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Role of calbindin-D28K in estrogen treatment for Parkinson's disease★

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Abstract

Studies have shown that estrogen has neuroprotective effects on the nigrostriatal system. The present study established a Parkinson's disease model in C57BL/6 mice by intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrapyridine. The mice were subjected to 17β estradiol injection into the lateral ventricle. Immunofluorescence double staining showed that estrogen increased tyrosine hydroxylase and calbindin-D28K expression and co-expression in dopaminergic neurons of midbrain substantia nigra pars compacta of model mice. Behavior experiments showed that estrogen improved swimming and hanging behaviors in this mouse model of Parkinson's disease.

Key Words

neural regeneration; neurodegenerative diseases; estrogen; calbindin-D28K; Parkinson's disease; dopaminergic neuron; tyrosine hydroxylase; photographs-containing paper; neuroregeneration

Research Highlights

(1) Estrogen can protect dopaminergic neurons from 1-methyl-4-phenyl-1,2,3,6-tetrapyridine-induced injury in the brain.
(2) The neuroprotective effects were achieved by upregulating the calcium-binding protein expression pathway.

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INTRODUCTION

Increasing evidence indicates that degenerative death of dopaminergic neurons occurs in the substantia nigra pars compacta of Parkinson's disease patients, leading to a reduction of dopaminergic neurons in the corpus striatum[1-2]. This indicates that death of dopaminergic neurons in the substantia nigra participates in Parkinson's disease occurrence. Estrogenic deficiency in postmenopausal women worsens Parkinson's disease symptoms. Estrogen treatment can attenuate the early symptoms of Parkinson's disease in female patients[3]. Animal studies have also confirmed that endogenous and exogenous estradiol can protect dopaminergic neurons against neurotoxicity[4]. Estrogen has been reported to protect dopaminergic neurons in Parkinson's disease [5-6].

Dopaminergic neurons containing calbindin-D28K exhibit a strong ability to antagonize degeneration[7-10].

Calbindin-D28K, a member of the calcium-binding protein family, 28 kDa, exists in cytoplasm[11]. Calbindin-D28K functions as a Ca^{2+} buffer. For example, it co-exists and interacts with calcium pumps on cell membranes to regulate Ca^{2+}

transcellular activities in the distal collecting duct of the kidney and intestinal absorptive cells to control insulin release from islet cells[12-13]. Recent evidence indicates that calbindin-D28K is rich in the entire central nervous system and can significantly alter hippocampal synaptic interaction and neurotransmitter release[11, 14]. Studies have demonstrated that calbindin-D28K can bind caspase-3[10], Ran-binding protein M[15], and inositol monophosphatase[16-17] and alter their activites. Moreover, calbindin-D28K can inhibit cell apoptosis and protect cells[7].

Tyrosine hydroxylase mainly distributes in dopaminergic neurons and is a specific enzyme in dopaminergic neurons and noradrenergic neurons. It is a key enzyme in dopamine biological synthesis. Tyrosine hydroxylase-mediated dopamine metabolic regulation is linked to Parkinson's disease. Thus, detection of tyrosine hydroxylase expression could help identify the quantity of dopaminergic neurons[2]. Calcium-binding protein can protect dopaminergic neurons *via* a variety of approaches; thus, we hypothesize that the protective effects of estrogen may be associated with calcium-binding protein. The present study induced injuries of 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP) dopaminergic neurons[18] and was followed by a specific dose of estrogen to investigate the cytoprotective mechanism of estrogen.

RESULTS

Quantitative analysis of experimental animals

Thirty-six C57BL/6 mice were randomly assigned to control, Parkinson's disease and 17β estradiol (E2) groups, with 12 animals in each group. Parkinson's disease and E2 groups were intraperitoneally injected with MPTP to establish Parkinson's disease models; the E2 group was additionally injected with E2 in the lateral ventricle. During model establishment, five mice from the Parkinson's disease group and three from E2 group died possibly because of sensitivity to MPTP. Finally, 36 mice (n = 12 each) were included in the final analysis following supplementation.

