

# Ellagic acid protects from rotenone-induced dopaminergic neuronal damage via activation of Nrf2 signaling in astroglia

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## Research

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# Abstract

## Background

Parkinson's disease (PD) is the second most prevalent central nervous system (CNS) degenerative disease. Oxidative stress is one of key contributors to the degeneration of dopamine (DA) neurons in the pathogenesis of PD. Nuclear factor erythroid-2-related factor 2 (Nrf2) is considered to be a 'master regulator' of many genes involved in anti-oxidant stress to attenuate cell death. Therefore, activation of Nrf2 signaling provides an effective avenue to treat PD. Ellagic acid (EA), a natural polyphenolic contained in fruits and nuts, possesses amounts of pharmacological activities, such as anti-oxidant stress and anti-inflammation. Recent studies have confirmed EA could be used as a neuroprotective agent in neurodegenerative diseases. However, the underlying mechanisms have not yet been entirely elucidated.

## Methods

In this study, mice subcutaneous injection of rotenone (ROT)-induced DA neuronal damage was performed to investigate EA-mediated neuroprotection. In addition, adult Nrf2 knockout mice and different cell cultures including MN9D-enciched, MN9D-BV-2 and MN9D-C6 cell co-cultures were applied to explore the underlying mechanisms.

## Results

The present study demonstrated EA protected DA neurons against ROT-induced neurotoxicity. Furtherly, activation of astroglial Nrf2 signaling pathway participated in EA-mediated neuroprotection as evidenced by the following observations. First, EA activated Nrf2 signaling pathway in ROT-induced DA neuronal injury. Second, EA generated neuroprotection with the presence of astroglia and silence of Nrf2 in astroglia abolished EA-conferred DA neuroprotection. Third, EA failed to produce DA neuroprotection in Nrf2 knockout mice.

## Conclusion

This study identified EA protected against DA neuronal loss via an Nrf2-dependent manner in astroglia.

## Background

To date, the pathogenesis of Parkinson's disease (PD) is intangible [1]. It has been verified that the increased and prolonged production of reactive oxygen species (ROS) plays critical roles in progress of PD [2]. ROS compromises the balances between oxidant and anti-oxidant systems [3]. The anti-oxidant defense system accounted for one of the underlying mechanisms that prevent oxidative stress-induced cell damage.

Nuclear factor E2-related factor 2 (Nrf2) regulates the physiological and pathophysiological processes of various diseases [4]. Importantly, Nrf2 increases the gene expressions of endogenous anti-oxidative enzymes, such as phase II detoxifying enzymes, quinone oxidoreductase 1 (NQO1) and NAD(P)H, heme oxygenase-1 (HO-1) [5]. A number of studies confirmed that Nrf2 is located and maintained in the cytoplasm in physiological conditions via a Kelch-like ECH-associated protein 1 (Keap1)-dependent ubiquitination-proteasomal degradation [6]. Upon stimulated by oxidants or electrophiles, Nrf2 compromises Keap1/Nrf2 interactions and then Nrf2 dissociates from Keap1 complex and finally enters the nucleus. This is followed by binding to the anti-oxidant response elements (ARE), a consensus gene sequence that encodes anti-oxidant enzymes [7]. Amounts of studies identified that Nrf2 signaling participates in PD pathogenesis [8]. Activation of Nrf2 evoked dopamine (DA) neuroprotection and downregulation of Nrf2 rendered DA neurons susceptible to oxidative stress damage [9]. Insufficient Nrf2 activation was highly involved in the progress of PD [10]. Thus, potent Nrf2 signaling activation is thought to be a potential promising strategy for PD treatment.

Ellagic acid (2, 3, 7, 8-tetrahydroxybenzopyrano [5, 4, 3-cde] benzopyran-5-10- dione, EA) is located abundantly in plant extracts [11]. EA presents a variety of pharmacological properties, such as anti-inflammatory, cardioprotective and anti-oxidant effects [12]. Recently, several lines of evidence verified that EA conferred neuroprotection against aging, nerve dysfunction and neurodegeneration. In sporadic Alzheimer's disease animal model, EA generated neuroprotection and cognitive enhancement [13]. In addition, EA protected against sodium arsenate-induced neurotoxicity in rats [14]. Moreover, EA was indicated to produce neuroprotection against ischemic stroke [15]. However, the underlying mechanisms remain unclear.

In the present study, subcutaneous injection of rotenone (ROT)-elicited PD mouse model was employed to investigate EA-exerted DA neuroprotection. Furtherly, adult Nrf2 knockout mice and various cell cultures were applied to illuminate the role of Nrf2 signaling in EA-mediated DA neuroprotection.

## Materials And Methods

### Reagents

Ellagic acid (purity > 95%) and rotenone were obtained from Sigma Chemical (St. Louis, MO, USA). Anti-tyrosine hydroxylase (TH, Catalog No. Ab113), hemeoxygenase-1 (HO-1, Catalog No. Ab68477), NADPH quinone oxidoreductase 1 (NQO1, Catalog No. ER1802-86), Nrf2 (Catalog No. Ab31163), PCNA (Catalog No. 10205-2-AP), HSP90 (Catalog No. GB11284-1) and  $\beta$ -actin antibodies were bought from Abcam (Cambridge, MA, USA). The small interfering RNA (siRNA) against Nrf2 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). SYBR green supermix (PCR) was obtained from Bio-Rad Laboratories (CA, USA). RNAiso plus was purchased from Takara Biotech Co., Ltd. (Dalian, China).

### Animals and Treatment

Wild type (WT) C57BL/6J male mice and homozygous Nrf2 knockout (Nrf2<sup>-/-</sup>) male mice (22–28 g, 10–12 weeks) were purchased from the Nanjing University Biomedical Research Institute (Nanjing, China; Permit number: SCXK2015-0001). All animals were kept in accordance with Chinese animal health and welfare norms. This study was approved by the Animal Care and Use Committee of Zunyi Medical University (Zunyi, China). WT and Nrf2 knockout mice were randomly divided into Control, EA (100 mg/kg), ROT (1 mg/kg), ROT + EA (20 mg/kg) and ROT + EA (100 mg/kg) groups. Mice were given subcutaneous injection of ROT (1 mg/kg) 6 times a week for consecutive 5 weeks followed by daily intragastric administration of EA for consecutive another five weeks.

