

## Original Article

# The effects and mechanism of estrogen on rats with Parkinson's disease in different age groups

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**Abstract:** Objective: In order to investigate the effect and mechanism of estrogen in rotenone-induced Parkinson's disease (PD) rats in different age groups. Methods: we established rat models of PD by rotenone at different interventions. Then, behavioral tests, immunohistochemistry, western blot, high-performance liquid chromatography-electrochemical detector (HPLC-ECD) and electron microscopy were performed. Results: Results revealed the following: (1) Rotenone significantly reduced rotarod latencies in senile rats, prolonged their climbing pole time, and decreased TH positive cells, DA and its metabolite, DOPAC. Estrogen ameliorated this effect, in which weaker effects were observed in younger rats compared with older rats. (2) Rotenone increased the expression of LC3-II in older rats, but estrogen and tamoxifen did not show the same effect. (3) Rotenone increased the number of autophagosomes, but estrogen increased the proportion of autolysosomes/autophagosomes in the rotenone-treated group. (4) U0126 could reduce the number of autophagosomes in the rotenone-treated group, but this did not change the proportion of autolysosome/autophagosome in combining rotenone with the estrogen group. Rapamycin did not increase the number of autophagosomes in the rotenone-treated group, but combining rapamycin with estrogen and rotenone was able to further increase the proportion of autolysosome/autophagosomes. Therefore, we speculate that the senile rat model of PD was more reliable than that in young rats. Conclusions: In addition, estrogen could promote autophagy maturation through the ERK pathway, and had an obvious therapeutic effect on the rat model of PD.

**Keywords:** Estrogen, Parkinson's disease, age, autophagy

## Introduction

The etiology of Parkinson's disease (PD) is multifactorial, which include oxidative stress, mitochondrial dysfunction and protein aggregation; and all of these are closely linked to autophagy. Accumulating evidence has revealed that the dysregulation of autophagy underlies the pathophysiology of several neurodegenerative diseases, especially PD [1]. Hence, the regulation of autophagy has become a new target for treating neurodegenerative diseases. It is known that autophagy is regulated by the activation of the phosphatidylinositol 3-kinase (PI3K) pathway and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, which are two well-known estrogen-activated signaling cascades [2-5]. Our previous study has demonstrated that IL-6 promoted autophagy maturation with the activation of the ERK pathway [6]. This was

consistent with the result in Corcelle's study [7], providing evidence that MAPK/ERK activation regulates the maturation of autophagy. Recently, accumulating evidence has indicated that 17 $\beta$ -estradiol (E2) has been implicated to have a neuroprotective effect against a variety of neurodegenerative disorders [8]. Furthermore, estrogen is involved in the regulation of the MAPK/ERK pathway [9, 10]. Barbati's study has shown that estrogen triggers ERK phosphorylation, which regulates autophagy and promotes SHSY5Y cell survival [10]. However, the mechanism underlying these protective effects remain uninvestigated; particularly its role in PD animal models and the mechanism on how autophagy is regulated.

Rotenone is both an herbicide and an insecticide. Rotenone exposure in rodents provides an important model for studying the mechanism of toxin-induced dopaminergic neuronal injury

[11]. The administration of rotenone has been shown to affect a number of mechanisms involved in the pathogenesis of PD such as the systemic inhibition of mitochondrial complex I activity, induction of oxidative stress, altered calcium signaling, loss of tyrosine hydroxylase, proteosomal dysfunction and the formation of Lewy bodies. These result in the degeneration of the dopaminergic pathway in the substantia nigra and striatum, and reproduces behavioral and motor deficits as clinical PD [12-15]. Compared to other models, the rotenone rat model of PD would be more suitable for experimental studies [16]. In the present study, an intraperitoneal rotenone-induced rat model was established through different age groups of SD rats to investigate the neuroprotective effect of estrogen and the mechanism.

### Materials and methods

#### *Animals and different interventions*

All experimental procedures were approved by the Animal Care and Use Committee of Jiangsu University School of Medicine (Zhenjiang, China). Two-year-old male SD rats weighing 500 g ( $n = 60$ ) and 12-week-old SD rats weighing 250 g ( $n = 40$ ) were purchased from the Pool BK Experimental Animal Co. (Shanghai, China). Rats were pair-housed in an environmentally controlled facility (12/12-hour light/dark cycle, temperature at  $22 \pm 2^\circ\text{C}$  and relative humidity of  $50 \pm 5\%$ ) and were provided with food and water *ad libitum*. Forty of the two-year-old rats were randomly divided into four groups: control group ( $n = 10$ ), rotenone-treated group ( $n = 10$ ), estrogen-treated group ( $n = 10$ ) and tamoxifen-treated group ( $n = 10$ ); the same was carried out for the 12-week-old SD rats ( $n = 40$ ). Rats in the control group received 1.5 ml of NS for three days by intraperitoneal injection; rats in the rotenone-treated group received 1.5 ml of rotenone solution (2 mg/kg/day, Sigma) for three days by intraperitoneal injection; rats in the estrogen-treated group received 0.75 ml of rotenone solution (2 mg/kg/day) and 0.75 ml of estrogen solution (1 mg/kg/day, Sigma) for three days by intraperitoneal injection; and rats in tamoxifen-treated group received 0.5 ml of rotenone solution (2 mg/kg/day), 0.5 ml of estrogen (1 mg/kg/day) solution and 0.5 ml of tamoxifen solution (1 mg/kg/day, Sigma) for three days by intraperitoneal injection. The remaining two-year-old rats ( $n = 20$ ) were ran-

domly divided into four groups: U0126 control group ( $n = 5$ ), rapamycin control group ( $n = 5$ ), U0126-treated group ( $n = 5$ ), and rapamycin-treated group ( $n = 5$ ). Rats in the U0126 control group received 1.5 ml of U0126 solution (20  $\mu\text{g/kg/day}$ , Sigma) for three days by tail vein injection; rats in the U0126-treated group received 0.5 ml of rotenone solution (2 mg/kg/day) and 0.5 ml of estrogen (1 mg/kg/day) by intraperitoneal injection and 0.5 ml of U0126 solution (20  $\mu\text{g/kg/day}$ ) by tail vein injection for three days; rats in the rapamycin control group received 1.5 ml of rapamycin solution (3 mg/kg/day, Sigma) for three days by gastric perfusion; rats in the rapamycin-treated group received 0.5 ml of rotenone solution (2 mg/kg/day) and 0.5 ml of estrogen (1 mg/kg/day) by intraperitoneal injection and 0.5 ml of rapamycin solution (3 mg/kg/day) by tail vein injection for three days.