Estrogen improved behaviors of Parkinson's disease mice

The hanging test and swimming test showed that the swimming ability and hanging duration were significantly reduced in the Parkinson's disease group compared with the control group (P < 0.05), while swimming ability and hanging duration were significantly increased in the E2 group compared with Parkinson's disease mice (P < 0.05), but were lower than the controls (P < 0.05; Figure 1).

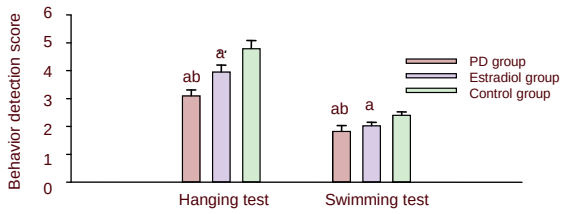

Figure 1 Influence of estrogen on behaviors of C57BL/6 mice with Parkinson's disease (PD).

In the hanging test, the time hanging at the metal bar was used as the index; in the swimming test, the floating duration was used to evaluate the behavior of mice. Low scores represent severe neurological deficits in the mice.

Data are expressed as mean ± SEM of 12 mice in each group. Intergroup differences were compared using the least significant difference *t*-test. [a]P < 0.05, *vs*. control group; [b]P < 0.05, *vs*. estradiol group.

Estrogen increased co-expression of tyrosine hydroxylase and calbindin-D28K in dopaminergic neurons of midbrain substantia nigra pars compacta of Parkinson's disease mice

Fluorescent immunocytochemistry showed that the number of cells co-labeled with tyrosine hydroxylase and calbindin-D28K was reduced in Parkinson's disease mice compared with the control group (P < 0.05), but was increased in mice injected with E2 (P < 0.05), which was lower than that in the control group (P < 0.05; Figure 2).

Estrogen increased protein expression of tyrosine hydroxylase and calbindin-D28K in dopaminergic neurons of midbrain substantia nigra pars compacta of Parkinson's disease mice

Western blot showed that tyrosine hydroxylase and calbindin-D28K protein expression in the midbrain substantia nigra was reduced in the Parkinson's disease group compared with the control group (P < 0.05); E2 injection increased tyrosine hydroxylase and calbindin-D28K protein expression in the midbrain substantia nigra (P < 0.05), but the expression remained lower than that in the control group (P < 0.05; Figure 3).

DISCUSSION

A number of studies have demonstrated the neuroprotective effects of estrogen in the nigrostriatal system[19]. However, the mechanism of action remains poorly understood. Pasqualini *et al* [20] reported that the protective effects of estrogen on dopaminergic neurons mainly contribute to effects of anti-oxidative stress,

increasing tyrosine hydroxylase activity to stimulate dopamine synthesis and block injury induced by toxicants such as 6-hydroxydopamine to dopaminergic neurons in the midbrain substantia nigra[21-22].

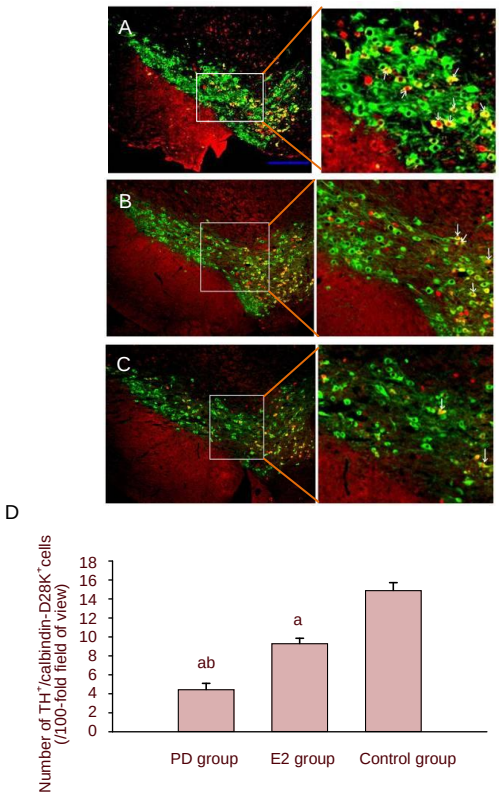

Figure 2 Co-expression of tyrosine hydroxylase (TH) and calbindin-D28K in dopaminergic neurons of midbrain substantia nigra pars compacta of C57BL/6 mice.