## Rotarod Test

The rotarod test, in which animals must balance on the rod, was widely used to evaluate motor deficit in neurodegenerative disease models. Before the trial, all mice received rotarod training until they stayed on the stick at least for the cut-off time. In the test, the started speed was 10 rpm and every 30 s increased 5 rpm until mice slid off the steps. Every mouse was tested 2 times a day and the average duration of stay on the rod was recorded.

## Open Field Test

Open field test was one of the most popular ethological tests to detect the anxiety-like behavior in animals. In the present study, mice were placed on the open field and each animal was located in a separate area and mice behavioral parameters were recorded during 5 min [16]. Before each round of testing, the device should be cleaned with a 75% alcohol solution to eliminate odor interference. After the end of the experiment, the total distance of mice movement was calculated.

## Immunohistochemistry Staining and Cell Counting

Mice brains were cut into 7 microns cross sections on a paraffin slicer and attached to a glass slide. The brain slices were then dried and dewaxed. Fixed brain slices were separately treated with 3% hydrogen peroxide and 0.1 M citrate buffer, and blocked with goat serum. Next, it was cultured overnight with an anti-TH antibody at 4 °C. Subsequently, brain sections were incubated with the secondary antibody working solution for 20 min followed by sections incubated with biotin for 15 min and developed with DAB developer. For morphological analysis, Olympus microscope (Tokyo, Japan) was applied to image TH-positive neurons in SN. Finally, TH-positive neurons were counted through counting the number of TH-positive neuronal cell bodies blindly by two investigators and the results were averaged.

## Western Blotting

Total protein was extracted from mice midbrain by RIPA lysis solution. Nuclear and cytosolic fractions were extracted using a nuclear-cell solute extraction kit (Solabio, Beijing, China). The protein concentrations were quantified by a BCA kit. Equal amounts of protein (10–30 µg/lane) were separated on a 10% Bis-Tris Nu-PAGE gel. Then, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, blocked with 8% skim milk for 2.5 h, and placed in the primary antibody at 4 °C overnight. The primary antibodies were: TH (1:2000), Nrf2 (1:1000), HO-1 (1:10000), NQO1 (1:1000), PCNA (1:5000) and

$\beta$ -actin (1: 3000). Next, membranes were incubated with anti-rabbit/mouse IgG secondary antibody at 1:2000 for 1 h and detected with ECL substrate. The results were statistically analyzed by quantitative analysis (Bio-Rad, Hercules, CA, USA) software.

## Real-Time RT-PCR Assay

Total RNA in the mice midbrain and cells were separately extracted by RNAiso plus reagent and purified with RNeasy Kits. The SYBR Green PCR Master Mix was used for real-time PCR analysis. The primers used were designed by Sangon Biotech (Shanghai, China). The relative differences of the target genes were first normalized to  $\beta$ -actin, and then calculated and expressed as a relative reduction or increase, setting the Control group at 100%.

## Cell Culture and Treatment

BV-2 microglia cell lines, C6 astroglia cell lines and MN9D DA neuron cell lines were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai). All cultures were maintained in DMEM/F12 media supplemented with 10% fetal bovine serum and 1% antibiotics. Three kinds of cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were seeded at 1 × 10<sup>6</sup>/well in poly-D-lysinecoated 24-well plates, respectively. Cells were treated with different concentrations of EA for 30 min followed by ROT (0.1  $\mu$ M) treatment for an additional 24 h. Then, cells and culture medium were collected for later detection.

## Nrf2-siRNA Transfection

C6 astroglia cell lines were seeds on 24-well plates for 24 h and then transfected with Nrf2-siRNA (50 nmol/L) or control-siRNA (50 nmol/L) for 6 h using siRNA transfection reagent (Thermo Fisher Scientific, Massachusetts, USA) using the manufacturer's protocol. The knockdown efficiency was evaluated by western blotting and real-time RT-PCR. After 6 h of transfection, the transfection solution was removed and cells were treated with EA for 24 h followed by ROT administration for 24 h. Afterwards, C6 cells were collected to detect the corresponding indicators.

## Reconstituted Neuron-Microglia and Neuron-Astroglia Co-Cultures Using Transwells

BV-2 cells or C6 cells were cultured in the upper chamber of transwell inserts for 24 h. The lower chambers contained MN9D cells were also cultured for 24 h. Then, BV-2 or C6 cells in transwells were transferred into MN9D cells and the reconstituted neuron-microglia (MN9D-BV-2) and neuron-astroglia (MN9D-C6) co-cultures were established. These co-cultures were treated with EA for 30 min followed by ROT application for 24 h. For MN9D-C6 co-cultures, the C6 cells in transwells were processed by Nrf2-siRNA for 6 h. Then, the MN9D cells in lower chambers were cultured for 24 h. Next, C6 cells were rinsed with fresh medium and transferred into MN9D cultures. The reconstituted neuron-astroglia co-cultures conditionally silencing astroglia Nrf2 were established. Finally, cultures were treated with EA for 30 min followed by ROT treatment for 24 h [17, 18]. DA neuronal damage was assessed by TH-positive neuron staining and TH protein expression.

# Cell Viability Assay

After EA treatment for 24 h, the upper chambers were moved and the lower chambers were incubated with MTT solution (5 mg/ml) for 4 h. Dimethyl sulfoxide (DMSO) was added to each well to determine cell viability. Absorbance values were measured at 490 nm.

# Immunocytochemical Staining

DA neurons were recognized with an anti-TH antibody. Cells were fixed with 4% paraformaldehyde followed by permeabilization using 0.03% Triton X-100 and closed with goat serum. DA neurons were labeled with an anti-TH (1:300) antibody at 4 °C overnight and then incubated with an anti-rabbit-IgG (1:2000) antibody for 1 h. TH-positive neurons numbers were calculated from four representative areas per well of the 24-well plate. In each condition, three wells were used for cell counting.

# Statistical Analysis

Data analyses were performed as mean  $\pm$  SEM. Statistical comparisons were analyzed using the SPSS statistical software by one-way ANOVA. Then, the Bonferroni's post hoc test was performed for all pairwise comparisons among means.  $p < 0.05$  was considered as statistically significant.

# Results

## EA protected against ROT-induced DA neuronal damage

To explore the neuroprotective effects of EA on PD, we established ROT-induced DA neuronal lesion in mice and determined whether EA could attenuate ROT-induced DA neurotoxicity. First, we examined behavior changes by open field and rotarod tests. As shown in Fig. 1a and b, ROT caused an apparent reduction in total movement distance and the time mice stayed on the rod. EA exhibited a significant improvement in locomotor dysfunction. Furthermore, ROT decreased TH-positive neuronal number and TH protein expression and EA ameliorated ROT-induced DA neuronal damage (Fig. 1c and d).