After eight days following the termination of treatment, all animals were sacrificed under anesthesia and directly decapitated. Rat mid-brains were acutely isolated on ice. All mid-brains were randomly divided into two groups. In one group, approximately 1-mm<sup>3</sup> sized brains that were isolated from the left side of the mid-brain were used for electron microscopic observation, while brains isolated from the right side were used for western blot assay. In the second group, the substantia nigra side was used for detection by immunohistochemical staining, and the striatum side was used for high-performance liquid chromatography (HPLC) analysis.

#### *Behavioral study*

In order to qualify specific Parkinsonism symptoms in rats, the rotarod test and climbing pole test were performed three days after termination of the treatment to determine behavioral changes. All behavioral tests were conducted in a quiet and well-lighted room that had a constant temperature and a fixed layout. Furthermore, these tests were secured by two investigators: one investigator was responsible for operating the instrument, and the other investigator was responsible for recording. (1) Rotarod test: Each rat was placed on a rotating rod (15 cm in diameter), and rotarod speed was eight cycles/minute. The stopwatch was started as the rat was placed on the rotating rod, and the time was noted as the rat dropped from the rotating rod (rotarod latency). This was

repeated three times for each animal at five-minute intervals, and results were averaged. Before the test, each rat underwent two adaptive trainings. (2) Climbing pole test: Before the test, each rat was guided to climb down from the peak of the staff (at a height of 50 cm) to the desktop twice. During the test, each rat was placed on the peak of the staff head down (the pole was wrapped with rope and sufficient friction was ensured). The stopwatch started as the rat was placed on the peak of the staff, and time noted as the rat climbed down to the sole plane (time to climb down pole). This was repeated three times for each animal at five-minute intervals, and results were averaged.

### *TH immunohistochemical examination*

Midbrains for immunohistochemical examination were dissected and post-fixed in 4% paraformaldehyde (PFA) + 2% sucrose for 4-6 hours. Then, these were immersed in 30% sucrose for 48 hours until the blocks sank to the bottom, embedded in OCT (Tissue-Tek, USA) at -25°C, cut into 5- $\mu$ m coronal sections using a microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany), and air-dried overnight. Prior to staining, the sections were placed at room temperature for 30 minutes and immersed in acetone at 4°C for 10 minutes. Then, the sections were incubated in 3% hydrogen peroxide for 30 minutes at room temperature to eliminate endogenous peroxidase activity. After antigen retrieval, the sections were treated with 0.5% Triton-X 100 for 30 minutes and 5% bovine serum albumin (Vector, USA) for 30 minutes to reduce nonspecific antibody binding. Next, the samples were incubated with rabbit anti-TH polyclonal antibody (1:1,000, Chemicon) at 4°C overnight and 37°C for 30 minutes. The sections were washed with PBS and incubated with anti-rabbit secondary antibody: peroxidase-conjugated goat anti-rabbit IgG (Vector, USA) for 30 minutes at room temperature, followed by incubation in a solution containing the avidin-biotin-peroxidase complex for 30 minutes at room temperature. The bound antibodies were visualized by placing the sections in a DAB solution at room temperature for 5-10 minutes. The reaction was stopped by washing with PBS. Finally, the sections were dehydrated, cleared and sealed for image capture. Images were captured with an inverted phase contrast microscope (Olympus Corporation, Japan).

### *Western blot analysis of TH and LC3*

Tissue samples were homogenized in lysis buffer (100 mg/1 ml; Betting Company, China) containing protease and phosphatase inhibitors. Lysates (10  $\mu$ g of protein) were separated using SDS-PAGE and electrophoretically blotted onto PVDF membranes (Millipore, Bedford, MA), which were blocked with 5% milk and incubated overnight at 4°C with the following primary antibodies: TH (anti-rabbit, 1:1,000; Chemicon), LC3 (anti-rabbit, 1:1,000; Sigma) and  $\beta$ -actin (anti-mouse, 1:1,000; Sigma). Subsequently, PVDF membranes were incubated for one hour at room temperature with an HRP-conjugated secondary antibody (1:10,000, Cell Signaling Technology). Signals were detected using a Kodak Professional Film Developer. Protein levels were normalized against  $\beta$ -actin levels and optical density of each band was quantified using "Image J" software.

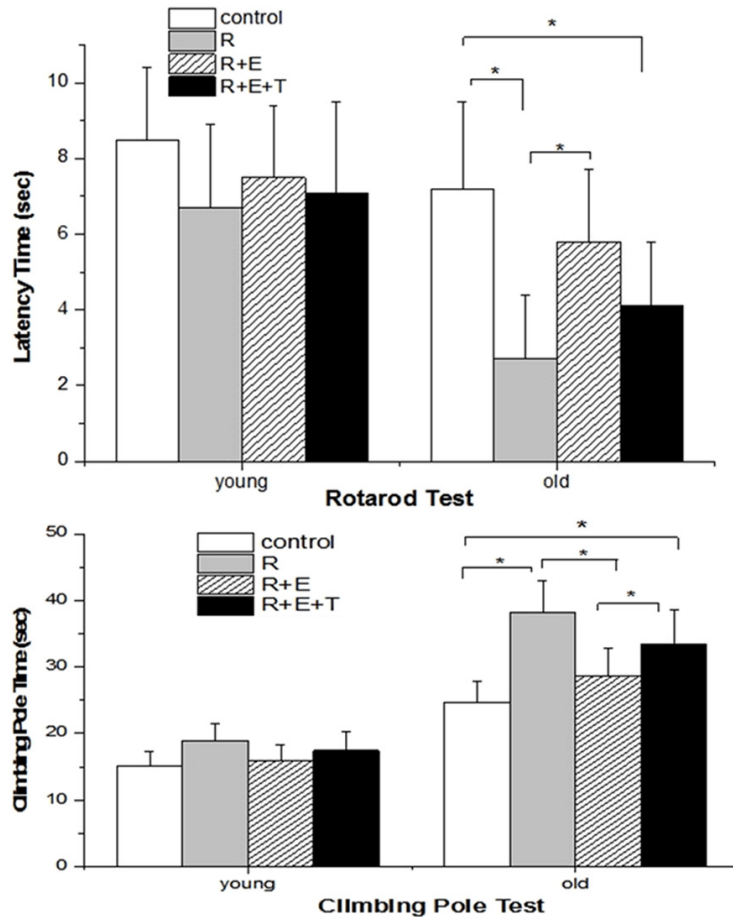
### *High-performance liquid chromatography (HPLC) analysis of monoamine neurotransmitter levels in the striatum*

