 ± 6.3%, $p = 0.000$) and GRP78/BiP (by 52.9 ± 5.9%, $p < 0.01$). The 4-PBA low dose

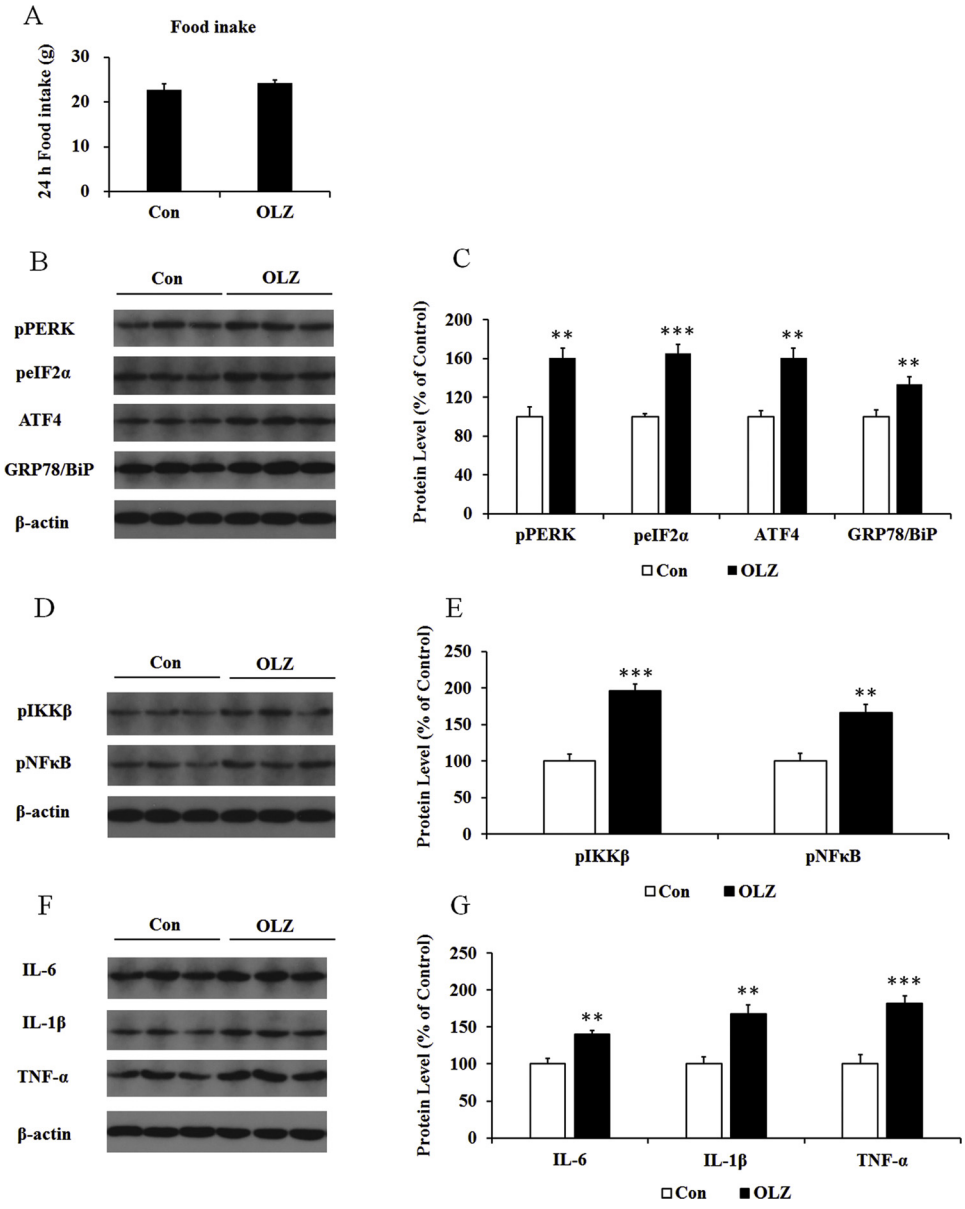

Fig. 5. Effects of 24 h olanzapine treatment on food intake and hypothalamic PERK-peIF2α signaling and IKKβ-NFκB signaling. (***A***) 24 h food intake of rats treated with olanzapine or vehicle. (***B, D*** and ***F***) Representative Western blot and densitometry analysis of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α in the hypothalamus of the olanzapine or vehicle-treated rats. (***C, E*** and ***G***) Western blot analysis of protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α in the hypothalamus. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (*$p <$ 0.05, **$p <$ 0.01, *** $p <$ 0.001vs. control). Con, control; OLZ, olanzapine.

