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Original Article

Downregulation of ATP13A2 in midbrain dopaminergic neurons is related to defective autophagy in a mouse model of Parkinson's disease

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Abstract: Parkinson's disease (PD) is one of the most common diseases of the nervous system characterized by movement disorders arising from loss of midbrain dopaminergic neurons. The relationship between PD and autophagy has received considerable attention. This study aimed to investigate the involvement of the ATP13A2 gene in damage of dopaminergic neurons induced by abnormal autophagy in a MPTP-induced PD mouse model. MPTP was intraperitoneally injected into C57BL mice at 40 mg/kg for 7 days in experimental group. Saline was injected into mice in the control group. After the injection, the mice were tested at different time points for abnormal limb movement by a swimming test. The brain tissue was collected on day 1, 5, and 7 to measure concentration of intracellular calcium. The expression of ATP13A2 was evaluated by real-time PCR. The expression of α-synclein, LC3, LAMP-2, and CaMKK protein was detected by western blot. We found significant motor dysfunction on day 7 in the experimental group, and the expression of α-synclein in the substantia nigra of the midbrain was significantly increased while the expression of ATP13A2 gene was reduced significantly compared with the control group. The concentration of intracellular calcium in the experimental group was significantly higher than in the control group. Autophagy associated proteins LC3-II and LAMP-2 were downregulated and CaMKK protein was upregulated in midbrain tissues of the experimental group compared to control group. In conclusion, our findings suggest that decreased expression of ATP13A2 may lead to defective autophagy and damage to midbrain dopaminergic neurons.

Keywords: ATP13A2, Parkinson's disease, autophagy, midbrain

Introduction

Parkinson's disease (PD) is the second largest neurodegenerative disease characterized by dopaminergic neuron loss and Lewy body (LB) accumulation in the substantia nigra neurons, and its pathogenesis is related to genetic and environmental factors [1]. It is widely believed that aggregation of toxic alpha-synuclein (α-Syn) plays an important role in PD [2]. The mechanism of α-Syn accumulation in dopaminergic nerve cells has not been fully clarified, but autophagy can promote α-Syn degradation and reduce α-Syn aggregation, and autophagy defects are important in the pathogenesis of PD [3]. Neuropathologic features in mice with the knockout of autophagy essential gene Atg7 are similar to brain tissue in PD [4].

The ATP13A2 gene is one of the genes involved in the pathogenesis of PD and is located in the Ip36 PARK 9 locus, encodes a lysosomal ATPase transporter and participates in protein degradation by the autophagy and lysosomal pathways [5]. ATP13A2 mutations can cause dysfunction in autophagic vacuole (AV)-lysosomal fusion, resulting in a significant loss of lysosomal-mediated degradation of intracellular proteins such as α-Syn, and toxic α-Syn polymer formation is related to PD dopaminergic nerve injury [6].

Therefore, we aimed to investigate the involvement of ATP13A2 gene in the damage of dopaminergic neurons induced by abnormal autophagy in a PD mouse model.

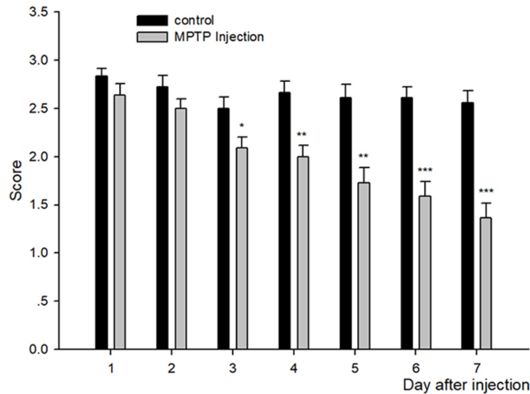


Figure 1. Motor function in mice tested by swimming test. The swimming score was compared between MPTP group and control group. *P<0.05 compared to control group at three days. **P<0.01 compared to the control group at four days and five days, and ***P<0.001 compared to control group at six days and seven days.