The striatum was removed, cleaned and frozen in liquid nitrogen, and stored at -80°C until analysis. Tissues were sonicated in 0.1 M of perchloric acid containing 50 ng/ml of dihydroxybenzylamine as the internal standard. Next, the samples were centrifuged at 12,000 $\times$ g for 15 minutes at 4°C. Then, 20  $\mu$ g of the supernatant was injected into a PM-80 ternary gradient pump (BAS Inc., USA) to detect DA, DOPAC, HVA and 5-HT. The mobile phase consisted of 0.15 mol/l of chloroacetic acid, 2.2 mmol/l of EDTA, 7.5 mmol/l of camphorsulfonic acid and 10% methanol. Flow rate was 0.4 ml/min. Peaks were detected by a BAS LC-4C electrochemical detector. Data were collected and processed using the BAS Chrom Graph 115101 chromatographic analysis system.

### *Electron microscopy*

Tissue blocks for electron microscopy observation were immersed in glutaraldehyde solution at 4°C for three hours. After washing with PBS, tissue blocks were immersed in 1% osmium tetroxide solution for 1-2 hours, dehydrated in gradient concentrations of ethanol (70%, 80%, 90%, 95% and 100%), and immersed in acetone for 20 minutes. Then, the tissue blocks

## Effect of estrogen on PD's rats



**Figure 1.** Comparison of the behavioral performance in different experimental groups. The latency time and climbing pole time compared in all experimental groups were recorded respectively and compared to the first day in all experimental groups. Values are expressed as mean  $\pm$  standard error of the mean.  $p < 0.05$  was considered as significance level. \* $p < 0.05$  represents significant difference between the two groups. Control = the control group; R = the rotenone-treated group; R+E = the estrogen-treated group; R+E+T = the tamoxifen-treated group; young = the 12-week-old rats; old = the 2-year-old rats. (Grouping methods and statistics in **Figures 2-5** are consistent with **Figure 1**).

were embedded in resin at room temperature overnight, and cut into 50-nm sections using a LKB-1 microtome. The sections were stained with lead citrate for 15 minutes, and 20-25 micrographs (primary magnification, 10,000 $\times$ ) were randomly taken from each sample. Then, the volume of the autophagic vesicles was estimated by morphometry using the Visilog program (Leica TCS SP2 CLSM; School of Medicine Electron Microscopy Center, Fudan University).

### Statistical analysis

Statistical analysis was performed using the SAS 6.12 software. Data were presented as the mean  $\pm$  standard deviation (mean  $\pm$  SD). One-

way analysis of variance (ANOVA) followed by post hoc Bonferroni test were used for comparison between two groups of different ages and between groups of different interventions, respectively. Student's *t*-test and Mann-Whitney nonparametric test were used when two groups of data were compared.  $P < 0.05$  was considered statistically significant.

### Results

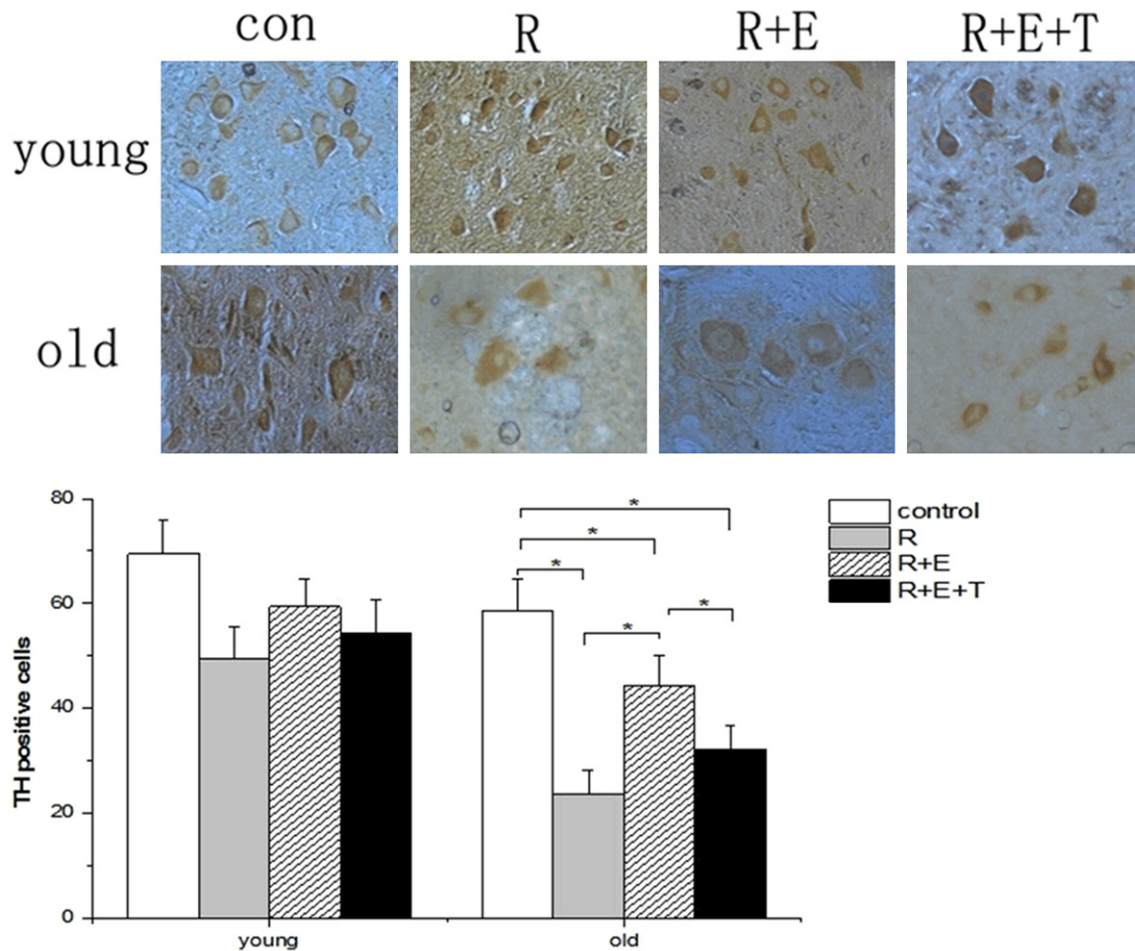
#### *Estrogen promotes behavioral improvement in older PD rats*