treatment significantly suppressed olanzapine-induced increased expression of pPERK (reduced by 56.6 ± 3.6% compared to the olanzapine-only group, $p <$ 0.05), peIF2α (by 108.6 ± 10.7%, $p =$ 0.000), ATF4 (by 77.6 ± 17.0%, $p <$ 0.05) and GRP78/BiP (by 30.7 ± 8.9%, $p <$ 0.05). The 4-PBA high dose treatment in normal rats significantly reduced the protein expression of pPERK (by 24.4 ± 2.6% compared with vehicle, $p <$ 0.05) and ATF4 (by 34.0 ± 6.5%, $p <$ 0.01), but not peIF2α and GRP78/BiP (all $p >$ 0.05). In terms of IKKβ-NFκB signaling, olanzapine significantly increased the protein expression of pIKKβ (by 147.5 ± 20.6%, $p =$ 0.000) and pNFκB (by 154.2 ± 17.1%, $p =$ 0.000). These effects were suppressed by co-treatment of 4-PBA high dose (pIKKβ: reduced by 99.7 ± 19.6% compared to the olanzapine-only group, $p <$ 0.01; pNFκB: reduced by 111.1 ± 2.7%, $p =$ 0.000, respectively) and low dose (pIKKβ: reduced by 76.9 ± 17.6% compared to olanzapine-only group, $p <$ 0.01; pNFκB: reduced by 76.2 ± 7.9%, p = 0.000, respectively (Fig. 7C–D). 4-PBA high dose treatment in normal rats tended to reduce the protein expression of pIKKβ (reduced by 13.1 ± 6.0% compared with vehicle, $p =$ 0.07) and significantly reduced the protein expression of pNFκB (by 24.7 ± 7.2%, $p <$ 0.05). Moreover, hypothalamic pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ and pNFκB expression were positively correlated with cumulative weight gain and feeding efficiency (Table 1). These findings indicated that activated ER stress PERK- eIF2α signaling and inflammatory IKKβ-NFκB signaling in the hypothalamus could be inhibited by an ER stress inhibitor. The inhibition of PERK-eIF2α signaling and its related IKKβ-NFκB signaling appears to reduce olanzapine-induced weight gain. Our data also revealed significant effects of olanzapine and 4-PBA treatment on hypothalamic IL-6 ($F_{[4,19]} =$ 39.542, $p =$ 0.000), IL-1β ($F_{[4,19]} =$ 127.649, $p =$ 0.000) and TNF-α ($F_{[4,19]} =$ 33.673, $p =$ 0.000). Olanzapine treatment evidentially increased the protein expression of IL-6 (increased by 231.2 ± 26.4% vs. vehicle (100%), $p =$ 0.000), IL-1β (by 251.6 ± 8.4%, $p =$ 0.000), and TNF-α (by 140.0 ± 18.5%, $p =$ 0.000) in the hypothalamus (Fig. 7E-F). 4-PBA co-treatment dose-dependently inhibited the protein expression of IL-6 (high dose: reduced by 121.4 ± 5.6% vs. olanzapine, $p =$ 0.000; low dose: by 73.4 ± 11.3%, $p <$ 0.001), IL-1β (high dose: by 189 ± 13.5%, $p =$ 0.000; low dose: by 83.8 ± 7.1%, $p =$ 0.000), and TNF-α (high dose: by 99.1 ± 7.7%, $p =$ 0.000; low dose: by 58.4 ± 8.2%, $p <$ 0.001). These findings suggest that olanzapine treatment increased hypothalamic inflammatory cytokines via ER stress signaling.