Materials and methods

Animals

Animal experiments were performed at No. 4 Affiliated Hospital of Jiangsu University and the protocols were approved by the Animal Care and Use Committee of No. 4 Affiliated Hospital of Jiangsu University. Forty healthy male C57BL mice (12 weeks old and weight 25-30 g) were purchased from Haipula Biotechnology Co., Ltd. and maintained with daily light 14 h and dark 10 h at 22°C with free access to food and water. The animals were randomly divided into two groups, A and B. Group A: intraperitoneal injection of 40 mg/kg 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) for 7 consecutive days; Group B: intraperitoneal injection with Brine for 7 consecutive days and was sacrificed on 7th day. Group A mice were randomly divided into 3 groups, which were 1st day, 5th day, and 7th day. At each time point, the midbrain specimens were collected.

Calcium concentration assay

Ca^{2+} concentration was measured using Calcium Assay Kit (Abcam) following the manufacturer's instructions.

Western blot analysis

The midbrain tissues were homogenized and the lysates were centrifuged at 12,000 rpm for 10 min. The supernatant was collected and subjected to SDS-PAGE, and then transferred onto PVDF membrane. The membrane was blocked in 5% skim milk and incubated with antibodies to LC3 (Abgent Biologicals, Littleton; 1:1,000), LAMP-2 (Cell Signaling Technology, Danvers, MA, USA; 1:500), CaMKK (Cell Signaling Technology; 1:500) and α-synclein (Abcam, USA; 1:500) overnight at 4°C. After washing the membrane was incubated with HRP-labeled goat anti-rabbit IgG (BioBMW, GB23303, 1:3,000) for 2 h at room temperature. After washing the membranes were developed with ECL method.

Real-time PCR

Total RNA was extracted from midbrain tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with a first-strand cDNA synthesis kit (Applied Thermoscientific, USA) according to the manufacturer's instructions. Next, fluorescence quantitative PCR was performed using SYBR Green PCR amplification master mix (TaKaRa, Dalian, China) according to the manufacturer's protocol. The reaction conditions were as follows: initial denaturation at 95°C for 30 s and then 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 34 s (ABI 7500 system, USA). The primers were synthesized by Songon (Shanghai, China). mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method and normalized to β-actin.

Statistical analysis

Data were expressed as mean ± sem, and one-way ANOVA was used for comparison among groups with SPSS19.0 software. P<0.05 was considered statistically significant.

Results

Establishment of MPTP mouse model

Behavioral test showed that C57BL mice injected with MPTP had a poor swimming score following prolonged days of injection, indicating that the MPTP model mice showed abnormal motor function compared with the control group (**Figure 1**).

Accumulation of α-synclein and downregulation of ATP13A2 in midbrain tissues of MPTP mice

The expression of α-synclein protein was detected by western blot in the midbrain tissues,