### EA activated Nrf2 signaling pathway in vivo

To determine whether EA could activate Nrf2 signaling pathway, the expressions of Nrf2, HO-1, and NQO1 were detected by real-time RT-PCR and western blot assay. As shown in Fig. 2a, the mRNA expressions of Nrf2, HO-1 and NQO1 were increased in the ROT and EA groups, in which EA induced higher mRNA expressions of these genes than ROT. To further determine the effects of EA on Nrf2 distribution, the cytosolic components and nuclear fractions were collected, respectively. The cytosol marker HSP90 was used to verify the purity of nuclear protein and the nuclear fraction was not contaminated by cytosol proteins as confirmed by the absence of HSP90. As shown in Fig. 2b, compared with control group, ROT activated Nrf2 and induced Nrf2 to transfer from cytosol to nucleus. Compared with ROT group, the increase in translocation of Nrf2 from cytosol to nucleus was more pronounced in EA group. Meanwhile,

the higher protein expressions of HO-1 and NQO1 in ROT + EA group were indicated than those in ROT group (Fig. 2c).

## **EA targeted astroglia to protect from ROT-induced DA neuronal damage**

To further explore which cell type EA targeted to produce DA neuroprotection, three cell culture systems including MN9D-enriched, MN9D-BV2 and MN9D-C6 co-cultures were performed using transwells. Cultures were treated with EA for 30 min followed by ROT treatment for 24 h. As shown in Fig. 3a, EA-mediated neuroprotection was indicated in MN9D-C6 co-culture system but not in either MN9D-enriched or MN9D-BV2 co-cultures. Similarly, from TH-positive neuronal immunofluorescence staining and TH protein detection assays shown in Fig. 3b and c, in MN9D-C6 co-culture system, EA attenuated ROT-induced decrease of TH-positive neuronal number and TH protein expression. However, in MN9D-enriched cultures and MN9D-BV2 co-cultures, this neuroprotection disappeared. These phenomenon demonstrated astroglia participated in EA-elicited neuroprotection.

### **EA activated Nrf2 signaling pathway in vitro**

To confirm the activation of Nrf2 signaling induced by EA treatment in which cell type, the expressions of Nrf2 signaling in MN9D, BV2 and C6 cells were detected. As shown in Fig. 4a, Nrf2 protein expression mainly concentrated in C6 cells. To further determine whether EA activated Nrf2 signaling in astroglia, the mRNA and protein expressions of Nrf2 signaling in C6 cells were examined. As shown in Fig. 4b, Nrf2, HO-1 and NQO1 mRNA levels were up-regulated in ROT and ROT + EA treatment groups, whereas EA enhanced these genes mRNA expressions compared with ROT administration. In addition, western blot assay showed the translocation of Nrf2 from cytoplasm to the nucleus was more obvious after EA treatment than that in ROT group (Fig. 4c). Also, EA induced the higher protein expressions of HO-1 and NQO1 than ROT (Fig. 4d).

## **EA protected DA neurons by the activation of astroglial Nrf2**

To explore the role of Nrf2 in EA-mediated DA neuroprotection, Nrf2 siRNA in vitro and Nrf2 knockout mice were performed. As shown in Fig. 5a, Nrf2 gene and protein levels were down-regulated in C6 cells transfected with Nrf2-siRNA with the silence ratio of 70%. Furtherly, in TH-positive neuronal counting and TH protein expression assays, EA-generated DA neuroprotection from ROT-induced neurotoxicity was neutralized by Nrf2-siRNA application (Fig. 5b and c).

Next, Nrf2 knockout mice were employed to confirm EA evoked neuroprotection via an Nrf2-dependent manner in vivo. First, the knockout efficiency was verified by Nrf2 protein level detection (Fig. 6a). Then, there was no significant difference of the genes and proteins expressions of HO-1 and NQO1 in the midbrain of Nrf2 knockout mice after EA treatment (Fig. 6b and c). In addition, EA failed to improve the time mice stayed on rod and locomotor distance in Nrf2 knockout mice (Fig. 6d and e). Meanwhile, TH-

positive neuronal counting and TH protein expression detection showed EA-mediated DA neuroprotection was abolished in Nrf2 knockout mice (Fig. 6f and g). Collectively, these results demonstrated EA protected DA neurons through the activation of astroglial Nrf2 signaling.

## Discussion

The present study aimed at investigating the neuroprotective actions of EA on ROT-induced DA neuronal damage and the underlying mechanisms as well. Results demonstrated that EA conferred neuroprotection against ROT-induced DA neurotoxicity. Activation of astroglial Nrf2 signaling was involved in EA-mediated DA neuroprotection, as evidenced by the following observations. First, EA activated Nrf2 signaling pathway in ROT-induced DA neuronal damage. Second, EA generated neuroprotection with the presence of astroglia and silence of Nrf2 in astroglia abolished EA-mediated neuroprotection. Third, EA failed to produce DA neuroprotection in Nrf2 knockout mice. Together, these results suggested EA-generated DA neuroprotection might be attributable to the activation of Nrf2 signaling in astroglia.

Oxidative stress has been considered as the central pathological event in the development of PD [19, 20]. For survival and development of neurons, the maintenance of redox homeostasis in the CNS was indispensable [21]. Nrf2 was an oxidative defense transcription factor which mitigated the toxic neuronal effects of parkinsonian agents, such as ROT, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) in vivo and in vitro [22]. Here, this study found that EA protected DA neurons, activated Nrf2 signaling pathway and initiated the Nrf2 stressor mechanism for its transfer from cytosol to the nucleus (Figs. 1 and 2). Further study indicated that EA-elicited neuroprotection were abolished in Nrf2 knockout mice (Fig. 6). These results suggested that EA-enhanced DA neuronal survival was dependent on Nrf2 activation.