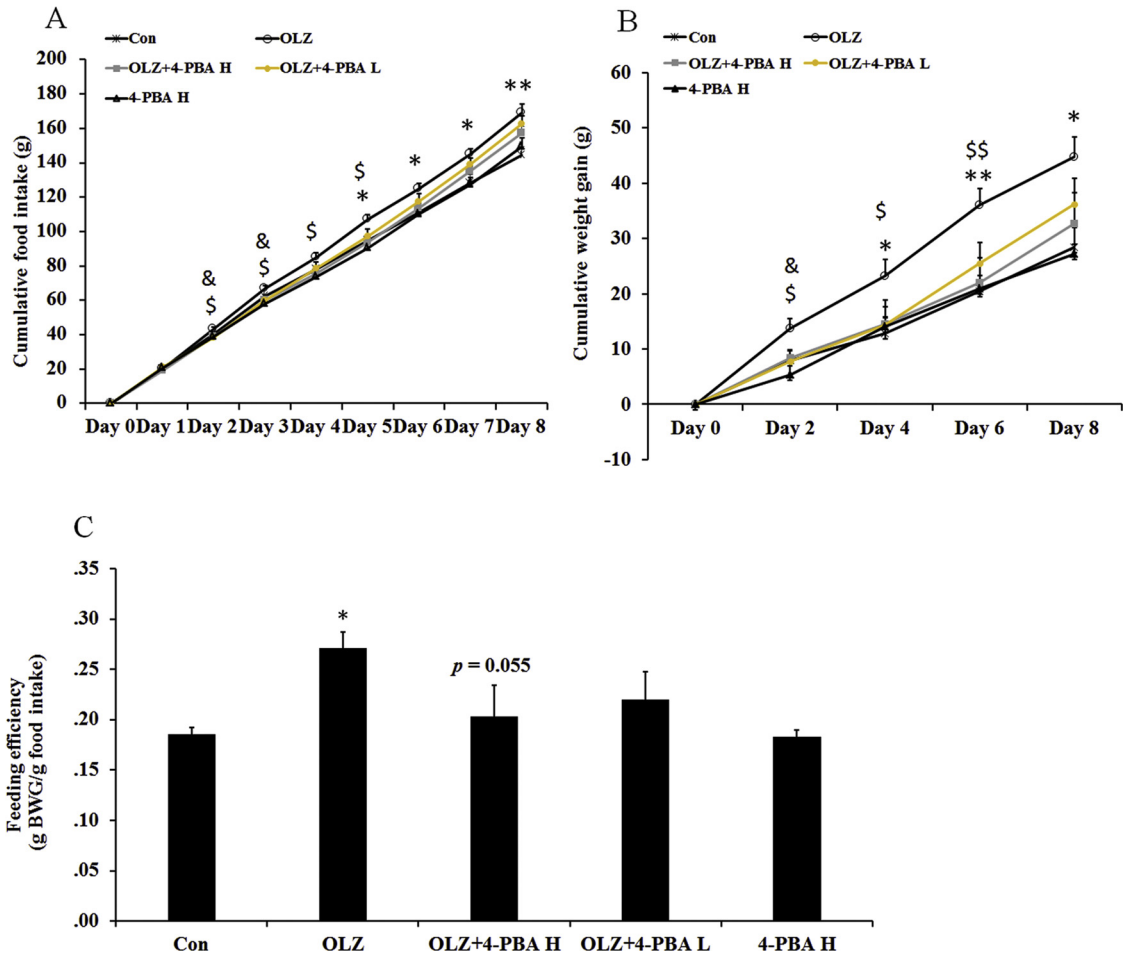

Fig. 6. Effects of an ER stress inhibitor, 4-PBA, on olanzapine induced increased food intake, weight gain and feeding efficiency (n = 8/group). (***A*** and ***B***) 4-PBA dose- and time-dependently inhibited olanzapine-induced hyperphagia and weight gain. (***C***) 4-PBA tended to decrease the feeding efficiency of olanzapine-treated rats. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, OLZ vs. Con; \$ $p < 0.05$, \$\$ $p < 0.01$, OLZ + 4-PBA H vs. OLZ; & $p < 0.05$, OlZ + 4-PBA L vs. OLZ). Con, control; OLZ, olanzapine; OLZ + 4-PBA H, olanzapine + 4-PBA high dose; OLZ + 4-PBA L, olanzapine + 4-PBA low dose. 4-PBA H, 4-PBA high dose.

4. Discussion

The present study firstly demonstrated that olanzapine induced aberrant activation of ER stress PERK-eIF2α signaling in human neuroblastoma SH-SY5Y cells, suggesting that olanzapine might directly act on the neurons to induce ER stress. The animal study provides the first evidence that sub-chronic olanzapine treatment activated the hypothalamic PERK-eIF2α signaling. The protein expression of pPERK positively correlated with food intake, weight gain and feeding efficiency. Previous studies indicated that activation of the hypothalamic ER stress PERK axis led to increased food intake and weight gain, while inhibition of the hypothalamic PERK axis reduced hyperphagia and weight gain in diet-induced obese mice (Zhang et al., 2008). These results indicated that hypothalamic ER stress may be largely related to olanzapine-induced obesity. Additionally, overfeeding has been proven to activate hypothalamic ER stress (Zhang et al., 2008). Our data reported that hypothalamic PERK-eIF2α signaling was activated after one-day olanzapine treatment, prior to hyperphagia onset. The evidence suggested that activation of the hypothalamic ER stress during olanzapine treatment may not be caused by hyperphagia. To further understand whether the hypothalamic ER stress was associated with olanzapine-induced weight gain, we examined whether an ER stress inhibitor could prevent olanzapine-treated rats from weight gain. Our study showed that the ER-stress inhibitor, 4-PBA, reduced olanzapine-induced weight gain, accompanied by reduced hypothalamic ER stress. The protein expression of pPERK, peIF2α, ATF4 and GRP78/BiP was positively correlated with weight gain. Therefore, it is possible that olanzapine activated the hypothalamic ER stress PERK- eIF2α signaling, leading to hyperphagia and weight gain during sub-chronic treatment. Inhibition of hypothalamic ER stress could be effective in preventing olanzapine-induced weight gain.

