Targeting the Nrf2-dependent mechanism of β-Ecdysterone in attenuating the motor dysfunction in the MPTP/Pro-induced Parkinson's disease mice model

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Abstract

Oxidative stress is a pivotal stimulating factor in neurocyte apoptosis and has been involved in the pathogenesis of Parkinson's disease (PD). In this study, we have demonstrated that the improvement in the motor disorder of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/Pro-induced mice caused by β-Ecdysterone (β-Ecd) treatment is due to its antioxidant properties. Using open field, rotarod, and pole climbing tests, we have found that β-Ecd alleviates motor disorder in MPTP/Pro-induced mice and ultimately reduces the impairment of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the substantia nigra (SN). Notably, these effects of β-Ecd were not observed in Nrf2-KO mice. In addition, β-Ecd significantly reduced the formation of ROS and the level of MDA, blocked the increase of LPO, and partially reversed the GSH/GSSG ratio in MPTP/Pro-induced WT mice; however, these results were also not observed in MPTP/Pro-induced Nrf2-KO mice. Mechanistically, β-Ecd enhanced the expression levels of heme oxygenase 1 (HO-1) and GCLc, but not NQO1 (NAD(P)H quinone dehydrogenase 1) and GCLm expression. Interestingly, β-Ecd failed to increase the protein and mRNA levels of HO-1 and GCLc in Nrf2-KO mice, suggesting that β-Ecd attenuates oxidative stress through an Nrf2-dependent mechanism. Furthermore, β-Ecd promoted the expressions of PI3K/Akt phosphorylation (activity) and GSK-3β phosphorylation (inactivity). Conversely, administration of β-Ecd markedly decreased Fyn phosphorylation levels. Collectively, our findings suggest that β-Ecd focuses on Nrf2 in reducing MPTP/Pro-induced oxidative stress and subsequent motor deficits by inhibiting its nuclear export through PI3K/Akt/GSK-3β/Fyn pathway regulation. These further indicate that β-Ecd may be an absorbing therapeutic agent for PD.

Key words: β-ecdysterone, nuclear factor erythroid 2-related factor 2 (Nrf2), motor dysfunction, Parkinson's disease.

Introduction

Parkinson's disease (PD) is an ordinary neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the midbrain substantia nigra pars compacta (SNpc). Unfortunately, despite numerous studies, effective treatments for preventing the progression of PD are still lacking [51]. Convincing evidence demonstrated that oxidative stress is a core contributor to cell senescence and neurodegenerative diseases, such as PD, and is also a key factor in neuronal apoptosis [24]. Therefore, oxidative stress was considered to be a driver of PD progression [6]. Hence, increasing anti-oxidative ability and inhibiting the apoptosis of dopaminergic neurons may be an important strategy for preventing the occurrence and development of PD.

The nuclear factor erythroid 2-related factor 2 (Nrf2)/ antioxidant response element (ARE) pathway is a main cellular defence mechanism against oxidative and electrophilic stress [31]. In addition, genetic studies have

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shown that variations in NFE2L2 modify the progression of PD, proposing another relation between oxidative stress and neurodegenerative disorders [56]. A recent animal study showed that Nrf2 knockout mice were susceptible to oxidants, electrophiles, and environmental insults [38]. Furthermore, it was found that the overexpression of Nrf2 through either genetics or pharmacological remedies contributes to neuroprotection [16]. Therefore, these studies indicate that the Nrf2/ARE pathway may be an attractive therapeutic target for PD [37,49].

Senescence is a crucial danger factor in the development of PD [58]. Along with aging, sex-related differences in PD patients have also been recognized [15]. Specifically, epidemiologic studies show that the incidence of PD is higher in males than in females [47]. Moreover, when oestrogen levels are lower, especially in pre-menopausal women during menstruation, PD symptoms gradually worsen. Furthermore, compared to pre-menopausal women, the severity of PD is higher in post-menopausal women [25,28]. An increasing body of evidence supports the notion that brain oestrogens cause a neuroprotective effect on dopaminergic neurons in the SN, and delay the development of PD [9,48]. Clinical studies indicate that oestrogen replacement therapy can alleviate the severity of early PD [18,33]. Coincidentally, phytoestrogens are used in selective hormone replacement therapy.

Phytoestrogens are polyphenolic plant-derived compounds that mimic the structure and function of mammalian oestrogens [17]. β-Ecdysterone (β-Ecd) (Fig. 1A) is a phytoestrogen compound. Several studies show that β-Ecd has various pharmacological functions, including anti-oxidative, anti-apoptotic, and anti-inflammatory activities [57]. However, limited studies have investigated the inhibitory effects of β-Ecd in the progression of PD *via* an anti-oxidative mechanism. Our previous study used specific inhibitors to confirm that β-Ecd attenuates MPP^+-induced PC12 neurotoxicity through the PI3K-Nrf2 signalling pathway [59]. Our present study aimed to ascertain whether β-Ecd ameliorates motor deficits in PD mice models and whether β-Ecd protects dopaminergic neurons in the SN against neurotoxicity through an Nrf2-dependent manner. To address these questions, we employed 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/Pro-induced WT and Nrf2-KO PD mice models (Fig. 1B) and explored how β-Ecd affects mice motor deficits and regulates the Nrf2/ARE signalling pathway. The findings presented in this study provide an original perception of the mechanism of β-Ecd in the clinical therapy of PD.

Material and methods

Animal models

Nrf2 knockout mice (Nrf2-KO, C57BL/6 genetic background) and wild-type (WT) C57BL/6 mice were obtained from the Laboratory Animal Centre of Qiqihar Medical University, Qiqihar, China. All of these mice (6-month-old) were raised in bioclean cages under alternating 12 h light and dark cycles, controlled temperature of 20-22°C, and unrestricted food and water. All possible methods were offered to reduce mouse discomfort.