Repeated administration of rotenone (2 mg/kg) produced significant impairments of locomotor activity in rats. On the second day after rotenone administration, all rats had less action against capture. The whole body of these rats was gradually shaken and locomotor activity was reduced. Furthermore, these rats moved slowly, were drowsy, had straight fur and tail, and had a hunched posture; which are similar to Parkinson syndromes. With the chronic injection of rotenone, the decrease in locomotor activity was gradually serious. Older rats had muscle tremors and rigidity of the limbs, which are similar to PD symptoms. The fur of rats

gradually became yellow and dirty. Some of the older rats even appeared to have clonic convulsions, systemic muscle tension, creeping and shortness of breath. The young rats had no significant muscle tremor or convulsions, and gradual improvements in motor function occurred within three days following the termination of the rotenone treatment; exhibiting a significant increase in locomotor activity. Motor function in older rats further declined following the termination of rotenone treatment, but no significant improvement was observed at the end.

As shown in **Figure 1**, rotarod and climbing pole test results revealed a significant decrease in





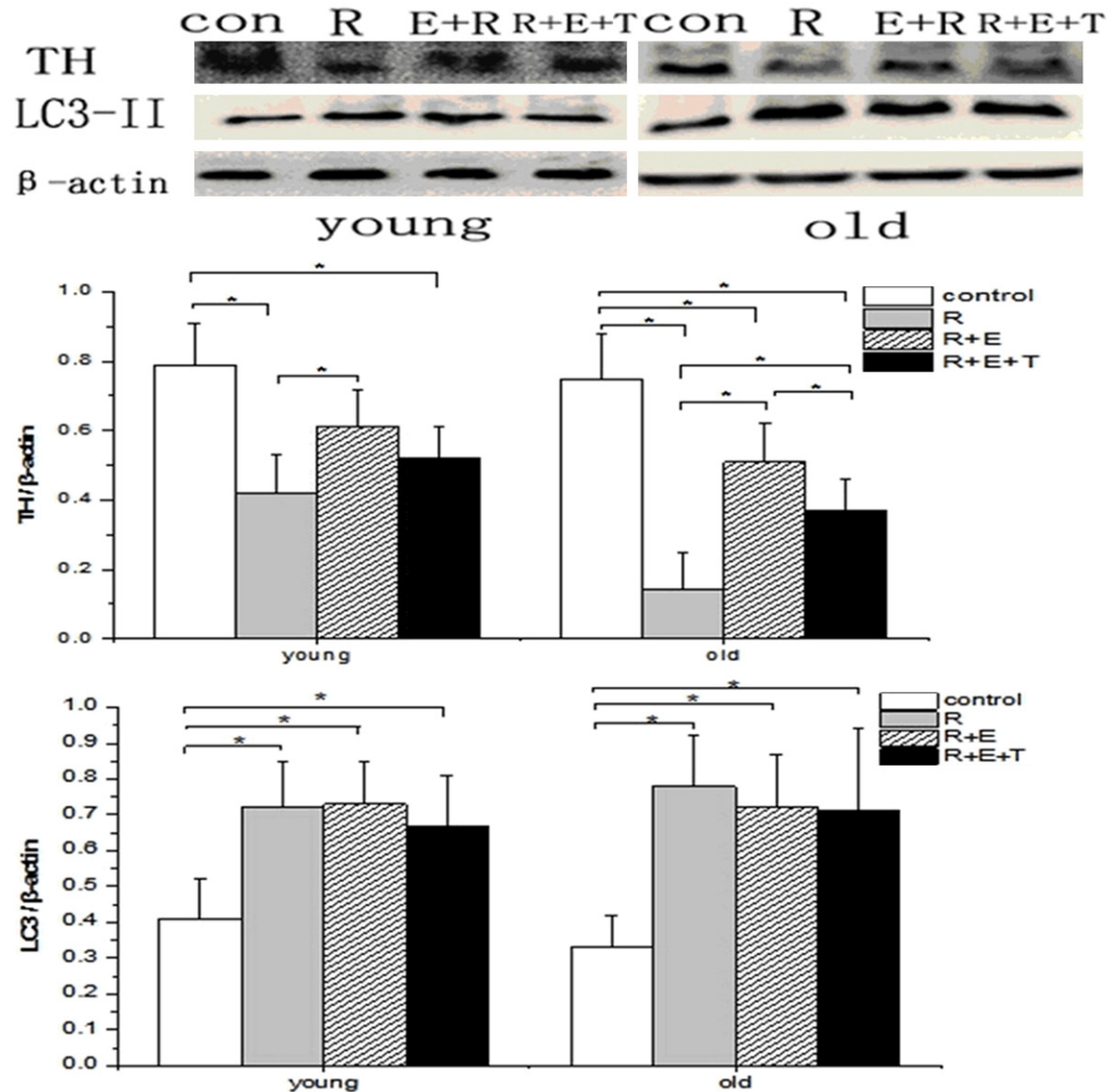
**Figure 2.** Effects of rotenone, estrogen, tamoxifen on the number of nigral TH-positive neurons in different experimental groups. TH-immunoreactive staining at ×400 magnification. Scale bar = 50 μm. Relative number of nigral TH-positive neurons in each group (n = 5 in each group). Ten homologous sections from each rat with the right side of the substantia nigra were investigated at ×400 magnification to determine the number of nigral TH-positive neurons.

performance in older rats in the rotenone-treated group, compared to control rats ( $P < 0.05$ ). Furthermore, estrogen reversed the decrease in performance in the rotarod and climbing pole tests (rotenone-treated vs. estrogen-treated,  $P < 0.05$ ); which means that estrogen prevented motor dysfunction in the rotenone-treated group. Tamoxifen could eliminate the effect of estrogen, but there was only a significant increase in the time to climb down pole of rats in the tamoxifen-treated group, compared with rats in the estrogen-treated group ( $P < 0.05$ ). However, weaker effects were observed in younger rats, compared with older rats. Furthermore, the rotarod test results revealed that there was no significant difference between younger and older rats, but the time to

climb down pole during the pole test was significantly longer in older rats compared to younger rats. Moreover, some of the older rats even fell down from the pole.

