ARTICLE
Neuroprotective effects of olanzapine against rotenone-induced toxicity in PC12 cells
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Olanzapine is an antipsychotic drug used to treat patients with schizophrenia due to its lower incidence of extrapyramidal symptoms. Previous studies have shown that olanzapine activates AMP-activated protein kinase (AMPK), and induce autophagy in SH-SY5Y cell line. In this study, we investigated whether olanzapine protected against rotenone-induced neurotoxicity in PC12 cells. We showed that treatment with olanzapine increased the phosphorylation of AMPK in both dose- and time-dependent manners in PC12 cells. In addition, olanzapine activated autophagy and increased autophagic vacuoles. Furthermore, olanzapine pretreatment could protect PC12 cells from rotenone-induced apoptosis. Besides, olanzapine pretreatment could suppress the rotenone-induced depolarization of mitochondrial potential and thus protect the cells. Moreover, pretreatment with specific AMPK inhibitor compound C or with autophagy inhibitor 3-methyladenine impaired the protective effect of olanzapine on rotenone-treated PC12 cells. In summary, our results show for the first time that olanzapine ameliorates rotenone-induced injury by activating autophagy through AMPK pathway.

Keywords: olanzapine; AMPK; autophagy; neuroprotection; rotenone; PC12 cells

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INTRODUCTION
The fat-soluble plant flavonoid rotenone is a well-known neurotoxic pesticide. It can pass through the blood–brain barrier, bind to complex I, impair mitochondrial function, mediate oxidative stress, induce inflammation, and ultimately lead to cell death [1, 2]. It has been reported that the mitochondrial dysfunction induced by rotenone is directly related to the inhibition of mitochondrial function in differentiated SH-SYSY cells [2]. In addition, rotenone can also inhibit autophagic flux, but its potential mechanism remains to be further studied. More importantly, identifying drugs that activate autophagic flux to prevent the neurotoxicity of rotenone may provide a promising clue for neuroprotection [3].

Olanzapine has been widely used to treat patients with schizophrenia due to its low incidence of extrapyramidal symptoms [4]. However, its use is hampered by side effects, such as obesogenic effects and associated metabolic effects [5–7]. The mechanisms underlying the adverse effects are the activation of the AMP-activated protein kinase (AMPK) [8–10] and autophagy-related mammalian target of rapamycin (mTOR) pathways [11]. As a major metabolic energy sensor, AMPK has been reported to induce autophagy by indirectly inactivating mTORC1 [12]. Olanzapine has been reported to induce autophagy in the SH-SYSY cell line, increasing autophagic flux, the number of autophagic vesicles, and the expression of autophagy-related genes (ATGs) [13]. In addition, olanzapine has been reported to inhibit the activity of nuclear factor kappa-B (NF-kB), a key factor in the inflammatory response and autophagy [14].

Autophagy is a basic homeostatic process that causes the degradation of damaged or misfolded proteins and organelles [15]. A decrease in autophagy leads to the accumulation of misfolded proteins in neurons [16] and results in early neonatal lethality [17, 18]. On the other hand, the induction of autophagy by the overexpression of the ATG beclin-1 or an autophagy inducer eventually attenuates neuronal pathology [19]. Taken together, these findings suggest that the enhancement of autophagy can activate protein degradation systems and accelerate the degradation of damaged mitochondria, thus alleviating nerve injury. Accordingly, autophagy induction may be a promising strategy for some disorders [20].

We aimed to verify the hypothesis that olanzapine initiates autophagy by activating AMPK, thus preventing neuronal injury induced by rotenone.

MATERIALS AND METHODS
Chemicals
Olanzapine was purchased from Selleckchem (Houston, Texas, USA). Rotenone, the AMPK inhibitor compound C, 6-(4-(2-piperidin-1-ylethoxy)phenyl)-3-pyridin-4-ylpyrazolo (1,5-a) pyrimidine and 3-methyladenine (3-MA, an autophagy inhibitor) were obtained from Sigma Chemical Company (Saint Louis, Missouri, USA). Olanzapine

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(200 mM), rotenone (10 mM), and the AMPK inhibitor compound C (10 mM) were separately prepared in dimethyl sulfoxide, and 10-μL aliquots were frozen at −80°C.