Male WT mice and Nrf2-KO mice were randomly and respectively classified into 4 groups: control group, β-Ecd alone group, MPTP/Probenecid (MPTP/Pro) group, and MPTP/Pro plus β-Ecd group. There were 12 mice in each group. In the chronic PD model experiments (Fig. 1B), Nrf2-KO and WT mice were treated with MPTP (M0896-100MG, Sigma) hydrochloride and Probenecid (sc-202773B, Santa Cruz Biotechnology, Inc) in dimethyl sulfoxide for 10 consecutive injections at 3.5-day intervals. Each mouse model was treated with an intraperitoneal injection of 250 mg/kg Pro, and 30 mins later, by a subcutaneous injection of 25 mg/kg MPTP. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine may result in an obvious but reversible loss of dopaminergic functions. However, co-administration of MPTP with Probenecid, displayed that it consolidates neurotoxicity by diminishing the clearance and metabolites of MPTP. With chronic MPTP/Probenecid administration, the loss of dopaminergic neurons would be even greater. On the basis of comparisons, the chronic MPTP/Pro model reveals substantial ameliorations over the traditional, subacute MPTP model. The alterations of the chronic MPTP/Pro model more closely correspond to human manifestations of PD [14]. For the MPTP/Pro plus β-Ecd group, an oral administration of 60 mg/kg of β-Ecd (Nanjing Jingzhu Biotechnology, Nanjing, China), dissolved in 0.5% carboxymethylcellulose sodium, was performed from day 7 of MPTP/Pro injection. The β-Ecd intervention groups were made once a day for 60 continuous days. Meanwhile, the mice were orally administered with the same volume of the vehicle solution as the control group. On day 64 of treatment, the motor ability of mice was trained. After the last treatment, the motor ability of each mouse was evaluated by open field, climbing, and rotarod tests. Then, the mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight) and mercy killing by cervical dislocation was performed. The SN region in each brain tissue was obtained after the mouse was sacrificed. The harvested SN tissues were used for downstream experiments. The animal models were prepared as previously described [8]. All procedures for animal use were approved by the Animal Care and Use Committee of Qiqihar Medical University and the Animal Ethical Care Committee of Qiqihar Medical University (QMU-AECC-2021-13).

Evaluation of oxidative stress and measurement of related biomarkers

The ROS level was assayed using the oxidation-sensitive fluoroprobe 2',7'-dichlorofluorescin diacetate

(DCFH-DA, E004-1-1, Nanjing Jiancheng Bioengineering Institute, China). The lipid hydroperoxides (LPO) content was measured using the lipid hydroperoxide assay kit obtained from Nanjing Jiancheng Bioengineering Institute (A016-1-1). The total glutathione (GSH) and oxidized GSH (GSSG) contents were measured using the GSH/GSSG-Glo™ assay kit (S0053, Beyotime Biotechnology Co. Ltd, Shanghai, China). The activity of superoxide dismutase (SOD) was evaluated using the superoxide dismutase assay kit (A001-3-2, Nanjing Jiancheng Bioengineering Institute, China). The TrxR activity was determined according to the protocol of the colorimetric assay kit (BioVision Inc, Milpitas, CA, USA). To assess the severity of the damage of the neurons, malondialdehyde (MDA) was measured using the OxiSelect™ Thiobarbituric Acid Reactive Substance assay kit (A003-1-2, Nanjing Jiancheng Bioengineering Institute, China). All tests were performed according to the manufacturer's protocol.

Motor ability test

An open-field test was performed to assess the autonomous motor ability of the mice models as previously described [54]. The relevant parameters, movement distance, rearing frequencies, and entries in zone-centre, were examined for 5 mins.

The rotarod test was performed according to previous methods [22]. Before the experiment, the mice were trained on the rotarod for 120 s each day for 3 consecutive days, at a speed of 15 rpm. For the test session, the initial rotational speed was set at 4 rpm, with an acceleration rate of 20 rpm/min. The maximum speed reached 40 rpm, and the test duration was 5 mins. The time it took each mouse to fall from the rotating rod was recorded. Each mouse was tested for 3 trials, with at least 1 hr interval between tests.

A 50 cm pole with a diameter of 1 cm was used in the pole climbing test. The time it took each mouse to climb down back to the floor was recorded. Each test was scored based on the following grading criteria: 1 point for the time of more than 6 s; 2 points for the time of less than 6 s but more than 3 s; and 3 points for the time of less than 3 s. Each mouse was subjected to 3 trials. In cases where the mouse fell off the pole, the test was considered invalid [32].

Immunohistochemistry

The expression of tyrosine hydroxylase (TH) in the SN was evaluated by immunohistochemistry (IHC) staining. Briefly, the deparaffinized tissue sections were first pretreated for antigen retrieval. Then, the samples were treated with 0.1% Triton X-100 for 15 mins. Subsequently, the tissue samples were blocked with 10% bovine serum albumin (BSA) for 30 mins, followed by 1 h incubation with the primary antibody. Afterwards, the samples were incubated with the secondary antibody for 1 h. Finally, the stained sections were evaluated using a confocal microscope. The number of TH-positive neurons in the SN samples was quantified by unbiased stereology.

Western blot

Total protein was extracted from mice SN tissue samples by using a nuclear and cytoplasmic protein extraction kit. Protein concentration was quantified using a BCA protein assay kit (ThermoFisher, Waltham, MA, USA). The target protein was obtained using SDS-PAGE and was then transferred to a nitrocellulose membrane at 75 V for 120 mins. The membranes were blocked with 5% non-fat milk and were then incubated with primary antibodies for HO-1, NQO1, GCLc, GCLm, Nrf2, DAT, PI3K, Akt, GSK-3β, Fyn, Keap1, and Bach1 for 4 h. After washing with TBS-T, the membranes were incubated with the secondary antibody for 2 hrs at 37°C. The housekeeping proteins, GAPDH and lamin B, were used as internal references. The list of antibodies is summarized in Supplementary Table I.

RT-PCR analysis

Total RNA was isolated from mice SN tissues using the TRIzol total RNA extraction kit (DRR037A, Takara Biotechnology, Dalian, China). The extracted RNA was reverse-transcribed to cDNA. The primer sequences referred to in the current study are listed in Supplementary Table 2. Gene expression analyses were performed using the comparative threshold cycle ($^{\Delta\Delta}$Ct) method.

Statistical analysis

All data are shown as mean ±SD. Statistical differences among multiple groups were determined using one-way ANOVA followed by Student-Newman-Keuls (SNK) post-hoc test. The SPSS17 statistical software was used for all statistical analyses. A p-value < 0.05 was considered statistically significant.

Results

β-Ecd alleviates motor deficits in MPTP/Pro-challenged WT mice

The open field, rotarod, and climbing pole tests were used to probe the potential effects of β-Ecd on the motor performance of MPTP/Pro-induced WT and Nrf2-KO mice. Compared with the MPTP/Pro-induced WT mice, treatment with β-Ecd significantly increased the distance in the zone, rearing frequencies, and entries in

zone-centre in WT mice (Fig. 1C-F). These results suggest that administration of β-Ecd improved movement activity, curiosity to a fresh environment, anxiety, and other emotions in MPTP/Pro-induced WT mice, but not in Nrf2-KO mice.