#### *Estrogen reduces dopaminergic neuronal loss in older PD rats*

PD is characterized by the loss of approximately 50-70% of dopaminergic neurons in the substantia nigra pars compacta. TH is a rate-limiting enzyme in the catalyzed synthesis of dopamine, and a transmitter and marker of dopaminergic neurons. The present study observed the effects of rotenone, estrogen and tamoxifen on the number of nigral TH-positive neurons in brains of rats in different ages. As



**Figure 3.** Comparison of TH and LC-3 expression in different experimental groups. The bands were normalized to beta-actin.

shown in **Figure 2**, the number of nigral TH-positive neurons was found to decrease in older rotenone-treated rats compared with control rats ( $P < 0.05$ ). Furthermore, estrogen reversed the decrease in the number of TH-positive neurons (rotenone-treated vs. estrogen-treated,  $P < 0.05$ ), which means that estrogen prevented neuronal loss in rats in the rotenone-treated group. Tamoxifen could eliminate the effect of estrogen ( $P < 0.05$ ). However, weaker effects were observed in younger rats compared with older rats, which mean the older rats are more sensitive to rotenone and suffer from more serious damage. In addition, estro-

gen greatly induced this protective effect in older rats, while tamoxifen eliminates the effect of estrogen.

As shown in **Figure 3**, the expression of TH decreased in older rats in the rotenone-treated group compared to the control group ( $P < 0.05$ ). Furthermore, estrogen reversed the decrease in expression of TH (rotenone-treated vs. estrogen-treated,  $P < 0.05$ ); which means that estrogen prevented neuronal loss in the rotenone-treated group, while tamoxifen could eliminate the effect of estrogen ( $P < 0.05$ ). However, weaker effects were observed in younger rats

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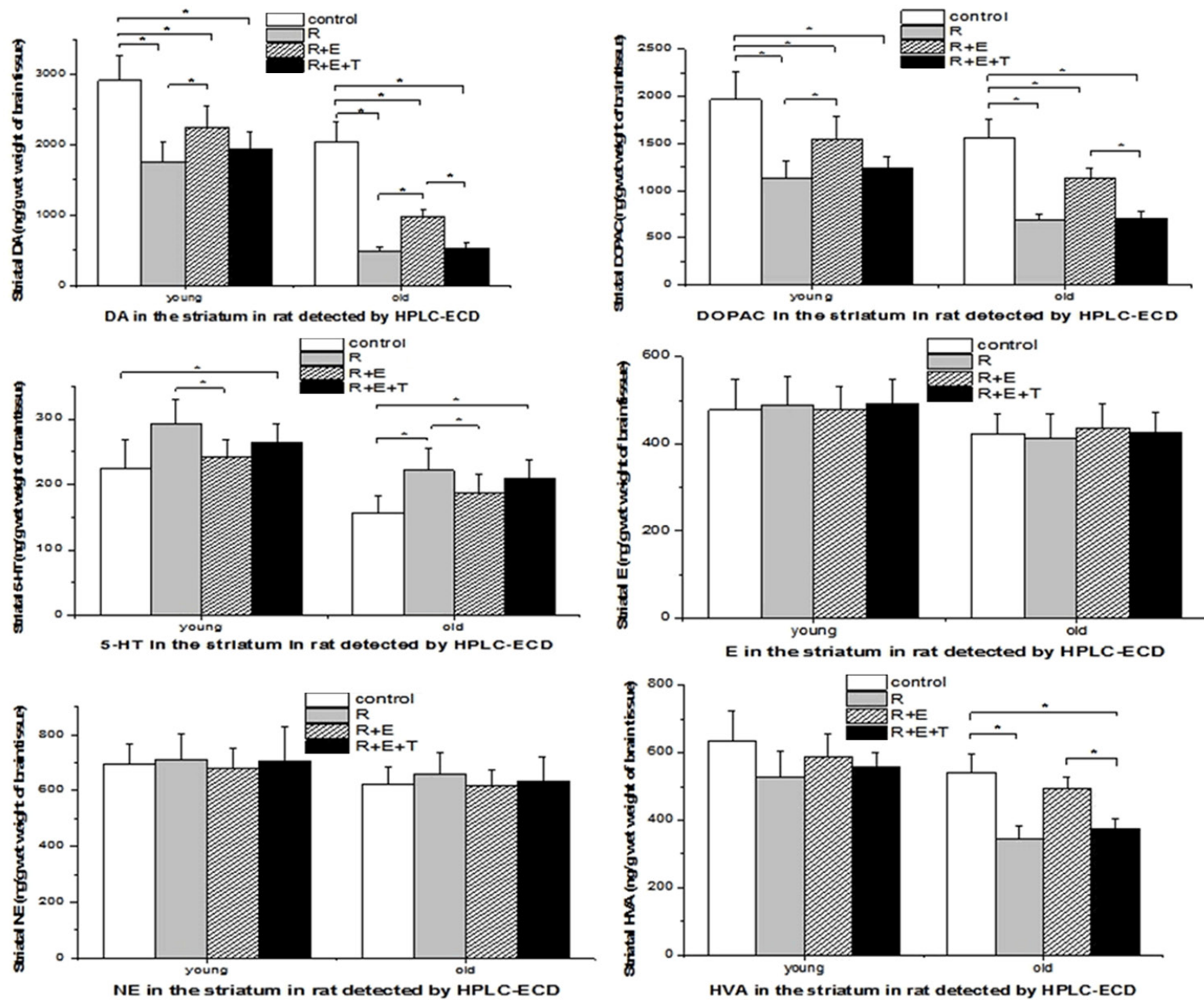
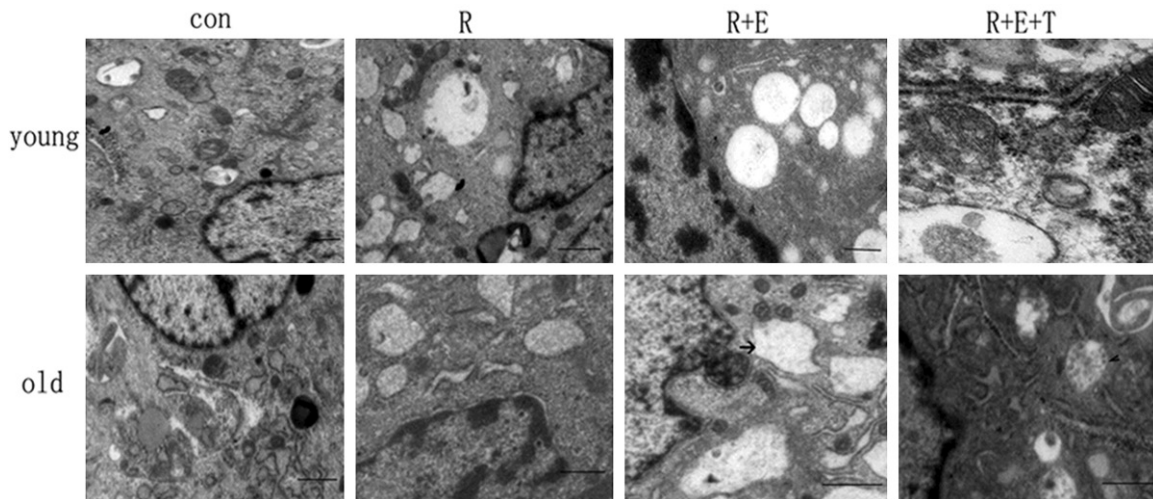