Cell culture and treatments
PC12 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and were routinely grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD, USA) and cultured at 37°C in a humidified 5% CO₂ atmosphere.

Immunoblotting assay
After the indicated treatment, the cells were harvested and lysed with ice-cold lysis buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration of the lysates was determined by a Protein Assay Kit (Thermo, Rockford, Illinois, USA), and equal amounts of protein were separated on a 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and transferred to a nitrocellulose membrane. Nonspecific protein binding was blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. The membranes were then incubated with specific AMPKα (1:10000), phospho-AMPKα (p-AMPK; Thr172; 1:1000), p62 (1:1000), poly-(ADP-ribose) polymerase (PARP), cleaved PARP (1:1000, Cell Signaling Technology, Massachusetts, USA), LC3 (1:1000), phospho-p70 S6 kinase (Thr389; 1:500, Abcam, Massachusetts, USA), cytochrome c (Cyt-c; 1:1000), Bcl-2 (1:1000), Bax (1:1000, Santa Cruz, State of California, USA), and β-actin (1:2000; MultiSciences, Hangzhou, China) primary antibodies overnight at 4°C. The membranes were washed the next day and incubated with secondary antibodies at room temperature for 1 h. After being washed with phosphate-buffered saline (PBS) three times, the membranes were incubated with secondary antibodies. The immunoblots were detected by the Odyssey Infrared Imaging System, and the data were quantified with ImageJ software.

Immunofluorescence staining
PC12 cells were seeded on coverslips. After specific treatments, the cells were fixed with methanol for 10 min at 4°C, washed with PBS and permeabilized with 0.3% Triton X-100 for 10 min. The cells were then incubated with blocking buffer (1% BSA and 0.1% Triton X-100) followed by a polyclonal anti-LC3 antibody overnight at 4°C. The cells were incubated with an Alex Fluor 488 (green)-conjugated rabbit antibody (1:500) for 1 h at room temperature after rinsing three times with PBS. Nuclei were counterstained with 4’,6-diamino-2-phenyl indole (DAPI) for 15 min at room temperature. Coverslips were mounted on slides with Vectashield mounting medium for fluorescence and analyzed by confocal microscopy (Carl Zeiss, Jena, Germany).

Transmission electron microscopy analysis
The cells were fixed with 3% glutaraldehyde for 1 h at 4°C. The cells were then washed and fixed again in aqueous 2% osmium tetroxide, dehydrated in ethanol, and embedded in epoxy medium. Ultrathin sections were cut and stained with uranyl acetate and lead citrate for examination under a transmission electron microscope.

Cell survival assays
Cell survival assays were performed using the Cell Counting Kit-8 (CCK-8; Beyotime, Haimen, China). Briefly, PC12 cells were seeded in 96-well plates at a density of 2 × 10⁴ cells per well and allowed to adhere overnight. After specific treatment, 10 μL CCK-8 solution was added to each well, and the plate was incubated at 37°C for 1 h. The optical density value of each well at 450 nm wavelength was measured using a microplate reader. The assay was repeated three times.

Hoechst 33258 staining
Treated cells grown in 24-well chamber slides were washed three times with PBS, fixed with methanol for 10 min at 4°C, and incubated with Hoechst 33258 (Beyotime, Haimen, China) for 10 min at room temperature. The nuclei were observed with a fluorescence microscope. Ten subfields from each slide were randomly selected to count and calculate the percentage of apoptotic cells in three independent experiments.

TUNEL assay
Treated cells were cultured on glass slides in a 12-well culture chamber, washed with PBS, fixed with methanol for 30 min, incubated with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) reaction mixture according to the manufacturer’s instructions (Beyotime, Haimen, China) in the dark (room temperature) for 60 min, washed with PBS three times, restained with DAPI, and washed, and apoptosis was observed by fluorescence microscopy.