To further elucidate the effects of β-Ecd in the MPTP/Pro-induced WT and Nrf2-KO mice motor deficits, these mice were subjected to rotarod and climbing pole tests. We found that β-Ecd treatment significantly increased the jungle gym scores compared with the MPTP/Pro-induced WT group (Fig. 1G). Furthermore, in rotarod tests, we found that MPTP/Pro-induced WT mice alone showed significantly lower suspension time compared with the control WT mice; whereas, β-Ecd treatment markedly increased the suspension time compared with the MPTP/Pro-induced WT group (Fig. 1H). Overall, these results indicate that β-Ecd treatment produces favourable attenuation of the motor disorder and enhances the motor coordination in WT mice with MPTP/Pro-induced PD. However, no obvious improvement in motor balance deficits of MPTP/Pro-induced Nrf2-KO mice caused by β-Ecd was observed.

β-Ecd alleviates neurotoxicity in MPTP/Pro-induced WT mice

To elucidate whether β-Ecd could protect the impaired dopaminergic neurons in the SN of MPTP/Pro-induced WT and Nrf2-KO mice, immunohistochemical staining, western blot, and RT-PCR were used to evaluate the level of TH and dopamine transporter (DAT) in the SN. Treatment of WT mice with β-Ecd for 60 consecutive days markedly relieved the MPTP/Pro-induced reduction of TH-positive neurons (Fig. 2A, B). This finding was corroborated by western blot and RT-PCR (Fig. 2C-E). Similarly, β-Ecd treatment significantly upregulated the protein and mRNA expression of DAT (Fig. 2F, G). We also tested the TH and DAT expression levels in Nrf2-KO mice. However, no significant difference was observed. Collectively, these results indicate that β-Ecd is beneficial to the survival of dopaminergic neurons in WT mice, but not in Nrf2-KO mice.

β-Ecd alleviates the oxidative stress in MPTP/Pro-induced WT mice by regulating the key antioxidant

Oxidative stress plays a crucial role in the loss of dopaminergic neurons during PD [10]. We investigated whether the antioxidant characteristics of β-Ecd can suppress the decrease in dopaminergic neurons of MPTP/Pro-induced WT mice. It was confirmed that an increased ROS directly leads to neuron death [44]. Hence, we wanted to determine the effect of β-Ecd on

ROS production in MPTP/Pro-induced mice. We found that β-Ecd partially, but remarkably, decreased the formation of ROS and the level of MDA in MPTP/Pro-induced WT mice, while no significant difference was observed in Nrf2-KO mice (Fig. 3A, B). Furthermore, the LPO assay results suggest that β-Ecd effectively blocked the increase of LPO in MPTP/Pro-induced WT mice, but failed to do so in Nrf2-KO mice (Fig. 3C). These results confirmed that β-Ecd has a potent antioxidant property. Interestingly, no notable difference in the activities of TrxR and SOD was noted in WT and Nrf2-KO mice treated with β-Ecd (Fig. 3D, E).

Generally, GSH principally exists in a reduced form, while the oxidized disulfide form (GSSG) has a lower concentration. Commonly, the GSH/GSSG ratio is considered an important indicator of oxidative stress. In this study, we further confirmed the antioxidant property of β-Ecd by determining the GSH/GSSG ratio. We found that the administration of β-Ecd significantly abolished the decrease in the GSH/GSSG ratio of the MPTP/Pro-induced WT mice, at least partly, compared with the MPTP/Pro-induced model group (Fig. 3F, G). Most interestingly, the administration of β-Ecd did not enhance the GSH/GSSG ratio in MPTP/Pro-induced Nrf2-KO mice. These findings are in line with the oxidative stress-related biomarker expression data. Overall, these findings suggest that β-Ecd attenuates the oxidative damage in MPTP/Pro-induced mice in an Nrf2-dependent manner.

β-Ecd promotes Nrf2 nuclear translocation and upregulates the level of HO-1 and GCLc in MPTP/Pro-induced mice

The Nrf2/ARE pathway is widely regarded as a vital cellular defence mechanism against oxidative stress [30]. Nrf2 is one of the important upstream regulators of the glutathione biosynthetic enzyme. Therefore, we were interested in elucidating whether Nrf2 translocates to the nucleus with β-Ecd treatment. Western blot results indicated that β-Ecd increased the levels of Nrf2 in the nucleus compared to the cytoplasm, suggesting that β-Ecd evoked Nrf2 nuclear localization (Fig. 4A, B). Interestingly, Nrf2 mRNA expression, a significant difference was not observed among groups (Fig. 4C).

In previous studies, Bach1 and Keap1 were shown to negatively regulate Nrf2 [1,4]. Therefore, we further determined the influence of β-Ecd on Bach1 and Keap1 protein expression. Western blot analysis showed that the protein expression of Bach1 and Keap1 were not distinct in the MPTP/Pro-induced mice treated with β-Ecd (Fig. 4A, D). These findings were consistent with the gene expression analysis (Fig. S1).
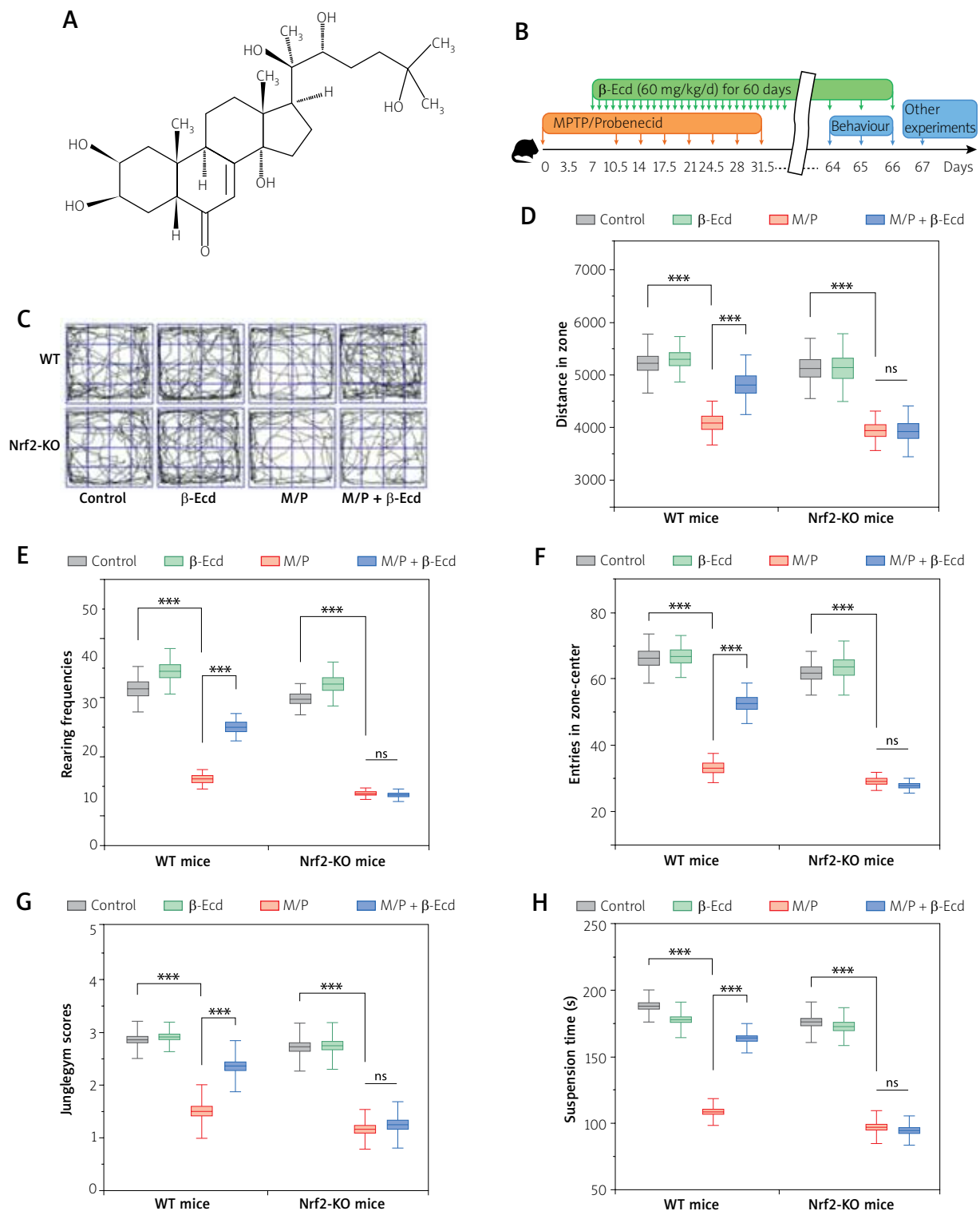
Fig. 1. β-Ecd improves motor incoordination in MPTP/Pro-challenged WT mice. **A**) The chemical structure of β-Ecd; **B**) Animal model preparation. The open-field, climbing pole, and rotarod tests were used to examine the motor ability. Open-field test: **C**) typical track plots of mice; **D**) distance in the zone; **E**) rearing frequencies; and **F**) entries in zone-centre; **G**) The scores of the climbing pole test; **H**) The suspension time of the rotarod test. All data were expressed as mean ±SD ($n = 12$). Statistical significance is denoted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ns for not significant.