Encouraged by the activation of Nrf2 signaling by EA, we further investigated Nrf2 signaling in which cell type participated in EA-mediated neuroprotection. It is interesting to note that EA targeted astroglia to produce neuroprotection since this protection was just indicated in the presence of astroglia (Fig. 3). Astroglia is the most abundant cell type in the brain [23]. They interacted with neurons in various aspects of signaling transmission, immune and oxidative defense, ion and water homeostasis, metabolite supply and neuroprotection [24]. In neurological disorders, astroglia themselves formed a defense barrier and promoted neuroprotection by producing anti-oxidants against inflammation, especially for excessive extracellular release of the excitatory neurotransmitter glutamate [25]. Stimulation of sulforaphane also prolonged Nrf2-mediated gene expression in astrocytes and prevented neuronal damage caused by superoxide [26]. In addition, besides anti-oxidants, astrocytes could release neurotrophic factors, such as BDNF and GDNF-mediated activation of the Nrf2 pathway in astrocytes, which increased the survival of DA neurons [27]. Recent research indicated that Nrf2-ARE pathway was preferentially activated in astrocytes, HO-1 and NQO1 were strongly expressed in astrocytes, sometime microglia, with more infrequent expression in neurons [28, 29]. Nevertheless, Pharmacological activation and overexpression of Nrf2 in astrocytes also clearly demonstrated its therapeutic potential for PD. In the mouse model of  $\alpha$ -

synuclein mutation, the specific overexpression of astrocyte Nrf2 was regulated, which improved the movement and non-motor dysfunction of the whole CNS, and delayed the process of  $\alpha$ -synuclein aggregation [30]. Also, Nrf2 overexpressed in astrocytes kept mice from mutated  $\alpha$ -synuclein to protect DA neurons [31]. Additionally, transplantation of astrocytes overexpressing Nrf2 into mouse striatum protected from DA neuronal loss [32]. Therefore, this was an important strategy for the support of neuroprotective functions of astrocytes to enhance neuronal survival and improve PD symptoms.

A large amount of evidence suggested Nrf2 activators could protect neurons and decrease the accumulation of aberrant proteins in vitro and in vivo in different neurodegenerative mouse models. In clinic, melatonin was found to activate Nrf2 to rescue hippocampal bioenergetics and improve cognitive function [33]. At present, the clinical trials on Nrf2 activators for the treatment of neurodegenerative diseases were increasing [34]. For example, pinostrobin could release HO-1 through Nrf2 activation to alleviate MPTP-induced neuronal loss in SN, and relieved zebra fish behavioral deficit disorder, and provided potential neuroprotective effects in vivo [25]. Moreover, Glaucocalyxin B was discerned to inhibit TLR/NF- $\kappa$ B pathway activation and induce Nrf2/HO-1 activation and further produce neuroprotection [35]. Also, dimethyl fumarate enhanced Nrf2 activation, thereby playing an important role in the defense of MPTP-induced loss of DA neurons in the SN [36]. In short, new Nrf2-based treatments might be considered as a viable strategy for future potential. Thus, this study found EA exhibited a promising therapeutic efficacy for PD via activating Nrf2 signaling pathway. These findings suggested that modulation of Nrf2 signaling by potential candidates, such as EA, might be beneficial for PD treatment. Despite this optimistic perspective for future administration of EA potential treatment for PD, most of the findings were derived from experimental models and in vitro data and therefore need be rigorously corroborated in clinical trials in future.

## Conclusions

This study demonstrated that EA protected DA neurons from ROT-induced neurotoxicity by activating astroglial Nrf2 signaling. These findings suggested EA might be an exciting option for treating PD.

## Abbreviations

EA	Ellagic acid
CNS	Central Nervous System
ARE	Anti-oxidant response elements
DA	Dopamine
ROT	Rotenone
HO-1	Heme oxygenase-1

NQO1	NADPH quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid-2 related factor 2
PCNA	Proliferating cell nuclear antigen
PD	Parkinson's disease
ROS	Reactive oxygen species
SN	Substantia nigra
TH	Tyrosine hydroxylase
WT	Wild type
Nrf2 KO	Nrf2 knockout
6-OHDA	6-hydroxydopamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

## Declarations

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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### Author contribution

FZ conceived and designed the experiments. All the authors participated in the experiment performance and data analysis. FZ, GQW and YZW wrote, revised and checked the article. All authors revised and

approved the final manuscript.

### **Ethics approval and consent to participate**

All experimental procedures were carried out in accordance with Chinese Guidelines of Animal Care and Welfare and this study received an approval from the Animal Care and Use Committee of Zunyi Medical University (Zunyi, China).

### **Consent for Publication**

Written informed consent for publication was obtained from all participants.

### **Competing interests**

The authors declared no conflicts of interests.