Figure 4. Comparison of monoamine neurotransmitter levels of striatum in different experimental groups using HPLC with electrochemical detection.





**Figure 5.** Effect of rotenone and estrogen on autophagy in the ventral neurons. Rotenone increased the number of autophagosomes, and estrogen increased the the proportion of autolysosomes/autophagosomes.

than in older rats, which suggest that older rats are more sensitive to rotenone and suffer from more serious damage. Estrogen reversed the effect of rotenone, and tamoxifen could eliminate the effect of estrogen. In addition, the expression of LC3 increased in the rotenone control group. However, estrogen did not affect the expression of LC3; and this is similar to tamoxifen.

#### *Estrogen regulated monoamine neurotransmitters in older PD rats*

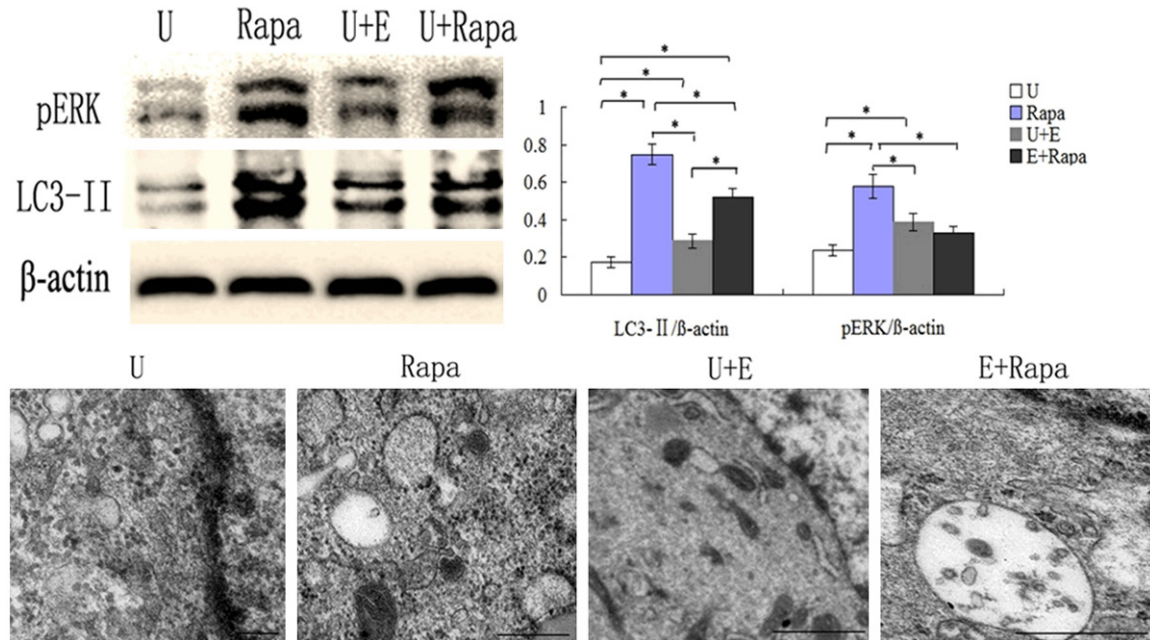
The detection of monoamine neurotransmitter levels in the substantia nigrostriatal system is an important biochemical indicator to assess DA metabolism, which are associated with motor dysfunctions of PD. Thus, we measured the monoamine neurotransmitter levels of the striatum. Results in **Figure 4** reveal that DA and its metabolite, DOPAC, significantly decreased, and 5-HT was elevated in the rotenone-treated group, compared to the control group ( $P < 0.05$ ). Estrogen downregulated this influence ( $P < 0.05$ ), and tamoxifen eliminated the effect of estrogen ( $P < 0.05$ ). The older group of rats revealed more obvious changes than in the younger group of rats. However, other monoamines did not significantly change. In addition, basic monoamine neurotransmitter levels in the striatum of older rats were lower compared to younger rats; which means that neurotransmitter levels declined with age or older rats have lower metabolism. These results show that older rats are more sensitive to rotenone

and suffer from more serious damage, while estrogen induces more protective effects on older rats.