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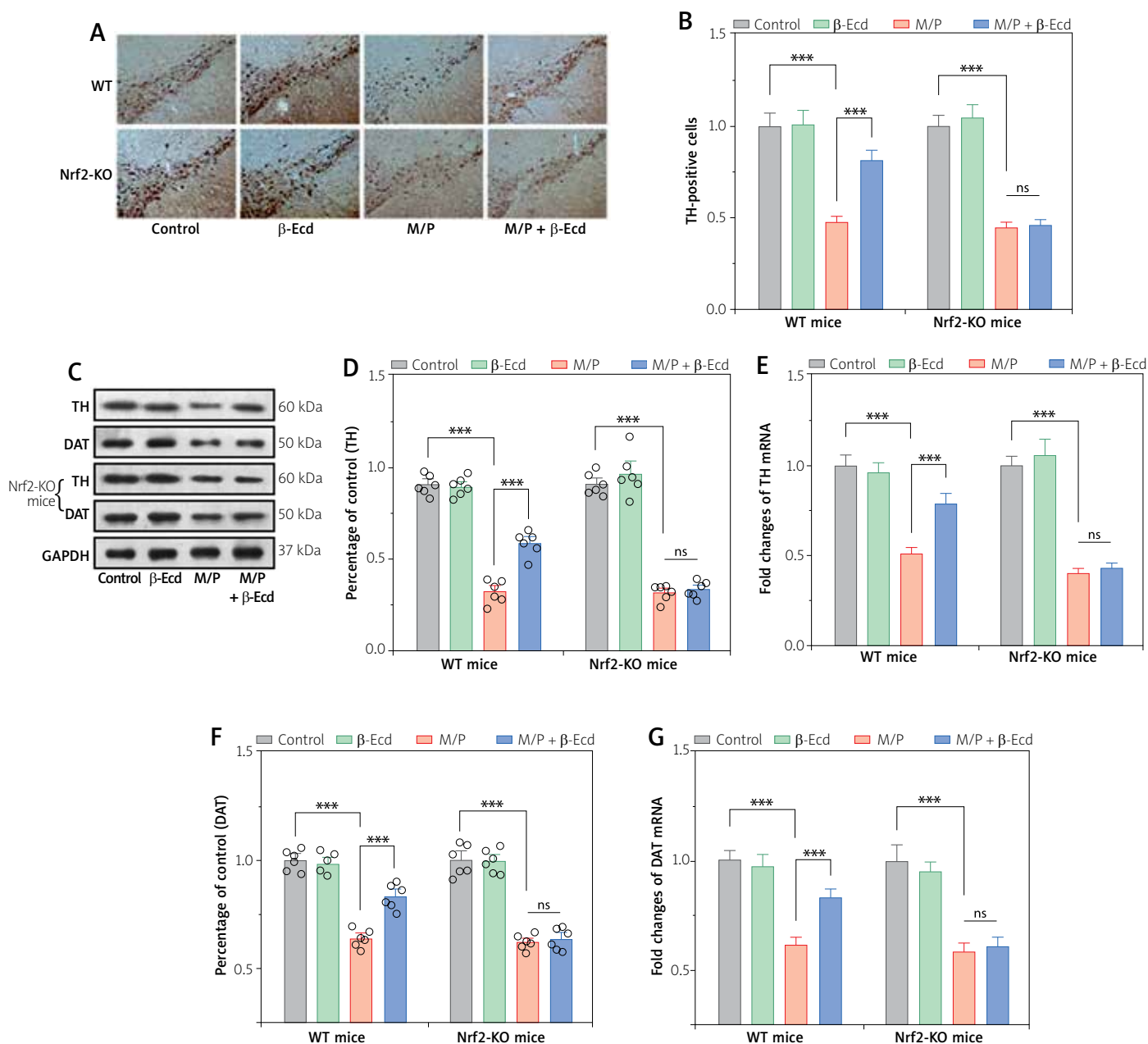
Fig. 2. β-Ecd treatment preserves the dopaminergic neurons in the SN of WT mice. **A, B**) The TH levels in the SN |of mice were examined by IHC staining. **A**) Qualitative (100×) and **B**) quantitative data from the IHC are shown. **C**) The TH and DAT protein levels in the SN of WT and Nrf2-KO mice. **D, F**) The quantitative analysis of TH and DAT. **E, G**) RT-PCR was used to evaluate the TH and DAT mRNA expression levels. All data were presented as mean ±SD ($n = 6$). Statistical significance is denoted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ns for not significant.