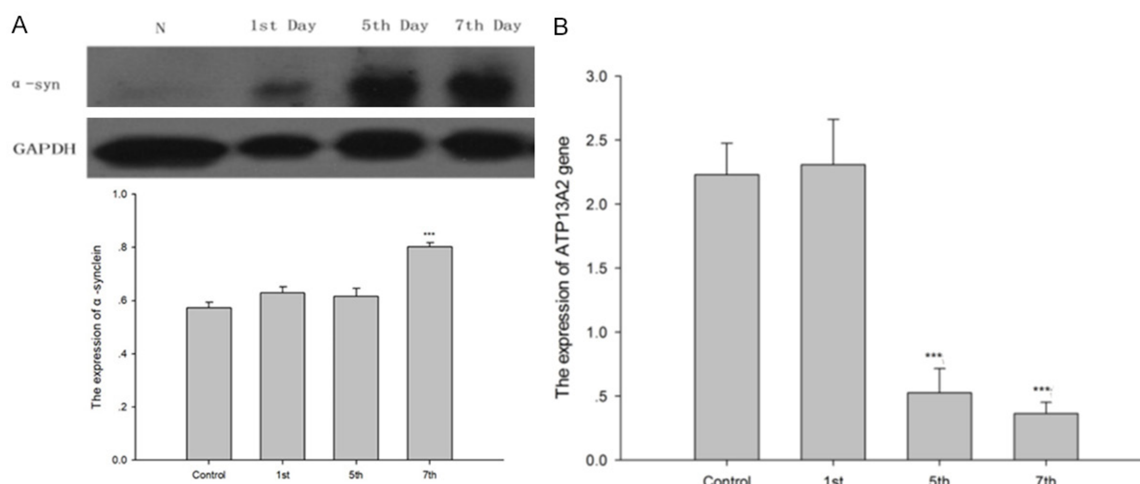


Figure 2. α-synclein and ATP13A2 expression levels in midbrain tissues. A. Western blot analysis of α-synclein protein expression in the midbrain tissues of control group and MPTP group. GAPDH was used as loading control. ***P<0.001 compared to control group (n=5). B. Real-time PCR analysis of ATP13A2 mRNA expression in the midbrain tissues of control group and MPTP group. ***P<0.001 compared to control group (n=5).

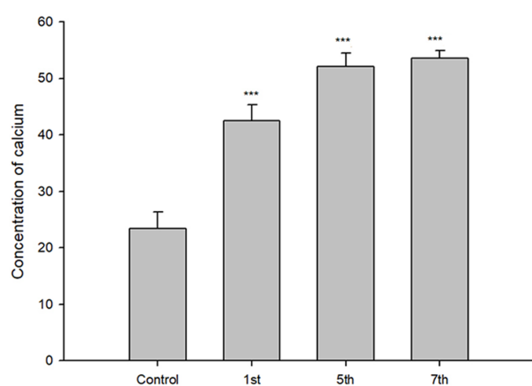


Figure 3. Concentration of calcium in midbrain tissues. Calcium concentration in the midbrain tissues of the control group and the MPTP group were measured. ***P<0.001 compared to control group (n=5).

and the results showed that the expression of α-synclein was significantly increased after continuous injection of MPTP for seven days, compared with the control group (**Figure 2A**). Real-time PCR was used to detect the expression of ATP13A2 in midbrain tissues. The results showed that ATP13A2 expression was significantly lower in the MPTP group than in the control group (**Figure 2B**).

Enhanced intracellular calcium release in midbrain tissues of MPTP mice

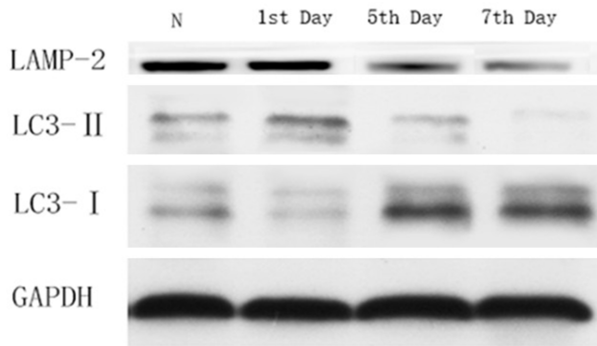
The concentration of intracellular calcium in midbrain tissue of mice with continuous injection of MPTP for 7 days was higher than that of the control group (**Figure 3**).

Downregulation of autophagy-related-protein LC3-II and LAMP-2 in midbrain tissues of MPTP mice

The microtubule-associated protein-1 light chain 3 (LC3) is an autophagy-related protein and the conversion from LC3-I to LC3-II is considered to be an important marker of autophagosome formation [7]. The lysosome-associated membrane protein (LAMP-2) is important in the maturation of autophagosomes and the fusion with lysosomes [8]. Therefore, we detected the levels of LC3-II/LC3-I and LAMP-2 in the midbrain tissues. The results showed that the levels of LC3-II/LC3-I and LAMP-2 in the midbrain tissues were significantly lower in the MPTP group than in the control group (**Figure 4**).