#### *Estrogen promoted maturation of autophagy in older PD rats*

Our results in **Figure 5** reveal that younger rats had neuronal nuclear membrane integrity, normal chromatin structure, and normal cytoplasmic distribution of mitochondrial morphology. There were no autophagosomes and lysosomes in the ventral midbrain. After rotenone administration, the chromatin edge was set, autophagosomes were formatted, and the number of lysosomes increased. Few mitochondria swelled and were deformatted. Moreover, mitochondrial cristae structures were complete. Estrogen increased the number of autolysosomes and the proportion of autolysosomes/autophagosomes, but the difference was not statistically significant. In the ventral midbrain of older rats, neuronal nuclear membrane integrity was observed, chromatin margination was set, cytoplasmic mitochondria morphology was normal, and there were a smaller number of lysosomes, compared with younger rats. After rotenone administration, the number of autophagosomes and lysosomes rapidly increased, but few autolysosomes were observed. Furthermore, mitochondria swelled and were deformatted, and the majority of mitochondrial crest structures were fuzzy and caviated. After estrogen treatment, there were fewer autophagosomes, and the number of





**Figure 6.** Effect of estrogen on both autophagy maturation and the ERK single pathway in rotenone-treated groups. U0126 could reduce the number of autophagosomes in rotenone treated group. Estrogen could reduce the number of autophagosomes and increased the proportion of autolysosomes/autophagosomes in rotenone-treated group by the ERK single pathway. U: U0126-added group; Rapa: rapamycin-added group; U+E: U0126 and estrogen-added group; U+Rapa: estrogen and rapamycin-added group.

autolysosomes and the proportion of autolysosomes/autophagosomes significantly increased; and this effect could be weakened by tamoxifen. It was suggested that the number of autophagosomes increased in the rotenone-treated group. However, the number of autolysosomes was significantly decreased, and mitochondria were damaged. After estrogen administration, the number of autophagosomes decreased, but estrogen increased the proportion of autolysosomes/autophagosomes. Moreover, mitochondrial morphology became normal. Tamoxifen could eliminate the effect of estrogen. However, these effects were weaker in younger rats than in older rats.

#### *E2 modulates autophagic maturation by the ERK signaling pathway*

In older rats, rotenone increased the LC3-II shift. Estrogen or U0126 had no effect on the expression of LC3-II, but estrogen could downregulate the expression of LC3-II in rats in the rotenone-treated group. Furthermore, U0126 could downregulate the expression of LC3-II in the rotenone-treated group. Rotenone induced the sustained activation of ERK1/2 and the abrogation of this activation by pretreatment with a specific MEK1/2 inhibitor; and U0126

(10 mmol/L) significantly decreased rotenone-induced vacuolation. Estrogen had no effect on the activation of ERK1/2. Rotenone increased the number of autophagosomes, and estrogen had no effect on the number of autophagosomes in the control group; but estrogen could reduce the number of autophagosomes and increase the proportion of autolysosomes/autophagosomes in the rotenone-treated group. U0126 could reduce the number of autophagosomes in the rotenone-treated group, but cannot change the rate of autolysosome/autophagosome in the rotenone-treated group. Combining U0126 with estrogen only reduced the number of vacuolation, but this did not increase the rate of autolysosome/autophagosome. Rapamycin increased the number of vacuolation and the proportion of autolysosome/autophagosome in all rotenone-administrated groups, and combining rapamycin with U0126 did not downregulate the proportion of autolysosome/autophagosome (Figure 6).

#### **Discussion**

PD is a chronic, progressive neurodegenerative disorder with increased prevalence in the aging population. The incidence of common move-

ment disorder' mild parkinsonian signs' (MPS) is approximately 50% over the age of 85 years [17]. In PD patients, the death of dopaminergic neurons in the substantia nigra has been considered to be associated with the accumulation of misfolded proteins, the delayed accumulation of abnormal proteins and neurotoxicity, and the decrease in autophagy [18, 19]. Autophagy, a lysosomal pathway for degrading organelles and long-lived proteins, is essential for the maintenance of cellular homeostasis; and can preclude death in stressed or diseased cells. Compelling evidence suggests that the dysregulation of autophagy results in the accumulation of abnormal proteins and/or damaged organelles, which is commonly observed in neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and PD [20]. Therefore, targeting autophagy has been proposed as a strategy for treating PD. Age-dependent alterations in the lysosomal system that leads autophagic activity to decline was responsible for the accumulation of damaged cellular components in neurodegenerative diseases [21-23], and all of these show a close association between aging and PD. Recently, increasing evidence have suggested that estradiol exerts neuroprotective effects in a variety of neurodegenerative disorders including stroke, Alzheimer's disease and PD. However, the mechanism underlying these protective effects remains uninvestigated, especially its role in animal models of PD. In the present study, an intraperitoneal rotenone-induced rat model was established using different ages of SD rats, in order to investigate the neuroprotective effect of estrogen and its mechanism. The intraperitoneal injection of rotenone was selected, because this type of administration can decrease mortality compared with other systemic injection methods and gradually resulted in movement disorders [24, 25].

Results of the behavioral tests indicate that rotenone induced motor impairments, which means that a model of PD has been successfully developed. Twelve-week-old groups had weaker effects than the two-year-old groups, showing that the motor function of rats declined with age. Furthermore, dyskinesia in the 12-week-old groups improved within a week, which was different from the two-year-old groups. After partial dopaminergic neuronal loss, the activity of residual tyrosine hydroxylase in-

creased to compensate for the lack of neurotransmitters in DA neurons and the like; and in order to meet the needs of the DA system for motor regulation. The decrease in the compensatory ability of older rats may have multiple causes. On one hand, exogenous toxins may cause more serious damage in the nervous system of older animals, leading to more DA neuronal death; and the remaining neurons were few with inadequate compensatory ability. On other hand, older animals have less nigra DA neurons; and the scope of functional compensation has been reduced to exhibit compensatory defects. Estrogen could improve motor function. However, the protective effect of estrogen on low age rats was weaker than in older rats. Probably, the basis level of estrogen in younger rats is higher than that in older rats; and the same dose of estrogen has more effective in older rats.