Numerous studies have shown that the use of endogenous phase II antioxidants from natural compounds are considered a hopeful strategy for improving oxidative damage-related diseases [46]. Hence, we evaluated the effects of β-Ecd to phase II antioxidants, including GCLc, HO-1, GCLm, and NQO1 in the SN of MPTP/Pro-induced WT and Nrf2-KO mice. Western blot analysis showed that β-Ecd significantly increased the contents of the glutathione biosynthesis enzyme HO-1 and GCLc, but not NQO1 and GCLm, in MPTP/Pro-induced WT mice (Fig. 4E, F, H, J). These findings are consistent with gene expression data (Fig. 4G, I, and
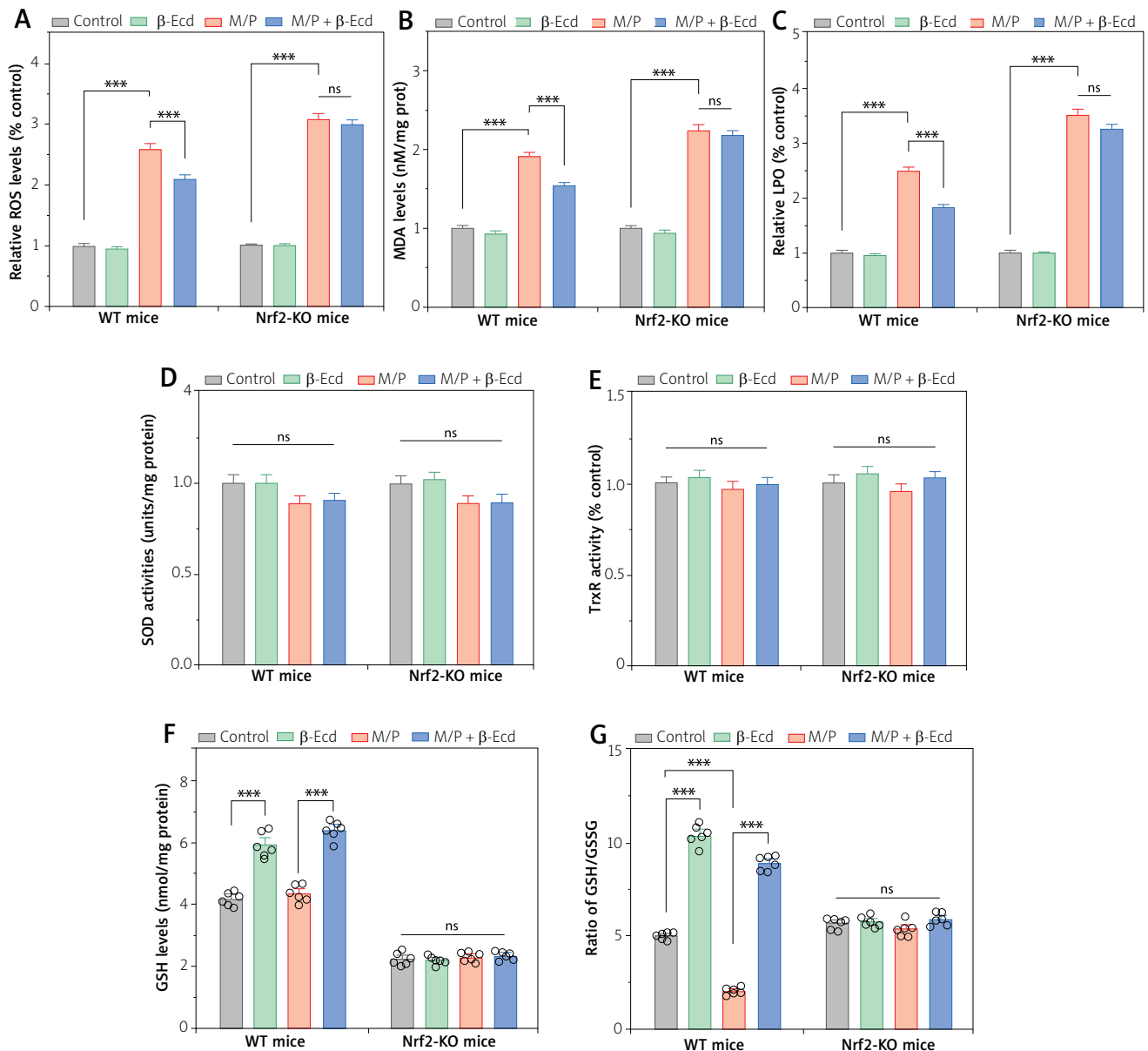
Fig. 3. β-Ecd suppresses oxidative stress by accelerating glutathione synthesis in the SN of MPTP/Pro-induced WT mice. **A**) Oxidation-sensitive H2DCF-DA fluorescent probes were used to measure ROS levels. **B**) The thiobarbituric acid method was used to determine MDA contents. **C**) LPO was measured by utilizing the redox reactions with ferrous ions. **D**) SOD activity was evaluated using the nitroblue tetrazolium assay. **E**) The colorimetric method was applied to detect TrxR activities. **F**) The total GSH and GSSG were determined by a fluorometric method. **G**) The reduced-oxidized GSH (GSH/GSSG ratio) is shown. All data were presented as mean ±SD (n = 6). Statistical significance is denoted as *p < 0.05, **p < 0.01, ***p < 0.001, and ns for not significant.

Fig. S2). In contrast, western blot and RT-PCR analysis in Nrf2-KO samples showed that β-Ecd did not affect HO-1 and GCLc expression. These findings reinforce the hypothesis that the glutathione biosynthetic enzymes HO-1 and GCLc were dependent on the presence of Nrf2.