RESULTS
Olanzapine activated AMPK and induced autophagy in PC12 cells
Olanzapine is usually administered at a starting dose of 5 mg/d (16 μM) and at a maintenance dose of 10 mg/d (32 μM). To investigate whether olanzapine activated AMPK, we treated PC12 cells with 0, 10, 20, or 40 μM olanzapine for 24 h. The expression of activated AMPK, as measured by the p-AMPK/total AMPK (p-AMPK/AMPK) ratio, increased in a dose- and time-dependent manner (Fig. 1a, b). Moreover, the peak p-AMPK/AMPK ratio was observed with 20 μM olanzapine and at 48 h. Therefore, olanzapine was administered 24 h before rotenone exposure in subsequent experiments.

Immunoblot assays revealed that the ratio of LC3-II/LC3-I was increased, while the protein level of p62 (the substrate of autophagy) increased, and the LC3-II/LC3-I ratio reached its peak 48 h after olanzapine treatment (Fig. 2a). The time course of p62 and p70-S6K signaling in the induction of autophagy by olanzapine. In addition, the LC3-II/LC3-I ratio increased in a dose- and time-dependent manner (Fig. 2b). The time course of p62 and p70-S6K expression was consistent with the changes in LC3-II. Immunostaining analysis of LC3 indicated that the treatment with olanzapine induced a distinct pattern of LC3 fluorescence in the cell cytoplasm (Fig. 2c). In addition, electron microscopy experiments showed that the number of autophagic vacuoles in cells treated with olanzapine was increased compared to that in untreated cells and that lysosomes were significantly activated in 96-well plates at a density of 2 × 10⁴ cells per well and allowed to adhere overnight. After specific treatment, 10 μL CCK-8 solution was added to each well, and the plate was incubated at 37°C for 1 h. The optical density value of each well at 450 nm wavelength was measured using a microplate reader. The assay was repeated three times.
Neuroprotective effects of olanzapine against rotenone-induced apoptosis in PC12 cells

We questioned whether olanzapine attenuated the damage to PC12 cells induced by rotenone. Rotenone has been reported to induce PC12 cell apoptosis, leading to cell damage [21]. Nuclear condensation and DNA fragmentation are hallmarks of cell apoptosis. To evaluate the protective effects of olanzapine on rotenone-induced nuclear condensation and DNA fragmentation, cells were stained with Hoechst 33258 and examined using microscopy. As illustrated in Fig. 3a, control PC12 cells had regular and round-shaped nuclei, while cells treated with rotenone for 24 h exhibited nuclear condensation and fragmentation, and were reduced in size. PC12 cells pretreated with olanzapine for 24 h exhibited many fewer condensed and fragmented nuclei than those treated with rotenone alone. Quantification analysis showed that the number of apoptotic rotenone-exposed cells was significantly increased by 35.42% compared with the number of apoptotic control cells. Olanzapine pretreatment, however, protected cells from rotenone toxicity by reducing the number of apoptotic cells to 14.79% (Fig. 3c). For further confirmation of rotenone-induced apoptosis, we also used a TUNEL mixture to stain PC12 cells. The results showed that red fluorescence was obvious in the rotenone group, indicating that rotenone-induced apoptosis, while olanzapine alleviated the apoptosis induced by rotenone (Fig. 2e).