ER stress is an important mediator of hypothalamic IKKβ-NFκB signaling. Central or peripheral administration of 4-PBA or TUDCA have been reported to reduce HFD-induced increased NFκB expression both in the hypothalamus and adipose tissue (Chen et al., 2016; Martinez-Sanchez et al., 2017). The in vitro study revealed that olanzapine-induced activation of PERK-eIF2α signaling and IKKβ-NFκB signaling were inhibited by 4-PBA treatment. These effects suggested that olanzapine could directly mediate the inflammatory pathway via ER stress signaling. Moreover, our in vivo study demonstrated that olanzapine activated hypothalamic IKKβ-NFκB signaling, and this effect was inhibited by 4-PBA. pIKKβ and pNFκB expression was positively correlated with weight gain. These results suggested that activation of hypothalamic IKKβ-NFκB signaling by olanzapine was at least partly induced by ER stress. The hypothalamic IKKβ-NFκB signaling activation may be largely related to olanzapine-induced weight gain.

Moreover, we have found that olanzapine increased the protein expression of inflammatory factors including IL-6, IL-1β and TNF-α both in SH-SY5Y cells and in 8-day olanzapine-treated rats, suggesting that olanzapine treatment was related to central inflammation. This is

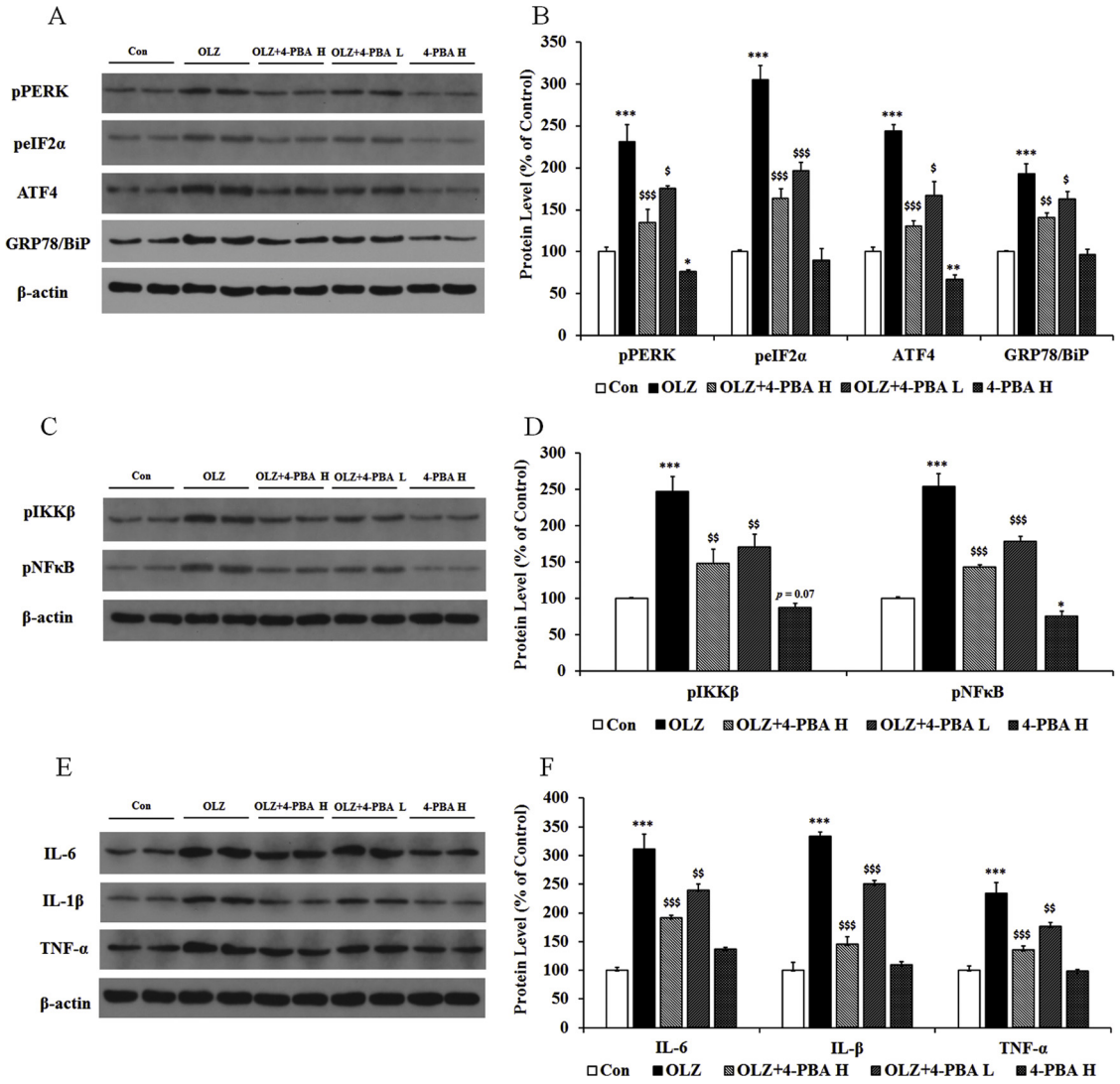

Fig. 7. Effects of 8-day olanzapine and 4-PBA treatment on hypothalamic PERK-peIF2α signaling and IKKβ-NFκB signaling. (***A, C*** and ***E***) Representative Western blot and densitometry analysis of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α in the hypothalamus of the olanzapine, vehicle or 4-PBA treated rats (***B, D*** and ***F***) Western blot analysis of protein expression of pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, IL-6, IL-1β and TNF-α in the hypothalamus of rats. All data are presented as mean ± SEM. Statistical significance was defined as $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ vs. Con; \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs. OLZ). Con, control; OLZ, olanzapine; OLZ + 4-PBA H, olanzapine + 4-PBA high dose; OLZ + 4-PBA L, olanzapine + 4-PBA low dose. 4-PBA H, 4-PBA high dose.

Table 1