Detecting monoamine neurotransmitter levels in the substantia nigrostriatal system is an important biochemical indicator to assess DA metabolism. Monoamine neurotransmitters include catecholamines (CA) and neurotransmitter serotonin (5-HT). CA includes neurotransmitter norepinephrine (NE), epinephrine (E) and dopamine (DA). Since DA metabolites include 3, 4-bis-hydroxy metabolite acid (DOPAC) and homovanillic acid (HVA) in neurons, the determination of DOPAC and HVA levels in the brain or cerebrospinal fluid would be useful to assess DA neuronal activity. HPLC analysis results on dopamine levels of the striatum revealed that DA and its metabolite DOPAC was significantly decreased in the rotenone-treated groups compared to the control groups. This may indicate the loss of dopaminergic neurons in the rotenone-treated groups. Estrogen could prevent the decrease in dopamine levels in the striatum. However, does this change in the level of neurotransmitters indicate its therapeutic effect on DA neurons? The answer is probably no. A similar result was also found in the report of Steyn S [26]. In order to further explore the pathological basis of behavioral variation, we detected the number of dopaminergic neurons in the substantia nigra using the quantitative morphological method. After rotenone administration, the number of TH positive neurons in the substantia nigra decreased, especially in 24-month-old rats. In this study, motor function declined with age; and TH-positive cells in the

substantia nigra also exhibited this decrease with age. Chu also found that TH-positive cells were lost with age in monkeys and humans, which is consistent with the results of our study [27]. Generally, the number of TH-positive neurons in the substantia nigra can reflect the levels of dopaminergic neurons. The serious loss of TH-positive cells in the substantia nigra and behavioral changes in older rats revealed that the same dose of rotenone caused more obvious neurotoxic effects and behavioral changes in aging subjects [28-30].

The enhanced sensitivity to rotenone in older rats may be related with the change in mitochondrial Complex I with age and the durative increase of oxidative stress. Rotenone is able to cross the blood-brain-barrier due to its hydrophobic nature, leading to systemic mitochondrial complex I inhibition. Nigrostriatal DA neurons have a specific sensitivity to rotenone, which is stronger compared with its surrounding tissues [31]. Age-associated changes in blood-brain-barrier permeability [32] could add to the enhanced sensitivity of rotenone in older rats. The function of mitochondrial complex I declines with age, and the already decreased activity of aged mitochondria is likely to be easily inhibited by the low dose of rotenone [33-35]. In addition, susceptibility to exogenous stressors (including neurotoxins) in aging individuals may be associated with L-type  $\text{Ca}^{2+}$  channels [36]. DA neurons have a high dependency on L-type Cav 1.3  $\text{Ca}^{2+}$  channels. When contacting exogenous stressors, young individuals can adjust the L-type Cav 1.3  $\text{Ca}^{2+}$  channels to maintain DA neuronal survival. On the contrary, aging individuals that lose this adjustment are susceptible to exogenous stressors, leading to neuronal degeneration and necrosis. Additionally, both rotenone and aging exacerbate oxidative stress [37, 38]. Combining these stressors may increase mitochondrial impairment and reactive oxygen species production, facilitating the degenerative process. All of these reasons caused older rats to be more sensitive to rotenone, and their model is more similar to human PD.

After estrogen administration, the rotenone effect was ameliorated. In this study, estrogen improved motor function and partially restored TH-positive cells loss; which means that this may have a neuroprotective effect against rotenone-induced toxicity in the rotenone-induced

rat model. Recently, accumulating evidence has indicated that estrogen can regulate autophagy by the MAPK/ERK pathway, leading to cell survival *in vitro*. In order to investigate the effect of estrogen on autophagy *in vivo*, electron microscopy was used to detect ultrastructural changes in ventral neurons. By electron microscopy analyses, we identified that rotenone increased the number of autophagosomes, but estrogen increased the proportion of autolysosomes/autophagosomes in the rotenone-treated group. Autophagy is a dynamic process, which begins with the formation of the double-membrane autophagosome that engulfs part of the cytoplasm [39, 40]. Subsequently, the outer membrane of the autophagosome fuses with late endosomes and lysosomes to become an autolysosome, where the content is finally degraded for the synthesis of new molecules and organelles. It is known that autophagy is regulated by the activation of the PI3K pathway and MAPK/ERK pathway, which are two well-known estrogen-activated signaling cascades [2-5]. Furthermore, estrogen is involved in the regulation of the MAPK/ERK pathway [9, 10]. Estrogen can regulate autophagy through the MAPK/ERK pathway, leading to cell survival. However, the mechanism on how autophagy is regulated remains uninvestigated. Our previous report provides evidence that the activation of MAPK/ERK regulates the maturation of autophagosomes [6], which is consistent with the result in the study of Corcelle [7]. In this study, rotenone increased the number of autophagosomes. This means that the initial step of the autophagic pathway did take place. However, there were few autolysosomes in the rotenone-treated groups; which means that the late step of autophagic maturation (i.e. fusion of autophagosomes with lysosomes) was disturbed. After estrogen administration, the number of autophagosomes decreased, but the number of autolysosomes and the proportion of autolysosomes/autophagosomes increased. These results reveal that estrogen improved the fusion of autophagosomes with lysosomes to become autolysosomes. In a word, estrogen induced autophagy maturation. With U0126, the ability of estrogen to increase the autolysosomes/autophagosomes ratio disappeared, which suggests that estrogen can promote the maturation of autophagy by regulating the ERK pathway. Although this result needs more investigation, it is probably due to some compensating mechanism; in

which estrogen has a neuroprotective effect against a variety of neurodegenerative disorders. The results of our study provide support that estrogen induces a protective effect in PD. It is worth noting that our study was the first to report on the usefulness of estrogen on autophagy maturation in the animal model of PD.

## Conclusion

This study confirms that age enhanced the sensitivity of dopaminergic substantia nigra neurons to rotenone. Considering the nature and magnitude of the changes observed in the substantia nigra of older animals, the senile rat PD model was more reliable and ethical. This study also clearly exhibited that estrogen could improve motor function and restore neuronal loss in animal models of PD. Moreover, its inductive effect on autophagy maturation in dopaminergic areas of the brain through the ERK pathway might be a reliable reason for its neuroprotective role in this model of PD.

## Disclosure of conflict of interest

None.

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