Inhibition of AMPK or autophagy abolished the neuroprotective effect of olanzapine

We also investigated the relationship between AMPK activation and autophagy induction. PC12 cells were pretreated with 2.5 μM compound C, a potent AMPK inhibitor that has been widely used to study AMPK signaling. We found that compound C prevented the olanzapine-induced activation of AMPK and that this effect was accompanied by a decrease in LC3 and an increase in p62 (Fig. 4c). Correspondingly, pretreatment with olanzapine reduced mitochondrial damage induced by rotenone, indicating that olanzapine had a neuroprotective effect against rotenone-induced toxicity in PC12 cells.
Moreover, 3-MA (1 mM) was used to determine whether autophagy affected the neuroprotective effect of olanzapine in PC12 cells. As shown in Fig. 6c, the rotenone-reduced expression of Bcl-2 was alleviated by the presence of olanzapine, but this effect was abolished by 3-MA pretreatment. Meanwhile, after 3-MA pretreatment, Bax and Cyt-c expression was still increased by rotenone, even under olanzapine exposure. We also measured the effect of olanzapine on the rotenone-induced depolarization $\Delta \Psi_m$ with or without 3-MA. We found that 3-MA significantly counteracted the repolarization effect of olanzapine against rotenone (Fig. 6b).

**DISCUSSION**

In this study, we demonstrated the neuroprotective effects of olanzapine against rotenone-induced injury in PC12 cells. The

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**Fig. 2** Ola induced autophagy in PC12 cells. a LC3 conversion, p62 and p70-S6K levels in PC12 cells treated with Ola at 0, 10, 15, and 20 µM for 24 h or b at 20 µM for 0, 12, 24, and 48 h were assessed by immunoblotting. c The staining of LC3 was examined by laser scanning confocal microscopy in PC12 cells. d Representative electron micrograph of PC12 cells were shown in i and ii with Ola treatment (20 µM, 48 h). The arrowheads indicated the presence of autophagic vacuoles. At higher magnification, autophagosomes containing lysosomes were found in cells treated with Ola (iii-v). e PC12 cells were treated with 3-MA (1 mM) before Ola treatment, LC3 and p62 levels were evaluated by immunoblotting assay (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control; #$P < 0.05$, ###$P < 0.001$ compared with the Ola group).
Effect of olanzapine was evidenced by increased cell viability, decreased cell apoptosis, and downregulation of cleaved PARP. We found that the phosphorylation levels of Thr172 in the active site of the AMPK catalytic subunit (p-AMPK) were increased in a dose- and time-dependent manner after olanzapine treatment (Fig. 1). These results were consistent with previous reports that olanzapine activates AMPK [8, 9].

Previous studies have suggested that AMPK directly inactivates mTORC1 by phosphorylating Raptor [23] and indirectly by phosphorylating TSC2 [24, 25], ultimately resulting in the induction of autophagy. In addition, a recent study indicated that AMPK initiates autophagy by phosphorylating ULK1 [26, 27]. Activated AMPK can induce autophagy in different ways. In this study, we confirmed that olanzapine-induced autophagy in PC12 cells in a dose- and time-dependent manner. Moreover, the finding that AMPK decreased the protein level of p70-S6K (Fig. 2), a downstream target of mTOR, suggests that AMPK initiated autophagy via the inactivation of mTOR kinase signaling. In addition, upon pretreatment with the AMPK inhibitor compound C, the effect on autophagy induction was blocked (Fig. 4), while 3-MA pretreatment had no effect on p-AMPK levels (data not shown), indicating that olanzapine initiated autophagy by activating AMPK.

AMPK is a well-known cellular energy sensor that is also involved in many other metabolic functions and can be affected by many drugs, such as olanzapine. Studies have reported that AMPK is activated by the antipsychotic drug olanzapine in acute settings, and research has shown that olanzapine antagonizes several receptors, including histaminergic, muscarinic, serotonergic, dopaminergic, and adrenergic receptors. Studies have shown that olanzapine significantly upregulates the mRNA and protein expression of the H1 receptor and that olanzapine-induced AMPK activation may be at least partially related to the antagonistic effect of olanzapine on the H1 receptor. These results suggest that olanzapine activates AMPK by antagonizing histamine H1 receptors, dopamine D2 receptors, and α1-adrenoceptors [28–32].