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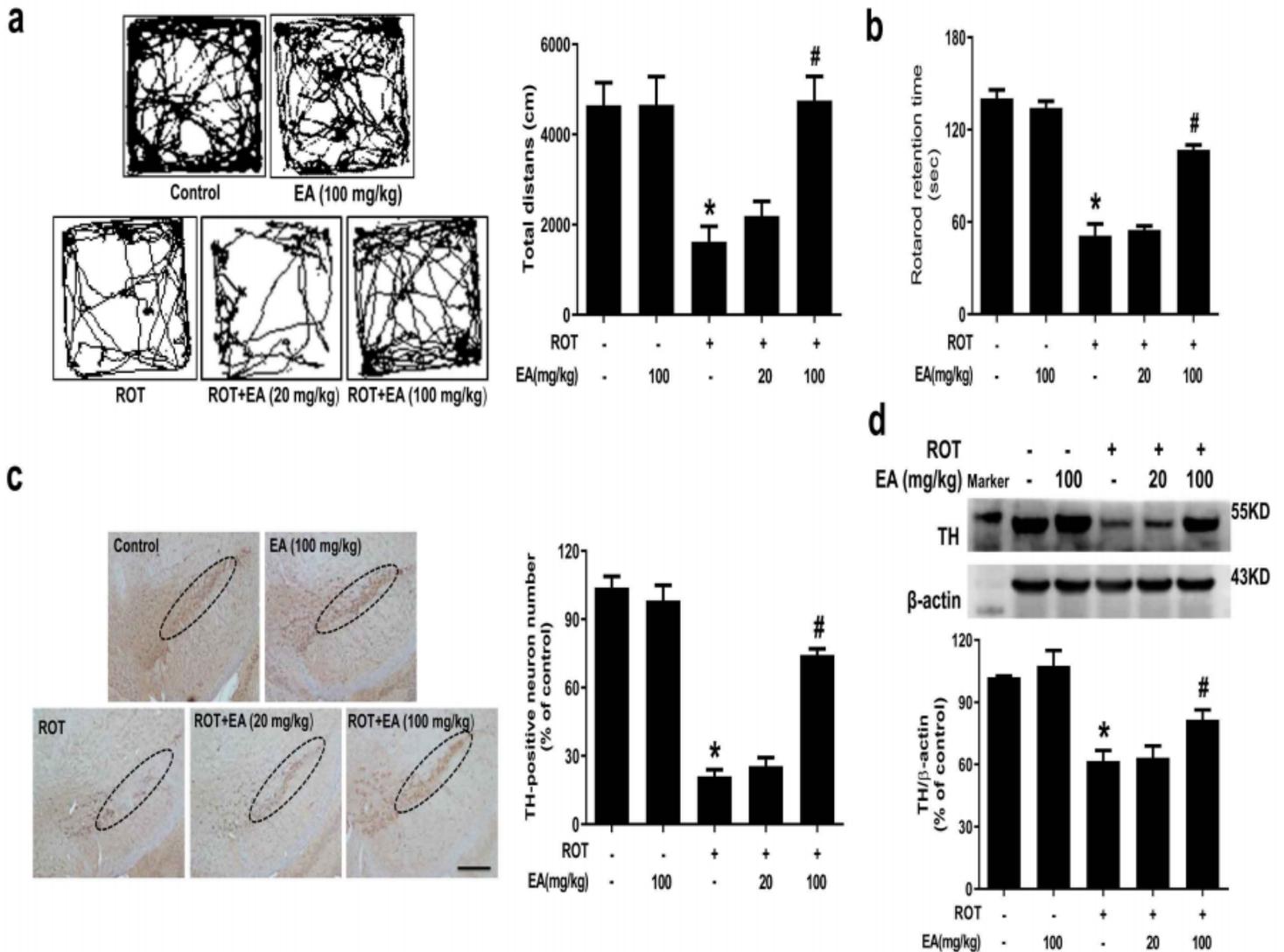
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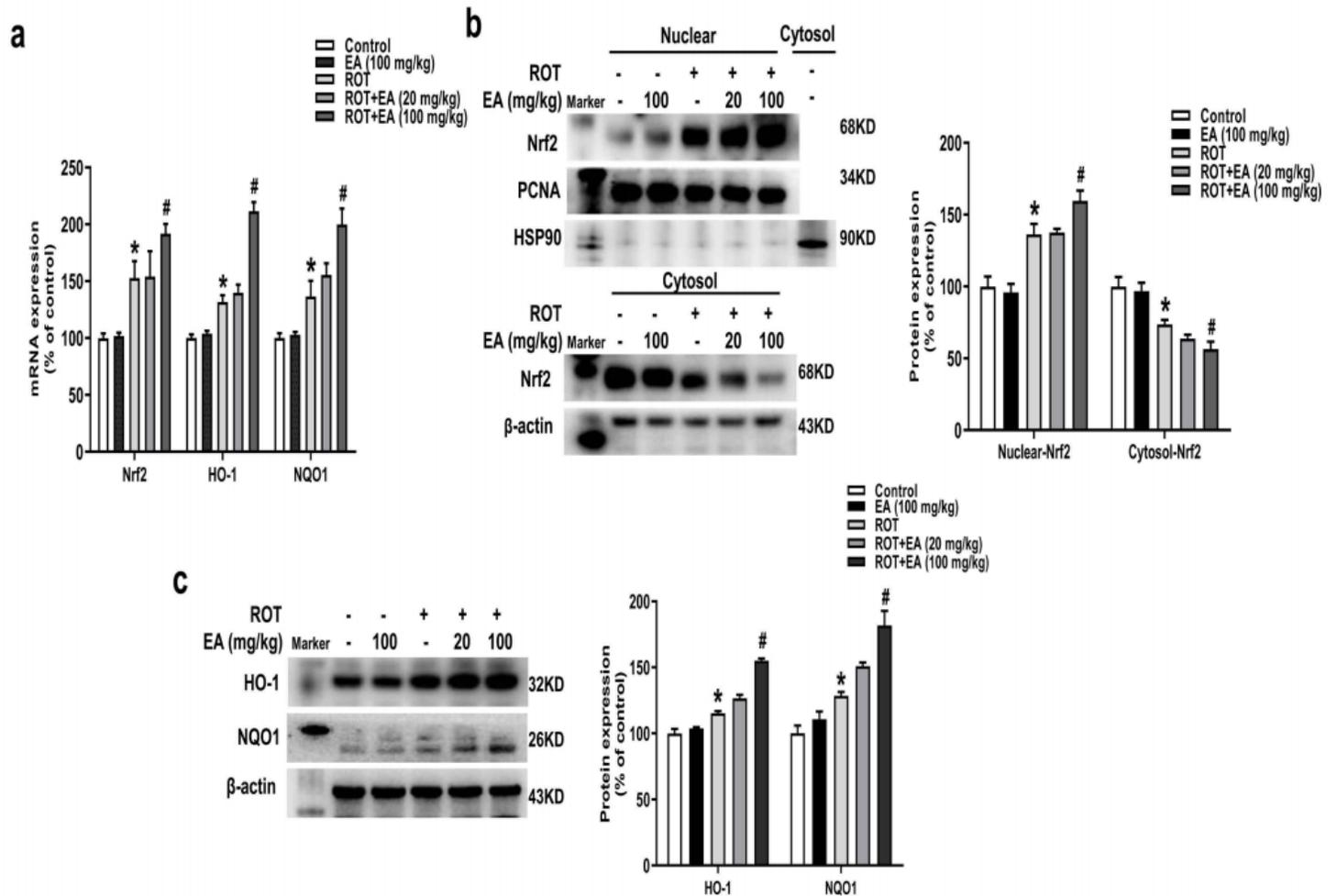
## Figures