β-Ecd treatment regulates PI3K/Akt/GSK-3β/Fyn signalling in mice

Several studies have found that phosphorylation of Fyn, a member of the Src kinase family, results in Nrf2 nuclear export, ubiquitination, and degradation [13,36].
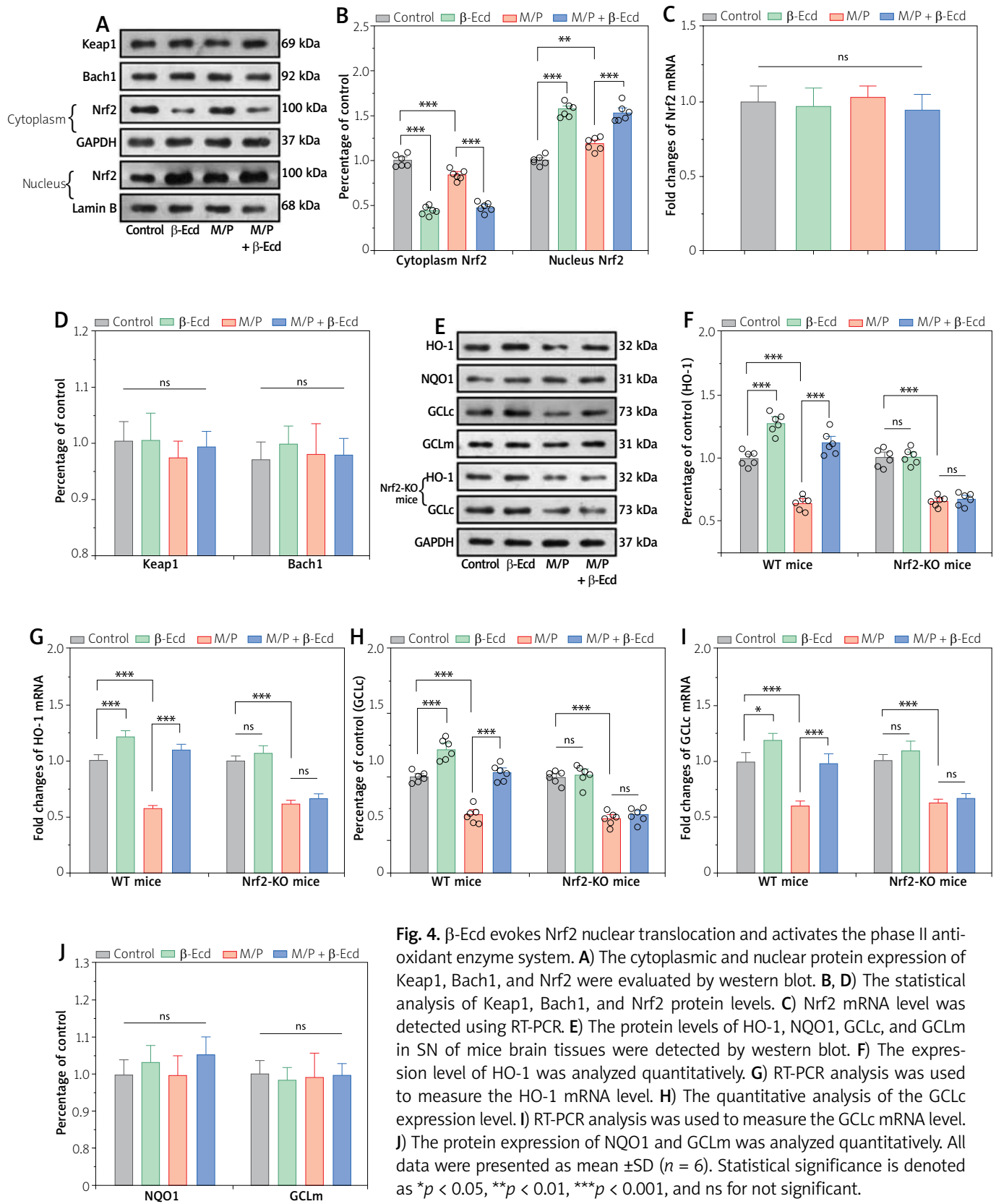
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Fig. 4. β-Ecd evokes Nrf2 nuclear translocation and activates the phase II antioxidant enzyme system. **A)** The cytoplasmic and nuclear protein expression of Keap1, Bach1, and Nrf2 were evaluated by western blot. **B, D)** The statistical analysis of Keap1, Bach1, and Nrf2 protein levels. **C)** Nrf2 mRNA level was detected using RT-PCR. **E)** The protein levels of HO-1, NQO1, GCLc, and GCLm in SN of mice brain tissues were detected by western blot. **F)** The expression level of HO-1 was analyzed quantitatively. **G)** RT-PCR analysis was used to measure the HO-1 mRNA level. **H)** The quantitative analysis of the GCLc expression level. **I)** RT-PCR analysis was used to measure the GCLc mRNA level. **J)** The protein expression of NQO1 and GCLm was analyzed quantitatively. All data were presented as mean ±SD ($n = 6$). Statistical significance is denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and ns for not significant.

In line with this, we tested if β-Ecd inhibits the phosphorylation of Fyn in MPTP/Pro-induced mice. Western blot analysis showed that β-Ecd robustly reduced the phosphorylation of Fyn in untreated normal and MPTP/Pro-induced mice (Fig. 5A, D). It has been shown that GSK-3β is located upstream of Fyn kinase, which in turn favours Nrf2 nuclear exclusion [35]. We then examined the influence of β-Ecd on the activation of GSK-3β in mice. We found that β-Ecd strongly induced the phosphorylation (inactivated form) of GSK-3β at Ser9 in the SN of mice, which did not depend on MPTP/Pro treatment; however, no significant difference in total GSK-3β protein was observed (Fig. 5A, D). We also determined the phosphorylation (activation) of PI3K and Akt in untreated normal and MPTP/Pro-induced mice. The phosphorylation levels of PI3K and Akt were significantly elevated by β-Ecd, which is consistent with the increase in GSK-3β phosphorylation at Ser9 (Fig. 5A, B). These findings were validated by gene expression analysis of PI3K, Akt, GSK-3β, and Fyn (Fig. 5C, E). In contrast, β-Ecd was not able to regulate

the expressions of the mRNA, protein, and the phosphorylation of PKCδ in the SN of mice (Fig. S3).