(A–C, × 100) Expression of TH and calbindin-D28K in dopaminergic neurons of midbrain substantia nigra of mice as observed by immunofluorescence staining in control, estradiol (E2), and Parkinson's disease (PD) groups. The magnification of blocks was × 300.

TH-positive cells were labeled with fluorescein isothiocyanate, stained green; calbindin-D28K-positive cells labeled with Cy3, stained red; yellow-stained cells (arrows) represent co-expression of TH and calbindin-D28K.

(D) Quantitative analysis of co-expression of TH and calbindin-D28K. Data are expressed as mean ± SEM. Intergroup differences were compared using least significant difference *t*-test. Experiments were conducted in triplicate. $^{a}P < 0.05$, *vs*. control group; $^{b}P < 0.05$, *vs*. E2 group.

But Sawada *et al* [23] proposed that estrogen protected dopaminergic neurons through anti-oxidation and anti-apoptosis. In addition, its influence on mitochondrial function may also be involved in the neuroprotection[24]. The present study utilized Parkinson's disease model mice and injected estrogen into the lateral ventricle. Results showed that the abilities of mice in swimming and hanging tests were significantly increased in the E2 group compared with Parkinson's disease mice but were lower than normal mice, indicating estrogen can attenuate MPTP-induced Parkinson's disease symptoms but cannot completely antagonize MPTP-induced injury in mice.

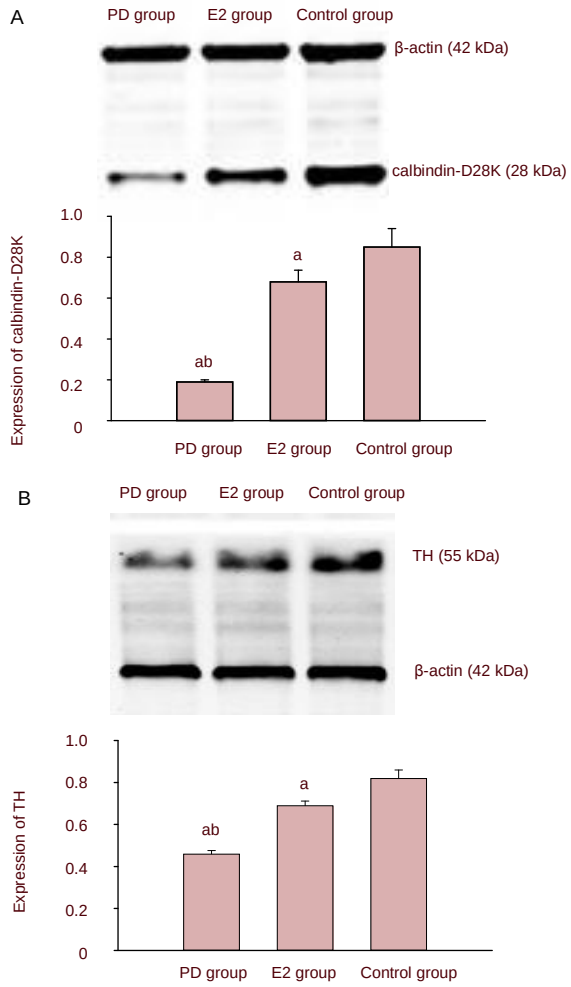

Figure 3 Expression of calbindin-D28K (A) and tyrosine hydroxylase (TH, B) in dopaminergic neurons of midbrain substantia nigra of C57BL/6 mice (western blot assay).