**Figure 1**

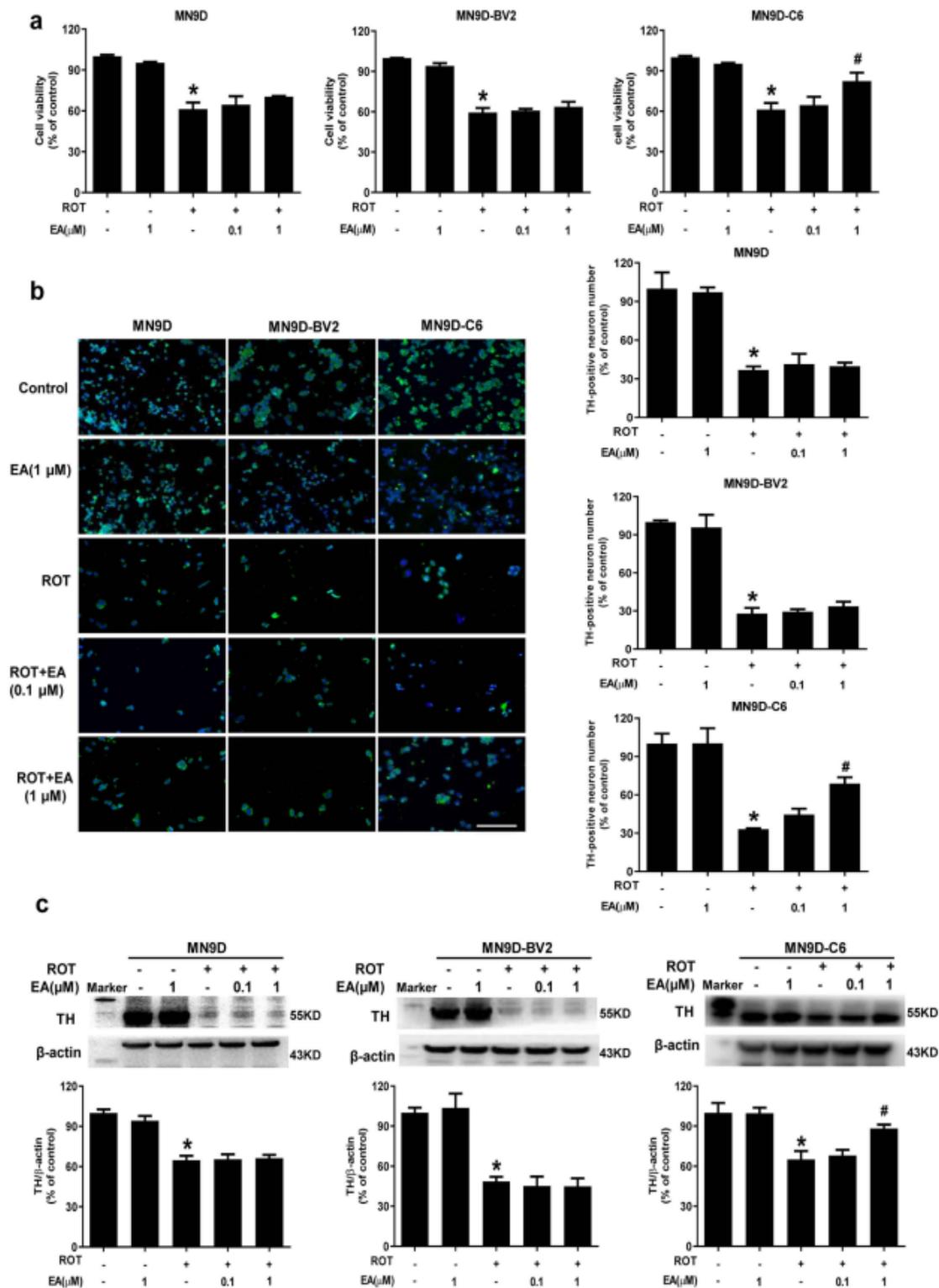
EA ameliorated ROT-induced DA neuronal damage. Male wild type mice received subcutaneous injection of ROT (1 mg/kg) six times a week for consecutive five weeks. EA were daily given by intragastric administration for consecutive another five weeks. a, b Open field test and rotarod test were performed to evaluate animal behavior changes. c TH-positive neurons number was counted after midbrain sections immunohistochemistry staining. Scale bar = 200  $\mu$ m. d TH protein expression was tested by western blot

assay. Results were represented as mean  $\pm$  SEM from 6 mice. \* $p < 0.05$  compared with the control group, # $p < 0.05$  compared with ROT-treated group.



**Figure 2**

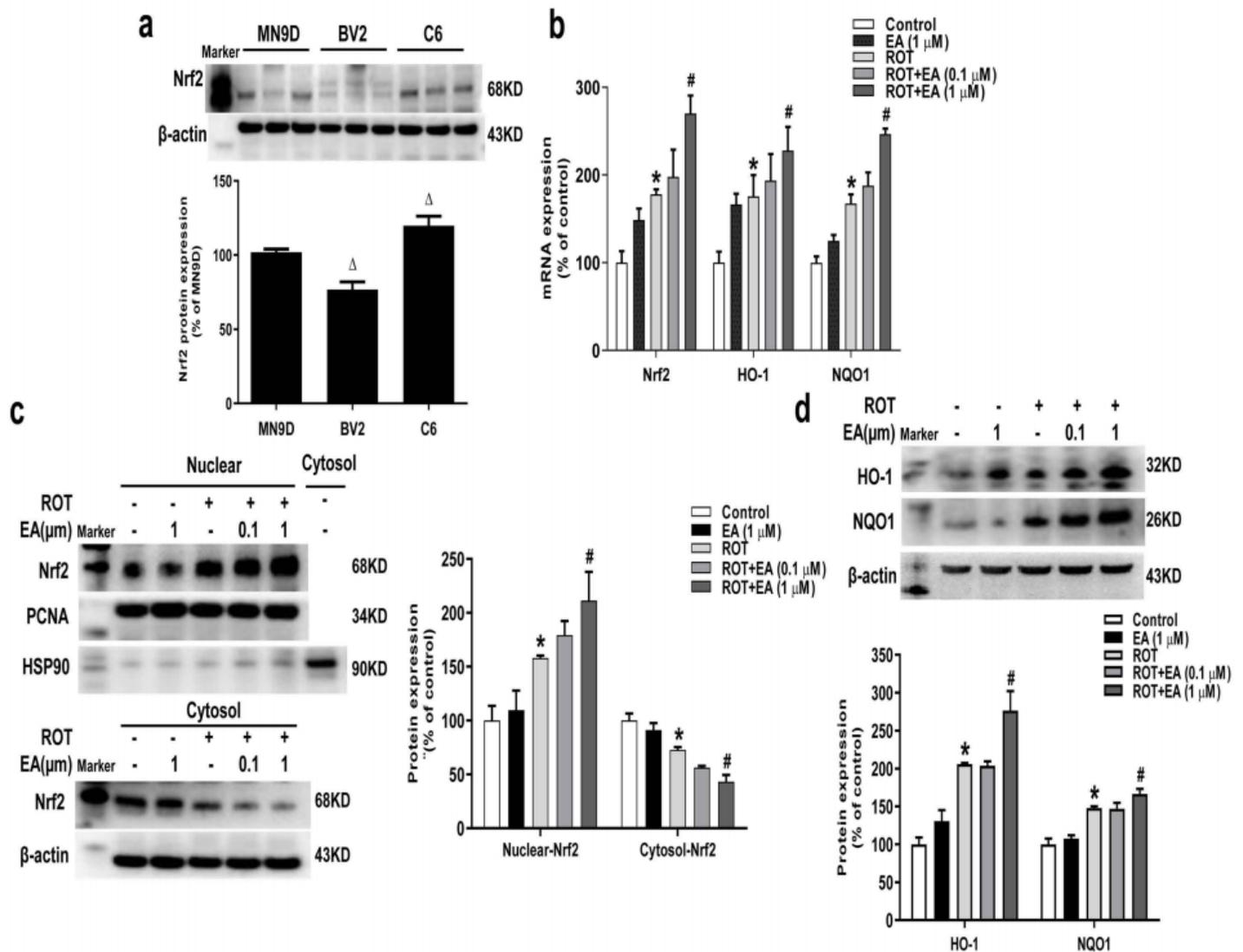
EA activated Nrf2 signaling pathway in vivo. a The mRNA expressions of Nrf2, HO-1, and NQO1 in mice midbrain were determined by real-time RT-PCR. b, c The protein expressions of nuclear Nrf2 and PCNA, cytosol Nrf2 and HSP90, whole cell HO-1 and NQO1 were measured by western blot assay. Results were mean  $\pm$  SEM from 6 mice. \* $p < 0.05$  compared with the control group, # $p < 0.05$  compared with ROT-treated group.