It should be noted that our results showed that olanzapine activated the AMPK pathway and that AMPK inhibition diminished the neuroprotective effects of olanzapine, suggesting that AMPK activation was involved in neuroprotection by olanzapine. As mentioned above, AMPK activation is required for the induction of autophagy. As AMPK inhibition blocked autophagy induction, we speculate that autophagy is involved in the neuroprotective effects of olanzapine. However, this protective effect was diminished by the AMPK inhibitor compound C, suggesting that AMPK activation is a prerequisite for the neuroprotective effects of olanzapine.

Autophagy is regarded as a survival mechanism that occurs under stress conditions to promote cell survival induced by various stimuli. The activation of autophagy may play a cell protective role through a variety of intracellular events, including the elimination of dysfunctional mitochondria and other damaged proteins. Mitochondrial dysfunction not only leads to the

**Fig. 3** Ola alleviated the rotenone-induced cell death and apoptosis. a Cells were pretreated with 20 µM Ola for 24 h, exposed to rotenone for another 24 h, then subjected to Hoechst 33258 staining, and b TUNEL staining. c Quantitative analysis of morphological changes in different groups. d CCK-8 assay. e △Ψm analysis using JC-1 (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control; #P < 0.05 compared with the Rot group)
breakdown of biological energy that directly damages neurons but also leads to the excessive generation of reactive oxygen species, resulting in oxidative stress, which further destroys the cellular functions of mitochondria. Thus, the timely removal of dysfunctional mitochondria can protect cells from environmental toxicity. Many studies have shown that mitochondria appear to be the target of many environmental toxins, such as rotenone [33]. The inhibition of mitochondrial complex I may lead to respiratory depression and increased cellular oxidative stress. Therefore, the activation of autophagy by olanzapine may be beneficial for the clearance of damaged mitochondria and thereby contribute to mitochondrial function.
and cellular homeostasis. Evidence from other studies has shown that the autophagy inducers rapamycin [19] and resveratrol [34] can prevent toxin-induced neuronal injury through the induction of autophagy. The effect of olanzapine on mitochondrial autophagy (mitophagy) has not been reported. The olanzapine-induced autophagic clearance of dysfunctional mitochondria was recently confirmed by electron microscopy and the colocalization of autophagosomal-associated MAP1LC3B and mitochondria [13]. In our study, we investigated whether mitophagy is involved in the effects of olanzapine. We found that olanzapine also activated mitophagy in PC12 cells, as evidenced by increases in Pink1 and Parkin, which are associated with mitochondrial autophagy.

A recent study showed that a decrease in \( \Delta \Psi_m \) may change membrane permeability and induce further apoptosis [35]. Therefore, cell apoptosis can be observed by measuring \( \Delta \Psi_m \). Our results showed that compared to the control group, the rotenone treatment group showed strong depolarization of \( \Delta \Psi_m \). This phenomenon was positively correlated with the expression of the apoptosis protein Bax and negatively correlated with the expression of Bcl-2, which is well known to control mitochondrial apoptosis. Cyt-c, which is released into the cytoplasm during cell apoptosis, was also measured and was found to be induced by rotenone. Upon olanzapine pretreatment, we observed that the rotenone-induced depolarization of mitochondrial potential, and the expression of Bax and Cyt-c were repressed. Using the Hoechst 33258 assay, TUNEL assay, and CCK-8 assay, we also found that olanzapine protected PC12 cells from rotenone-induced cell apoptosis. The results indicated that olanzapine attenuated rotenone-induced apoptosis. Nevertheless, further animal and clinical experiments are required to verify whether autophagy is required for the neuroprotective effects of olanzapine.

In conclusion, the results obtained in our study show for the first time that olanzapine ameliorates rotenone-induced injury by activating the AMPK pathway and that autophagy, as a downstream event, may be involved in this effect.

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AUTHOR CONTRIBUTIONS
ZQL designed the research; YJX, YZS, YZ, WQZ, XS, YFZ, WJW, and YLL performed the research; JCW and ZQL contributed new reagents or analytic tools; YJX, YZS, and YZ analyzed the data; and YJX and YZ wrote the paper.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

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