Upregulation of CaMKK in midbrain tissues of MPTP mice

Calmodulin-dependent protein kinase kinase (CaMKK) is an upstream signaling protein of AMPK. Activation of CaMKK can activate AMPK to inhibit the synthesis of autophagy-associated proteins [9]. The expression of CaMKK protein in midbrain tissues of the mice in two groups was detected. The results showed that the expression of CaMKK was significantly increased in mice continuously injected with



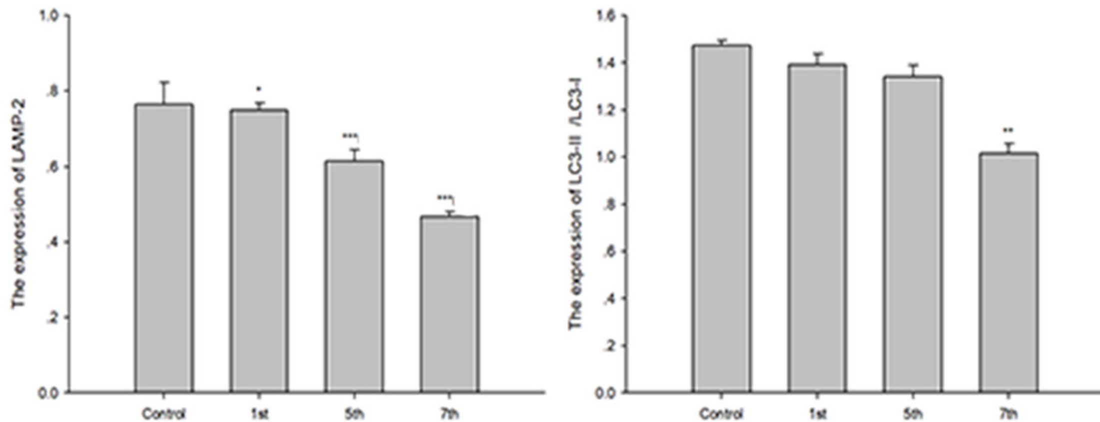
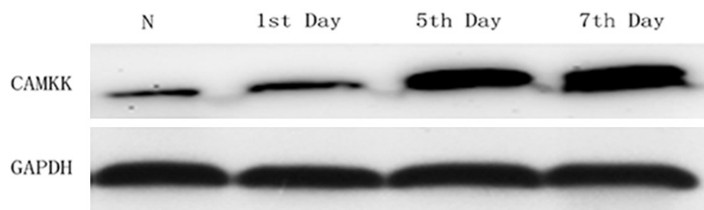


Figure 4. LAMP-2, LC3-II and LC3-I levels in midbrain tissues. Western blot analysis of LAMP-2, LC3-II and LC3-I protein expression in the midbrain tissues of control group and MPTP group. GAPDH was used as loading control. *P<0.05, **P<0.01, ***P<0.001 compared to control group (n=5).



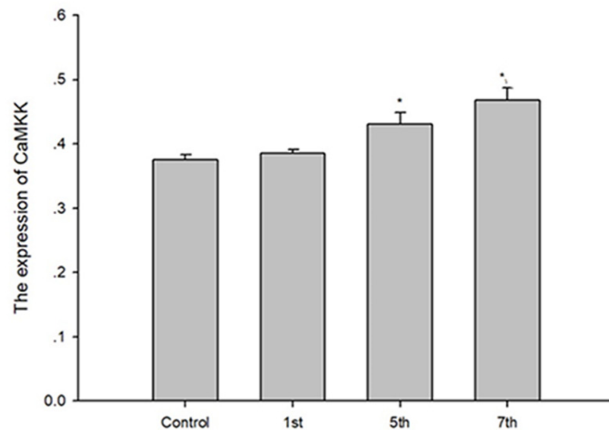


Figure 5. The expression of CaMKK in midbrain tissues. Western blot analysis of CaMKK protein expression in the midbrain tissues of control group and MPTP group. GAPDH was used as loading control. *P<0.05 compared to control group (n=5).