Correlations between pPERK, peIF2α, ATF4, GRP78/BiP, pIKKβ, pNFκB, cumulative food intake, weight gain and feeding efficiency after 8-day olanzapine and 4-PBA treatment.

	pPERK r (P-value)	peIF2α r (P-value)	ATF4 r (P-value)	GRP78/BiP r (P-value)	pIKKβ r (P-value)	pNFκB r (P-value)
Cumulative food intake	0.451(0.069)	0.377(0.136)	0.324(0.205)	**0.506(0.038)**	0.469 (0.057)	0.306 (0.232)
Weight gain	**0.650(0.003)**	**0.590(0.008)**	**0.545(0.016)**	**0.679(0.001)**	**0.641 (0.003)**	**0.501(0.029)**
Feeding efficiency	**0.735(0.001)**	**0.715(0.001)**	**0.683(0.002)**	**0.754(0.000)**	**0.729 (0.001)**	**0.659(0.003)**

Statistical significance was defined as $p < 0.05$. Significant correlations are indicated in bold.

supported by our previous findings that the mRNA expression of IL-6, IL-1β and TNF-α in the rat hypothalamus was increased after different terms (8-, 16- and 36-day) of olanzapine treatment (Zhang et al., 2014a). Numerous studies have reported that hypothalamic IL-6, IL-1β and TNF-α levels were elevated during obesity development (Le Thuc et al., 2017; Ropelle et al., 2010; Wu et al., 2014). Recently, it has been suggested that hypothalamic inflammation is a unifying mechanism of obesity pathogenesis (Dorfman and Thaler, 2015; Jais and Bruning, 2017). Our acute study reported that one-day olanzapine treatment (rats were not hyperphagic and overweight) increased the protein expression of IL-6, IL-1β and TNF-α, suggesting that hypothalamic inflammation occurs as early as the 24 h treatment, prior to weight gain onset. Moreover, the increased inflammatory markers were decreased by an ER stress inhibitor, 4-PBA, both in vitro and in vivo, suggesting that olanzapine-induced increased IL-6, IL-1β and TNF-α expression were related to ER stress activation. Furthermore, activation of the hypothalamic inflammatory IKKβ-NFκB signaling results in the production of inflammatory cytokines including TNF-α, IL-1β, and IL-6 (De

Souza et al., 2005; Roth et al., 2016). Hypothalamic TNF-α, IL-1β, and IL-6 also mediate IKKβ -NFκB signaling in body weight regulation (Romanatto et al., 2007; Ropelle et al., 2009). The cause-effect relationship between IKKβ-NFκB signaling and increased inflammatory cytokines in antipsychotic induced weight gain should be investigated in further studies.