Data are expressed as mean ± SEM. Intergroup differences were compared using least significant difference *t*-test. $^{a}P < 0.05$, *vs*. control group; $^{b}P < 0.05$, *vs*. estradiol (E2) group. Experiments were conducted in triplicate.

Studies demonstrated that estrogen can rapidly activate PI3-K/Akt signaling pathway[24-26], rapidly phosphorylating cAMP response element-binding protein, inhibiting glycogen synthase kinase 3 activity, and upregulating Bcl-2 protein expression[27-29]. Among various brain injuries, estrogen may exert neuroprotective effects *via* immunological factors[30]. Recently, a kind of seven-transmembrane domain receptor, G-protein-coupled receptor 30, has been used as an estrogen receptor[31-32] and functions by binding E2[33]. Sun *et al* [34] reported that the neuroprotective

effects of calcium-binding protein involve the PI3-K/Akt signaling pathway. Calcium-binding protein synthesis is associated with neuronal cytoskeleton formation, which is Ca^{2+} dependent; excessive intracellular Ca^{2+} can destroy cytoskeleton formation[35]. Ultrastructure showed that calbindin-D28K distributes in the intracellular matrix and cytoskeleton. It can bind intracellular Ca^{2+} to reduce the influence of a high concentration of Ca^{2+} on neurons and protect neuronal activity and function.

To further investigate the mechanism of estrogen neuroprotection, the present study utilized immunofluorescence and western blot assay to observe the influence of estrogen on dopaminergic neurons in the substantia nigra. The number of dopaminergic neurons and calbindin-D28K content were significantly increased in Parkinson's disease mice following administration of estrogen compared with the Parkinson's disease group but were less than in normal mice. This indicates that a large number of dopaminergic neurons are maintained following estrogen injection, and calbindin-D28K can protect dopaminergic neurons from injury. However, the mechanism by which estrogen upregulates calbindin-D28K requires further investigation.

In conclusion, estrogen can protect dopaminergic neurons by promoting calbindin-D28K expression. The present study provides experimental evidence for further investigation of the neuroprotective effects of estrogen.

MATERIALS AND METHODS

Design

A randomized, controlled, animal study.

Time and setting

The experiment was performed in the Laboratory of Neurobiology, Xuzhou Medical College, China from March 2010 to January 2012.

Materials

A total of 50 male C57BL/6 mice of clean grade, aged 8–9 weeks, weighing 20–25 g, were provided by the Laboratory Animal Center of Xuzhou Medical College (license No. SCXK (Su) 2005-0005; use No. SYXK (Su) 2005-0018). They were housed in a shielded environment at 24 ± 3°C with a day/night cycle and were allowed free access to food and water. Experimental procedures were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[36].

Methods

Establishment of a subacute injury model in dopaminergic neurons

MPTP injection in mice can induce degenerative changes in dopaminergic neurons of the substantia nigra pars compacta[18]. E2 and Parkinson's disease groups were intraperitoneally injected with MPTP (Sigma, St. Louis, MO, USA), 30 mg/kg, at 8:00 a.m. daily for 5 consecutive days. The control group was intraperitoneally injected with normal saline.

Estrogen injection in the lateral ventricle

In addition to MPTP, the E2 group was injected with E2 (Sigma; 0.2 μg/μL) into the lateral ventricle[37-38] daily, 5 μL, for 14 consecutive days.

Behavior tests

Behavior tests were conducted at 14 days after E2 injection.

Swimming test: The mice were placed in a water tank of organic glass, 20 cm × 30 cm × 20 cm, filled with water to a depth of 10 cm at 22–25°C. Score grading was as follows: continuously swimming in 1 minute for 3.0 scores; swimming for most of the time, with occasional floating for 2.5 scores; floating for half of the testing time for 2.0 scores; swimming occasionally for 1.5 scores; floating at one side of the tank and swimming occasionally with the hind limbs for 1.0 score. The testing was conducted five times, with a 1-minute interval between the two tests. The mean value was calculated[39].