MPTP compared with the control group (**Figure 5**).

Discussion

PD is the most common type of neurodegenerative disease, and it is mainly caused by a decrease in dopaminergic activity in the substantia nigra region and the aggregation of α-synclein in the perinuclear space [5]. PD is also accompanied by a variety of non-motor symptoms, including sleep, autonomy, sensory, cognitive, and mental disorders. Our results demonstrated significant behavioral abnormalities on day 7 after intraperitoneal injection of MPTP, consistent with symptoms of the exercise system of PD. We also found that expression of α-synclein was significantly higher in the substantia nigra region in mice injected with MPTP, suggesting that the model can mimic the characteristics of pathologic damage in PD patients. In addition, we found that the concentration of intracellular calcium in the midbrain dopaminergic neurons increased after the injection of MPTP.

Autophagy is implicated in a variety of diseases including PD [10, 11]. We examined the levels of autophagy-associated protein LC3-II and LAMP-2 and found that LC3-II/LC3-I and LAMP-2 levels in the midbrain tissues of MPTP mice were significantly lower than in the control group. These results suggest that there is an autophagy defect in the substantia nigra of MPTP mice, which can cause a clearance disorder of intracellular toxic α-synclein.

ATP13A2 is mainly distributed in the substantia nigra of the midbrain, and its abnormal expression is related to the pathogenesis of PD [12]. Our experiment revealed that the expression of ATP13A2 in the midbrain tissues of MPTP mice was significantly lower than in the control group. A previous study reported that overexpression of ATP13A2 in the rat embryonic midbrain can protect dopaminergic nerve against α-synclein toxicity, which suggested that decreased expression of ATP13A2 may lead to the formation of toxic α-Syn polymer [13]. Dehay et al. found that in the fibroblasts of patients with ATP13A2 gene mutations the fusion of intracellular autophagic vacuoles (AV) with lysosomes was impaired, resulting in a significant loss of lysosomal-mediated degradation of intracellular proteins [3]. In human dopaminergic neurons, the expression of α-Syn was increased and cell viability was decreased after ATP13A2 knockdown, while overexpression of wild-type ATP13A2 protein can rescue lysosomal defects in ATP13A2 mutant fibroblasts [14]. It has been reported that lysosomal dysfunction, α-Syn accumulation, and α-Syn neurotoxicity are caused by a deficiency in ATP13A2 in the cortical structure [15]. These results suggest that cells with ATP13A2 gene mutation or downregulation may be characterized by decreased autophagosome clearance and increased accumulation of toxic α-Syn polymer, which may lead to dopaminergic nerve injury in PD.

ATP13A2 encodes a lysosomal P-type ATPase involved in the selective active transport of cation transmembranes [16]. ATP13A2 is resistant to Ca^{2+} induced cytotoxicity [17]. Clinical studies have found that the release of endoplasmic reticulum Ca^{2+} is manifestly elevated in the fibroblasts in PD patients. We observed an increase in intracellular Ca^{2+} concentration in MPTP-induced PD mice, and the expression of ATP13A2 was decreased compared with control group, suggesting that the downregulation of ATP13A2 gene in the midbrain of MPTP model leads to intracellular calcium release.

Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) is an important kinase to regulate autophagy in metabolic stress [18]. The upstream kinase Ca^{2+}/Calmodulin-dependent protein kinase kinase (CaMKK) is mainly expressed in the nervous system, where increased intracellular Ca^{2+} can activate CaMKK and inhibit the synthesis of autophagy-related proteins and intracellular autophagy pathway [19]. In this study, we found that CaMKK expression was increased in MPTP mice, consistent with the higher Ca^{2+} concentration and the inhibition of apoptosis in these mice.

In summary, our findings suggest that decreased expression of ATP13A2 may lead to defective autophagy and damage to midbrain dopaminergic neurons.

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Disclosure of conflict of interest

None.

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