The mechanisms by which ER stress is activated by olanzapine both in vitro and in vivo is currently unknown. It has been reported that injection of adenoviruses encoding AMPKα-DN in the rat hypothalamus results in inhibition of the ER stress PERK axis in the ventromedial hypothalamus (VMH) (Martinez-Sanchez et al., 2017). In contrast, injection of adenoviruses encoding AMPKα-CA in the VMH of hyperthyroid rats significantly reversed the hyperthyroid-induced inhibition of the hypothalamic ER stress PERK axis, and these effects are involved in energy balance regulation (Martinez-Sanchez et al., 2017). Previous studies reported that olanzapine significantly induced AMPK activation in the rat hypothalamus via olanzapine's antagonistic effect on the histamine H1 receptor, and this effect contributes to olanzapine-induced weight gain (He et al., 2014; Lian et al., 2014; Liu et al., 2015). Therefore, olanzapine-induced activation of hypothalamic ER stress may be related to H1 receptor-AMPK signaling. Moreover, evidence has been reported that CB1 receptor antagonism could inhibit palmitic acid-induced activated ER stress in human renal proximal tubular cells, and this effect plays an essential role in the treatment of diabetic nephropathy (Lim et al., 2010). This evidence suggested that CB1 receptor signaling is also significantly related to ER stress. Chronic olanzapine treatment (15 days) decreased CB1 receptor binding density in the hypothalamus of rats (Weston-Green et al., 2012). CB1 receptor inverse agonists (rimonabant or NESS06SM) treatment attenuated olanzapine-induced weight gain (Lazzari et al., 2017). Therefore, the olanzapine-induced activated ER stress PERK axis may also be due to reduced CB1 receptor expression.

Recently, besides the cannabinoid (Lazzari et al., 2017), histaminergic (He et al., 2014) and ghrelin (Zhang et al., 2014b) system, serotonergic (Gressier et al., 2016; Lord et al., 2017) and opioidergic (Kurbanov et al., 2012; Sun et al., 2018) neurotransmission have been proven to significantly contribute to antipsychotic (in particular clozapine and olanzapine)-induced food intake and weight gain. Moreover, studies in rats reported that chronic olanzapine (1, 2, 5 and 11 weeks) or clozapine (3 weeks) treatment increased the expression of orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP), and reduced the expression of anorexigenic POMC in the hypothalamus, and these effects were related to olanzapine-induced hyperphagia (Martins et al., 2010; Rojczyk et al., 2015; Weston-Green et al., 2012; Zhang et al., 2014b). Interplay between hypothalamic NPY/AgRP neurons and POMC neurons is well known in feeding control (Morton and Schwartz, 2001; Waterson and Horvath, 2015). It is worth noting that inhibition of brain ER stress decreased NPY expression in rats (Castro et al., 2013). In AgRP neurons, GRP78/BiP immunoreactivity was activated by starvation, indicating the importance of ER stress in AgRP-related feeding regulation (Henry et al., 2015). Additionally, the ER stress pathway in POMC neurons regulates feeding and energy balance (Williams et al., 2014). Activation of hypothalamic ER stress blocked the post-translational processing of POMC by inhibiting alpha melanocyte stimulating hormone (α-MSH) production (Cakir et al., 2013). Our study revealed activation of hypothalamic ER stress PERK-eIF2α signaling by olanzapine treatment. It is possible that ER stress may be a potential regulatory mechanism of olanzapine-related changes in NPY, AgRP and POMC in the hypothalamus. However, further studies are required. Furthermore, olanzapine treatment for 14 days increased the mRNA expression of GAD65 (enzyme for γ-aminobutyric acid (GABA) synthesis) in the rat hypothalamus (Weston-Green et al., 2012). GABAergic neurons have been described to interact with NPY/AgRP neurons and inhibit feeding (Ito et al., 2013; Wu et al., 2009). A previous study reported that GABAB receptors and ATF4 are coclustered and suggested an interaction between the ATF4 and GABAergic neurons in mediating feeding (Maurin et al., 2014). Therefore, olanzapine-induced activation of ER stress signaling in the present study could also be related to reduced GABAergic signaling. Further studies that investigated the role of ER stress on hypothalamic expression of NPY, AgRP and POMC, and GABA release in olanzapine-induced obesity are warranted.