Discussion

Dopamine replacement therapy has always been regarded as the prevailing treatment for PD. Unfortunately, it does not hinder the progression of PD [29]. Natural products can be a rich source of new drug candidates for PD [3]. β-Ecd is obtained from the root of a traditional Chinese medicinal plant *Achyranthes bidentata* BI. We demonstrate that β-Ecd ameliorates motor dysfunction in MPTP/Pro-induced mice models through its antioxidant property. This study illustrates the favourable effects of β-Ecd against motor disorder in MPTP/Pro-induced mice using the open field, rotarod, and pole climbing tests. Surprisingly, Nrf2 KO abolished the antioxidative property of β-Ecd in MPTP/Pro-induced mice. Mechanistically, the PI3K/Akt/GSK-3β/Fyn pathway blocked Nrf2 nuclear exclusion through β-Ecd-mediated antioxidation in MPTP/Pro-induced mice. If these findings translate to human patients,
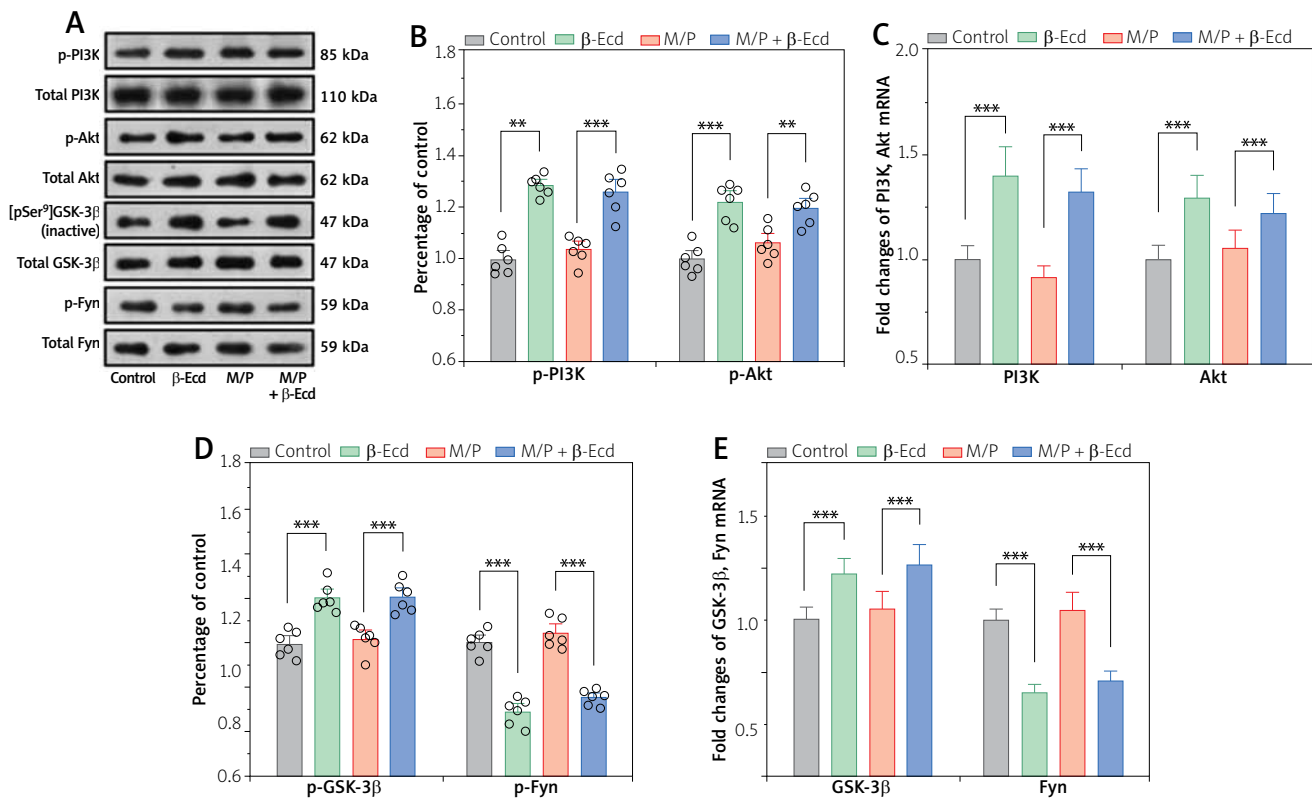

Fig. 5. β-Ecd restricts Nrf2 nucleus exclusion through the PI3K/Akt/GSK-3β/Fyn pathway. **A**) Western blot analysis of PI3K, Akt, GSK-3β, and Fyn expression. **B**) The expression levels of PI3K and Akt were analyzed quantitatively. **C**) RT-PCR was used to determine PI3K and Akt mRNA levels. **D**) The quantitative analysis of the protein levels of GSK-3β and Fyn. **E**) The mRNA expression levels of GSK-3β and Fyn were determined by RT-PCR. All data were presented as mean ±SD ($n = 6$). Statistical significance is denoted as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.
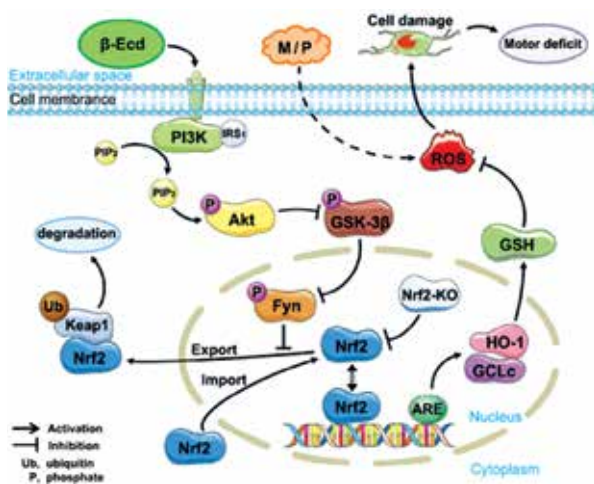
Fig. 6. Working model showing the molecular mechanism by which β-Ecd inhibits MPTP/Pro-induced oxidative stress. β-Ecd activates the PI3K/Akt signalling pathway, which in turn suppresses GSK-3β activation *via* phosphorylation and restrains Fyn activation through dephosphorylation. Subsequently, the nuclear exclusion of Nrf2 is impeded, and Nrf2 induces the transcription of ARE-driven target genes, including HO-1 and GCLc, to counteract the MPTP/Pro-induced oxidative stress.

β-Ecd could be a hopeful candidate for the treatment of neurobehavioral deficits in PD.

In the last few years, natural products have always been a dominating source for obtaining pharmacologically active antioxidants beneficial to some diseases, including PD [2]. Several studies have focused on plant-derived oestrogen antioxidants to mimic the structure and function of mammalian oestrogens, and determine their protective role in neurodegenerative diseases, such as PD. Shen *et al.* found that estradiol significantly modulates the level of GSH, ROS, and TRAP in the 6-OHDA-induced PD model [42]. Notably, both estradiol and β-Ecd can potentially alleviate oxidative stress in PD models; however, their mechanism of action is different. In our previous study, we found that plant-derived oestrogen, β-Ecd, could prevent the damage in chronic MPTP-induced PC12 cell model [59]. *In vivo* studies are well accepted and considered an essential method in PD research. Moreover, these studies provide an effective reference and guidance for clinical application and treatment [52]. The prime objective of drug therapy is to improve motor dysfunction in PD. The motor features of MPTP/Pro-induced mice are similar to the signs and symptoms of PD, suggesting that these are suitable models for discovering new drug candidates for PD [50].

Furthermore, long time administration of MPTP/Pro induces a large loss of dopaminergic neurons and significant motor dysfunction in chronic PD mouse models [20,26,27,55]. In this study, we found that β-Ecd attenuates the motor deficits in MPTP/Pro-induced WT mice. However, we did not observe the same results in MPTP/Pro-induced Nrf2-KO mice.