**Figure 3**

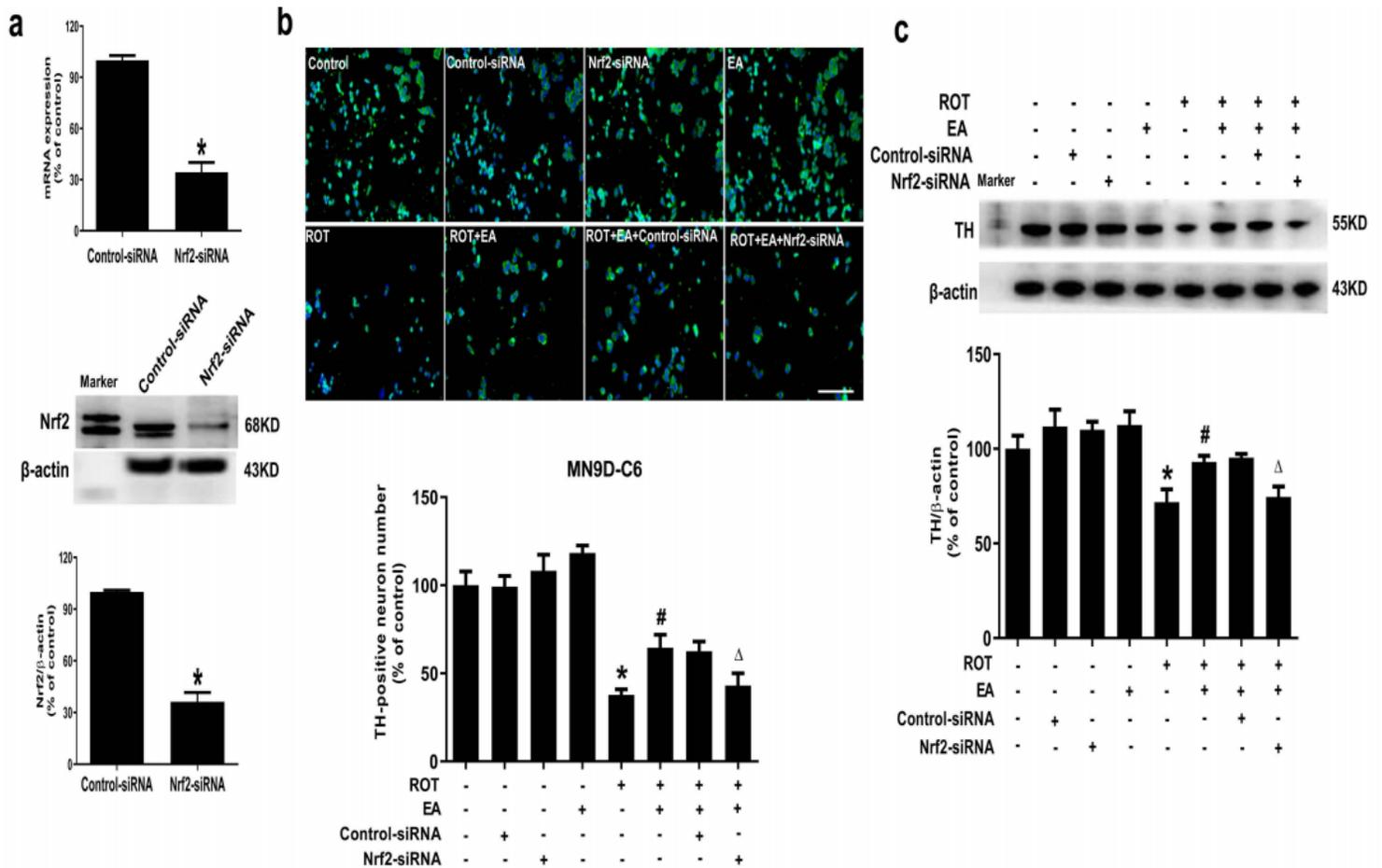
EA targeted astroglia to protect against ROT-induced DA neuronal damage. MN9D-enriched cultures, MN9D-BV2 and MN9D-C6 co-cultures were cultured for 24 h, respectively. EA was pretreated for 30 min and then incubated with ROT for another 24 h. a The neurotoxicity of ROT and EA on cell viability in MN9D, MN9D-BV2 and MN9D-C6 cultures was determined by MTT assay. b DA neuron number in these 3 cultures was counted after TH-positive neurons immunofluorescence staining. Scale bar = 100 μm. c TH

protein expression in 3 cell systems was detected by western blot assay. Results were mean  $\pm$  SEM from three independent experiments. \* $p < 0.05$  compared with the control cultures, # $p < 0.05$  compared with ROT-treated cultures.