Our study showed that 4-PBA significantly reduced olanzapine-induced food intake from day 2–5, suggesting that 4-PBA mainly inhibits olanzapine-induced obesity by reducing food intake during short-term treatment. However, as treatment extended to 6–8 days, 4-PBA treatment no longer significantly reduced olanzapine-induced hyperphagia, but the weight gain of the 4-PBA + olanzpaine treated group was lower than that of the olanzapine-only group. The findings suggested that 4-PBA may also regulate pathways other than food intake to regulate body weight. A previous study reported that central administration of an ER stress inhibitor, TUDCA, or specific administration of adenoviral particles encoding GRP78 in the VMH significantly alleviated the HFD-induced increased hypothalamic ER stress, accompanied by decreased food intake, increased body temperature, increased BAT temperature and increased uncoupling protein 1 (UCP1) protein expression in BAT (Contreras et al., 2016). Pharmacologic activation of hypothalamic ER stress by ER stress activators, thapsigargin and tunicamycin, inhibited lipolysis, fatty acid oxidation and thermogenesis in brown adipocytes (Qiang et al., 2017). On the contrary, inhibition of ER stress by TUDCA ameliorated HFD-induced ER stress in BAT (Qiang et al., 2017). These findings indicated that activated hypothalamic ER stress regulated energy expenditure partly by mediating BAT thermogenesis. Previous studies also showed that acute treatment of obesogenic APs including olanzapine, clozapine and quetiapine regulate energy expenditure by suppressing sympathetic activity (Blessing et al., 2006; Monda et al., 2006; Stefanidis et al., 2009). Chronic treatment with olanzapine (16 and 36 days) decreased BAT thermogenesis and UCP-1 expression in BAT (Zhang et al., 2014c). Possibly, the activation of hypothalamic ER stress and IKKβ-NFκB signaling by olanzapine may also contribute to decreased energy expenditure, resulting in obesity. Further studies that investigate whether inhibition of hypothalamic ER stress reverses olanzapine-induced decrease in BAT thermogenesis and UCP-1 protein expression are warranted. Moreover, we could not exclude the possibility that olanzapine's effect on the hypothalamic ER stress PERK axis and IKKβ-NFκB signaling could be involved in other metabolic disorders induced by olanzapine. It has been reported that activation of the hypothalamic ER stress PERK axis and IKKβ-NFκB signaling leads to dysfunction of lepin (Ramirez and Claret, 2015) and insulin pathway (Melo et al., 2014), leading to leptin and insulin resistance. Numerous clinical studies and animal models have demonstrated that AP treatments (particularly olanzapine) induced hyperleptinemia and hyperinsulinemia (Ebenbichler et al., 2003; He et al., 2014; Ikegami et al., 2013; Weston-Green et al., 2018). These factors play a key role in diabetes during AP treatment (Chen et al., 2017). It is possible that the activated ER stress and IKKβ-NFκB signaling in the hypothalamus by olanzapine were associated with AP-associated diabetes. Our next study would investigate the role of hypothalamic ER stress and its related inflammatory signaling in AP-associated diabetes and hyperleptinemia.

In the present study, the dosages of olanzapine used for the in vitro experiment were chosen based on previous studies which reported that 0.2–160 μM olanzapine are not toxic and do not reduce the metabolic activity of SH-SH5Y cells (Heiser et al., 2007; Lee et al., 2010; Vucicevic et al., 2014). Olanzapine treatment of 50 and 100 μM have therapeutical effects against serum withdrawal-induced cell death in SH-SY5Y cells (Kim et al., 2008). In the clinic, the therapeutic serum level of olanzapine is about 0.1-0.64 μM, based on patients receiving 5–30 mg day^{-1} olanzapine treatment (Bergemann et al., 2004; Mauri et al., 2014; Olesen and Linnet, 1999; Rao et al., 2001). It has been reported that in post-mortem blood samples, average olanzapine concentration is 358 ng/L ranging up to 5200 ng/L (about 1.15 μM-16.2 μM) (Robertson and McMullin, 2000). Moreover, antipsychotics