The oxidative stress resulting from the overproduction of ROS is hypothesized as a common mechanism that causes neurological function disorder [23]. Since the brain possesses a higher metabolic rate and lower cell regeneration ability, neurons are more vulnerable to the effects of ROS [7]. Generally speaking, the brain is more susceptible to oxidative injury as compared to other organs [34]. This may lead to the accumulation of various neurotoxins in the brain, which in turn contribute to neurodegenerative diseases [43]. Imbalances between oxidants and antioxidants cause oxidative stress, hence, it is necessary to apply antioxidant resistance to neurotoxins. Antioxidant therapy is a promising strategy to prevent and treat diseases caused by excessive exposure to ROS [21].

Dopamine is an important neurotransmitter in the brain. Lipid peroxidation can exacerbate the loss of dopaminergic neurons [5]. Epibiotic dopaminergic neurons in PD patients fail to effectively counteract the overproduction of ROS. This leads to dopaminergic neuron necrosis and the eventual progression of PD. It has been shown that in PD patients, levels of malondialdehyde, a product of lipid peroxidation, were significantly higher than normal levels [12]. In this study, we found that β-Ecd significantly reduced the loss of TH-positive dopaminergic neurons, and increased DAT expression in the SN of the ventral midbrain of MPTP/Pro-induced WT mice. β-Ecd also markedly restrained the increase of ROS, MDA, and LPO contents in the neurons of MPTP/Pro-induced WT mice. These results demonstrate that β-Ecd can counteract the oxidative stress in MPTP/Pro-induced mice. Furthermore, the reduced/oxidized GSH ratio observed in this study verifies the antioxidant potential of β-Ecd.

Nrf2 is important for cellular protection against oxidative damage [39]. It is composed of 605 amino acid residues in humans and is a specific transcription factor for ARE. Under normal conditions, the high-affinity ETGE motif and the lower-affinity DLG motif of the Neh2 domain of Nrf2 specifically bind to Keap1, resulting in the ubiquitination and degradation of Nrf2, and thus a decrease in Nrf2 stability [11]. In the presence of electrophiles and/or oxidative conditions, Keap1 undergoes a conformational change that blocks the Keap1-Cul3-E3 ubiquitin ligase complex from ubiquitinating Nrf2. The more stable Nrf2 moves into the nucleus and binds to ARE regions to regulate the tran-

scriptional activation of important downstream antioxidant enzymes. In this study, β-Ecd increased the nuclear levels of Nrf2 while decreasing its cytoplasmic levels. This indicates that the nuclear accumulation of Nrf2 plays a vital role in the antioxidant activity of β-Ecd. Simultaneously, Nrf2-KO mice were used to validate whether β-Ecd prevents the MPTP/Pro-induced oxidative stress and neuron apoptosis through Nrf2. Interestingly, in MPTP/Pro-induced Nrf2-KO mice, β-Ecd failed to improve the motor disorder and protect damaged TH-positive dopaminergic neurons. Furthermore, β-Ecd also failed to covalently modify the cysteine residues of Keap1. These data demonstrate that the Nrf2 signal participated in the neuroprotective effect of β-Ecd.

Increasing evidence has shown that the Nrf2/ARE signalling pathway is aberrant in PD. Consequently, it has been shown that the activation of Nrf2/ARE signalling may be a promising therapeutic strategy for PD patients [41]. HO-1 and GCLc are important endogenous phase II antioxidant enzymes, which play protective roles against free-radical damage. In the MPTP-induced PD mouse model, the Nrf2/ARE-driven genes, such as GCLc and HO-1, were up-regulated after caffeic acid phenethyl ester derivative intervention [19]. In this study, we evaluated whether β-Ecd enhances the expression of GCLc, GCLm, HO-1, and NQO1 in the SN. Surprisingly, β-Ecd only increased HO-1 and GCLc expression, but not NQO1 and GCLm, suggesting that some level of specificity was involved in the β-Ecd-mediated glutathione biosynthesis mechanism. Furthermore, the findings that β-Ecd was able to enhance the levels of HO-1 and GCLc mRNA in MPTP/Pro-induced WT mice, but failed to do so in the MPTP/Pro-induced Nrf2-KO mice, further solidified the hypothesis that β-Ecd specifically induces HO-1 and GCLc to participate in glutathione biosynthesis through an Nrf2-dependent manner. Overall, the findings of this study powerfully indicated that the antioxidant effects of β-Ecd were targeting the Nrf2 gene.

Numerous signalling pathways play important roles in regulating Nrf2 nuclear translocation. However, until now, it has still been unclear which of these pathways are vital. Previous studies have revealed that MAPK, PKC, PI3K/Akt and GSK-3β are involved in the regulation of Nrf2/ARE-driven cytokine expression [40,45]. Several findings showed that GSK-3β-mediated Nrf2 nuclear export is vital for blocking the antioxidant defensive reaction after brain damage [53]. Our results supported the hypothesis that β-Ecd affects the Akt/GSK-3β/Fyn signalling pathway. More specifically, we found that β-Ecd suppresses the activation of GSK-3β, which in turn reduced Fyn phosphorylation and subsequently restrained Nrf2 nuclear exclusion in MPTP/Pro-induced mice. The nucleus cumulation of Nrf2 activated

the ARE-driven HO-1 and GCLc gene transcription and translation and induced the *de novo* synthesis of GSH. It is quite clear that we cannot affirm causality among Akt, GSK-3β and Fyn after β-Ecd treatment. However, we found that β-Ecd increased the phosphorylation of PI3K/Akt and GSK-3β and decreased the phosphorylation of Fyn in untreated normal and MPTP/Pro-induced mice. Taken together, these findings demonstrate that β-Ecd activates the PI3K/Akt/GSK-3β/Fyn signalling pathway through a stimulus-independent manner.

In conclusion, we found that β-Ecd improved dyskinesia and reduced oxidative damage in MPTP/Pro-induced mice through an Nrf2-dependent manner. We propose a mechanistic model where β-Ecd inhibits Fyn phosphorylation *via* a GSK-3β-dependent manner, which further abolishes Nrf2 nuclear export, induces ARE-mediated HO-1 and GCLc factor transcription and translation and increases the synthesis of GSH (Fig. 6). Although the real mechanism of action is potentially more complicated than our proposed model, our findings, including our previous *in vitro* study data, suggest that β-Ecd may be a promising therapeutic candidate for PD.

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Ethics

Report from Animal Ethical Care Committee of Qiqihar Medical University (QMU-AECC-2021-13)

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Disclosure

The authors report no conflict of interest.

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