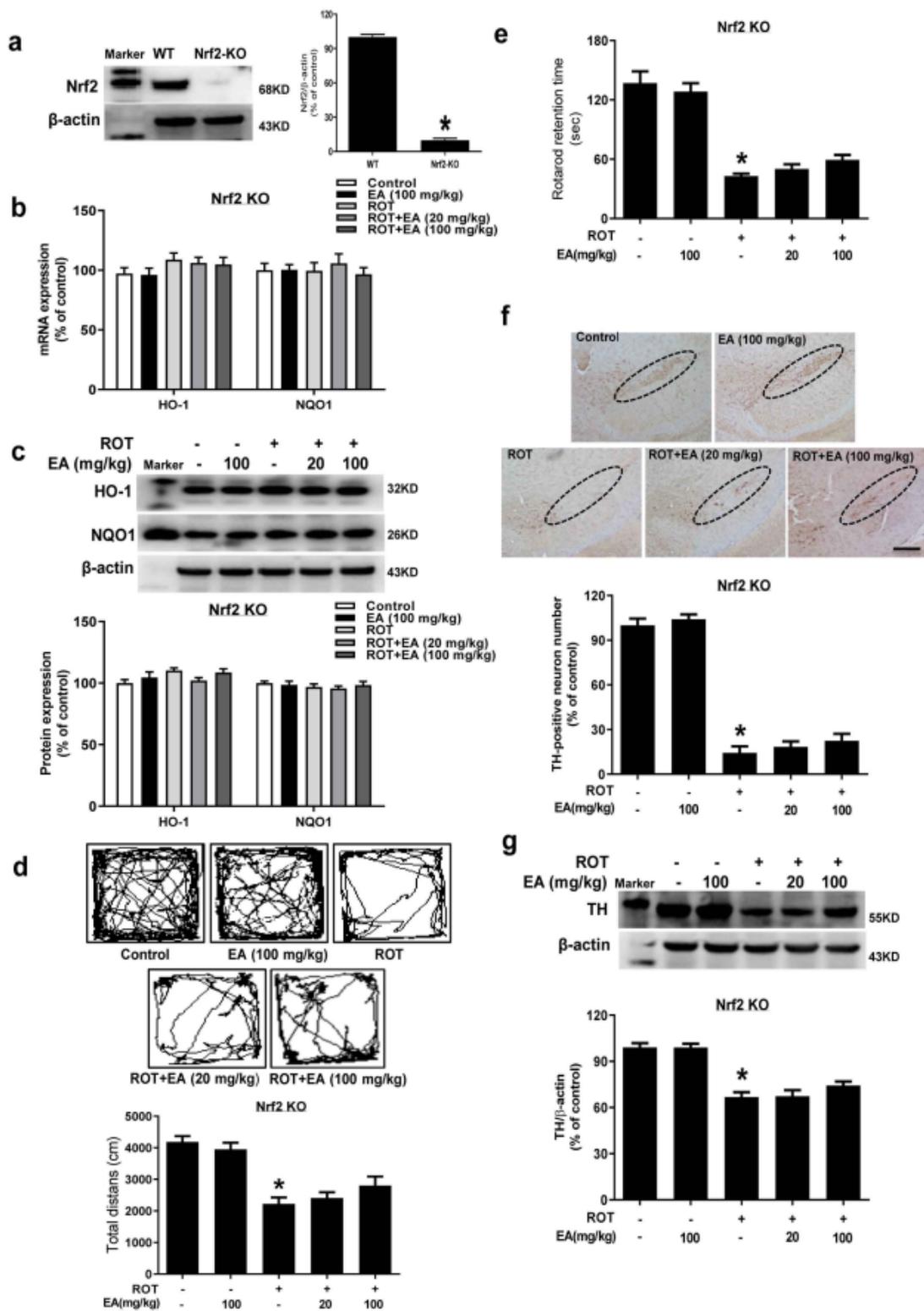
**Figure 4**

EA activated Nrf2 signaling pathway in vitro. a Protein expression of Nrf2 in MN9D, BV2 and C6 cultures was detected by western blot assay. C6 cells were pretreated with EA (0.1 and 1  $\mu$ M) for 30 min and then stimulated by ROT (0.1  $\mu$ M) for 24 h. b The mRNA expressions of Nrf2, HO-1 and NQO1 were determined by real-time RT-PCR. c, d The protein expressions of nuclear Nrf2 and PCNA, cytosol Nrf2 and HSP90, whole cell HO-1 and NQO1 were measured by western blot assay. Results were mean  $\pm$  SEM from three independent experiments performed in triplicate. \* $p < 0.05$  compared with the control cultures, # $p < 0.05$  compared with ROT-treated cultures,  $\Delta p < 0.05$  compared with MN9D cell cultures.



**Figure 5**

Role of Nrf2 in EA-mediated DA neuroprotection. C6 cells were treated with Nrf2 siRNA (50 nmol/L). After 6 h of transfection, the transfection solution was removed and cells were rinsed with PBS. a The silence efficiency was validated by real-time RT-PCR and western blotting. Then, C6 cells conditionally silencing Nrf2 were seeded in transwells upper chamber followed by MN9D cells seeded in the lower chamber to establish MD9D-C6 co-cultures and then treated with EA for 30 min and ROT for another 24 h. b, c DA neuronal damage was determined by TH-positive neuronal number counting and TH protein expression detection. Scale bar = 100  $\mu$ m. Results were mean  $\pm$  SEM from three independent experiments performed in triplicate. \* $p$ <0.05 compared with the control cultures, # $p$ <0.05 compared with ROT-treated cultures,  $\Delta$  $p$ <0.05 compared with ROT+ EA cultures.



**Figure 6**

EA-elicited neuroprotection was abolished in Nrf2 knockout mice. Nrf2 knockout mice received subcutaneous injection of ROT (1 mg/kg) six times a week for consecutive five weeks followed by intragastric administration of EA for another five weeks. a Western blotting was used to test knockout efficiency in Nrf2 knockout mice. b, c The mRNA and protein expressions of HO-1 and NQO1 in mice midbrain were detected via real-time RT-PCR and western blot assays. d, e Mice behavior changes were

assessed by open field and rotarod tests. f Brain sections were immunostained and the number of TH-positive neurons in mice SN was counted. The “ellipse” presented the area of SN. Scale bar = 200  $\mu$ m. g TH protein expression in SN was measured by western blot assay. Results were mean  $\pm$  SEM from 6 mice. \* $p < 0.05$  compared with the control group.