accumulate in brain tissue at 20–30 fold higher than serum levels (Aravagiri et al., 1999; Kornhuber et al., 1999). Therefore, it is possible that olanzapine reaches levels in the brain sufficient to produce effects at 2 μM-20 μM in patients. However, it is worth noting that there is a known difference in the olanzapine dosages between studies in the neuronal cell line and clinic. Based on the literature, olanzapine dosages in most studies in the neuronal cell line ranged from 10 to 200 μM, in which 50–200 μM olanzapine treatments normally cause significant changes in signaling pathways (Kim et al., 2008; Lee et al., 2010; Vucicevic et al., 2014). We found that olanzapine 50 μM did not significantly induce activation of ER stress (p values were between 0.085-0.364 vs. control group), possibly because 50 μM olanzapine treatment in vitro failed to significantly activate AMPK (Takami et al., 2010), which is an activator of brain ER stress (Martinez-Sanchez et al., 2017). However, the treatment period may also be a reason for non-significant activation of ER stress by olanzapine since olanzapine leads to changes in different signal pathways in the neuronal cell line during a range of 2–72 h treatment (Lee et al., 2010; Vucicevic et al., 2014). In terms of the correlation between the dosages of olanzapine used in cells and in rats, no significant correlation was found in our study. In rats, we chose a dose of 3 mg kg^{-1} day^{-1} according to our previous research (He et al., 2014; Weston-Green et al., 2011b). It has been reported that olanzapine (3 mg kg^{-1} day^{-1}) treatment for 15 days induced a steady state serum concentration of 10 ng/mL (˜30 nM) and brain concentration of ˜180-300 nM olanzapine in rats (Aravagiri et al., 1999). In SH-SY5Y cells, the dosage of olanzapine was chosen since a range of 50–100 μM of olanzapine was commonly used in neuronal cell lines and is within the therapeutical dosage of olanzapine (Kim et al., 2008; Lee et al., 2010; Vucicevic et al., 2014). Moreover, 100 μM olanzapine treatment has been proven to prevent phencyclidine (commonly used in creating a model for schizophrenia)-induced reduction in neurite outgrowth in the primary cultured cortical neurons (Zhang et al., 2016). Therefore, the biologically effective dose of olanzapine may vary in different types of models.

In the clinic, patients treated with olanzapine are commonly associated with increased food consumption and changed feeding behaviours, leading to dramatic weight gain, as reviewed in (Benarroch et al., 2016). In rodents, olanzapine-induced changes in food intake show sex differences. As shown in (Benarroch et al., 2016), olanzapine-induced hyperphagia and weight gain are mainly observed in females but not in males. Therefore, the female rats were used in the present study to mimic the clinical situation of olanzapine-induced food intake and weight gain. Our study in female rats suggested that olanzapine activated the hypothalamic PERK-eIF2α signaling, and this effect may be related to olanzapine-induced hyperphagia and weight gain. However, in male rats, the effect of olanzapine on hypothalamic ER stress is currently unknown. It has been reported that acute central delivery of olanzapine activated hypothalamic AMPK in male rats, and this effect contributes to olanzapine-induced hepatic insulin resistance independent of weight gain (Martins et al., 2010). The ER stress is regulated by AMPK (Martinez-Sanchez et al., 2017). Therefore, in male rats, hypothalamic ER stress may be affected by olanzapine possibly via AMPK. Moreover, it has been reported that in male rats, olanzapine treatment significantly induced dysfunction of hepatic insulin signaling, including increased plasma insulin level and activation of the insulin receptor substrate (IRS)-phosphoinositide 3-kinase (PI3K) pathway (Ren et al., 2018), reduced IRS 2 expression, reduced phosphorylation of glycogen synthase kinase 3α (GSK3α) and increased GSK3β expression in the liver (Mondelli et al., 2013). Hypothalamic ER stress contributes to the development of insulin resistance (Cnop et al., 2012). It is possible that hypothalamic ER stress may play a role in olanzapine-related diabetes in male rats. Further studies are warranted to examine the effect of central olanzapine and 4-PBA treatment on hypothalamic ER stress in male rats, and its possible relationship with olanzapine-induced hepatic insulin signaling dysfunction.

5. Conclusions

In conclusion, our study revealed that olanzapine treatment markedly activated the hypothalamic ER stress PERK-eIF2α signaling and inflammatory IKKβ-NFκB signaling, and these effects were related to olanzapine-induced weight gain. Co-treatment with an ER stress inhibitor, 4-PBA, inhibited olanzapine-induced weight gain via suppressing PERK-eIF2α signaling and the related inflammatory IKKβ-NFκB signaling in the hypothalamus. This study may shed light on future possibilities for development of pharmacological solutions to reduce AP-induced metabolic disorders by focusing on hypothalamic ER stress and its related inflammatory signaling.

List of author contributions

Prof T Sun and X-F Huang designed this study. Drs M He, J Li, G Gao, Ms T Zhou, W Li, J Hu and J Chen performed and managed this project study. M He and J Li undertook the statistical analysis. M He wrote the draft of the manuscript. Drs Sun, Huang and He contributed in the discussion of interpretation of the results. All authors have contributed to and have approved the final manuscript.

Conflicts of interest

None.

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