Hanging test: The hanging box was made of organic glass, with a horizontal metal rod 115 mm in diameter and 30 cm from the ground. There was a cover 1 cm above the metal rod to prevent the mice sitting on the rod. The mouse was placed hanging on the metal rod, and duration to falling to the ground was recorded: 0–4 seconds, 0 score; 5–9 seconds, 1 score; 15–19 seconds, 3 scores; 20–24 seconds, 4 scores; 25–29 seconds, 5 scores; and more than 30 seconds, 6 scores. The test was conducted every 2 minutes, five times. The mean value was calculated[39].

Immunofluorescence double-labeling detection for tyrosine hydroxylase and calbindin-D28K expression in the substantia nigra of mice

At 2–3 days after administration, 12 mice from each group were subjected to behavior tests. Following the tests, six mice from each group were selected and subjected to left ventricle-ascending aortic cannulation

and perfusion, washed with normal saline, and perfused with 4% paraformaldehyde. Following perfusion, the mice were sacrificed, and the brain was harvested. The midbrain was dissected through the mammillary body and superior and inferior colliculus of midbrain. The midbrain samples containing the substantia nigra were collected, postfixed in 4% paraformaldehyde for 12–24 hours, washed with water, dehydrated, embedded, and sectioned using a paraffin microtome at 6 μm thickness. The sections were dewaxed, rehydrated, retrieved with microwave, blocked with goat serum, incubated with rat anti-mouse tyrosine hydroxylase monoclonal antibody (1:1 000; Sigma) and rabbit anti-calbindin-D28K polyclonal antibody (1:500; Sigma), diluted with PBS, and stored in a wet box at 4°C for one or two nights. The sections were then washed with 0.01 M PBS, three times at 5 minutes each, incubated with Cy3-labeled goat anti-rabbit antibody (1:600; Sigma) and fluorescein isothiocyanate-labeled goat anti-rat antibody (1:100; Sigma) at 4°C overnight in the dark, rewarmed at 37°C for 1 hour, washed with 0.01 M PBS, 5 minutes each for five times, and mounted with fluorescence mounting medium. The number of cells co-labeled with tyrosine hydroxylase and calbindin-D28K was quantified under light microscope (Olympus, Shinjuku, Japan).

Western blot assay for tyrosine hydroxylase and calbindin-D28K expression in dopaminergic neurons of the substantia nigra of mice

Mice were sacrificed 2–3 days following administration, and the substantia nigra tissues were harvested immediately and stored at –80°C. The brain tissues were weighed, homogenized with homogenate (100 mg brain tissues: 1 mL homogenate), and centrifuged at 10 000 × *g* at 4°C for 10 minutes. The supernatant was collected, and protein was determined and packaged. According to a previously described method[14], equal volumes of protein samples were electrophoretically separated using 10% sodium dodecyl sulfate polyacrylamide gel, electrotransferred to nitrocellulose membranes. The membranes were blocked using 5% defatted milk powder, incubated with rat anti-mouse tyrosine hydroxylase monoclonal antibody (1:1 000; Sigma), rabbit anti-calbindin-D28K polyclonal antibody (1:500; Sigma), and rat anti-mouse β-actin monoclonal antibody (1:100; Sigma) at 37°C for 1 hour. The membranes were washed, and protein gray scale was scanned using the Odyssey laser imaging system (LI-COR, USA). Results were represented by the gray scale ratio to β-actin.

Statistical analysis

Data were expressed as mean ± SEM and analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used for statistical analysis, and intergroup comparison was conducted using least significant difference *t*-test. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Chunhua Wang conceived and designed the study and provided and integrated the experimental data. Chao Jiang analyzed the experimental data, wrote the manuscript, and provided technical support. Honghua Yuan and Dianshuai Gao revised the manuscript. Chenghua Xiao was in charge of funds and statistical analysis. Dianshuai Gao guided the study. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the the Animal Care and Research Committee of Xuzhou, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

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