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Original Research

Icariin Inhibits Overexpression and Aggregation of α-Synuclein in A53T α-Synuclein Transgenic Mice by Regulating Parkin and PLK2

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Abstract

Background: Synucleinopathies, which are major pathological features of Parkinson's disease (PD), are characterized by misfolded aggregates of α-synuclein in the peripheral and central nervous system. Icariin (ICA) is the main active component of *Epimedium* flavonoids. Our previous study found that ICA decreases α-synuclein expression in APPV717I transgenic mice. **Methods**: The aim of the present study was to examine the potential applications and mechanisms of ICA in PD using A53T α-synuclein transgenic (A53T Tg) mice. After 3 months of intragastric ICA administration, rotarod and pole tests were used to assess behavioral changes in A53T Tg mice at 8 and 13 months of age. SH-SY5Y cells over-expressing wild-type α-synuclein were used to further examine the pharmacological effect and underlying mechanism of ICA. Western blotting and immunocytochemistry were used to detect the expression levels of α-synuclein and its related proteins. **Results**: ICA significantly improved the impaired motor function and coordination in A53T Tg mice. It also decreased the expression, Ser129 phosphorylation, and aggregation of α-synuclein in SH-SY5Y cells transfected with α-synuclein and the striatum of A53T Tg mice. Moreover, ICA increased the expression of parkin, which is associated with the ubiquitin-proteasome system (UPS), and decreased the level of polo-like kinase 2 (PLK2), an enzyme that phosphorylates α-synuclein. **Conclusions**: ICA alleviated motor impairments in A53T mice, an effect which may be associated with the decreased phosphorylation and aggregation of α-synuclein through PLK2 and parkin regulation.

Keywords: icariin; synucleinopathy; α-synuclein; motor impairment; parkin; PLK2

1. Introduction

α-Synuclein plays an important role in the pathogenesis of several neurodegenerative disorders, including Parkinson's disease (PD), multiple system atrophy, and dementia with Lewy bodies. These disorders are also known as "synucleinopathies", which are characterized by abnormally misfolded and aggregated α-synuclein in the nervous system [1]. Currently, only palliative treatments addressing dopaminergic deficits have been approved, and no disease-modifying options are available for PD and related synucleinopathies [2]. However, strategies targeting misfolded α-synuclein aggregates are being considered as new therapeutic approaches [3].

α-Synuclein is a neuronal presynaptic protein regulating neurotransmitter release. Structurally, α-synuclein is a protein formed by 140 amino acids encoded by α-synuclein (*SNCA*). The A53T point mutation in *SNCA* was the first reported pathogenic modification associated with PD that underlies disease initiation [4]. Post-translational modifications of α-synuclein, such as phosphorylation, favor aggregation. In the healthy brain, only few α-synucleins are phosphorylated, whereas in pathological inclusions most α-synucleins are phosphorylated at serine 129 (Ser129), as in the Lewy bodies of the PD brain [5]. The polo-like kinase 2 (PLK2), which is a member of polo-like kinase (PLK) family, has been reported to phosphorylate α-synuclein at Ser129 and modulate its aggregation [6,7]. The ubiquitin-proteasome system (UPS) can eliminate unfolded or misfolded proteins including misfolded α-synuclein [8] and parkin plays a key role in the degradation of α-synuclein through the UPS degradation process [9]. Moreover, the expression level of parkin has been reported to decrease due to the overexpression of α-synuclein [10,11]. Thus, PLK2 and parkin might partly be involved in the modification and/or degradation of α-synuclein in synucleinopathies, which we investigated in this study.

Herba Epimedii is the dried leaf of the medicinal plant *Epimedii*, named Yinyanghuo in Chinese. Flavonoids extracted from *Epimedium* constitute the main active ingredient, showing neuroprotective and anti-inflammatory effects [12,13]. Among these flavonoids, icariin (ICA) is the most prominently active flavonoid. In recent years, ICA has been reported to show beneficial effects in several diseases of the central nervous system, including Alzheimer's disease (AD), PD, and multiple sclerosis [14–16]. Our previous study indicated that ICA decreases α-synuclein expression in the hippocampus of APPV717I transgenic mice, indicating a potential effect on PD and other synucleinopathies [17].

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Mutant A53T α-synuclein transgenic (A53T Tg) mice, which express the mutant A53T α-synuclein, were used to investigate mechanisms and pharmacology [18]. A53T Tg mice show obvious motor impairments at 8 months of age due to progressive pathological changes in α-synuclein [19,20]. As we have previously identified the potential effects of ICA on APPV717I transgenic mice, we used A53T α-synuclein transgenic mice of varied ages to observe the behavioral and pathological changes after ICA treatment to investigate the potential effects and mechanisms of ICA on the pathology of α-synuclein. Moreover, we used wild-type α-synuclein-transfected SH-SY5Y cells to investigate the pharmacological effect and potential mechanism of ICA on synucleinopathies.

2. Materials and Methods

2.1 Drugs

For animal studies, ICA (purity >98%) was purchased from Scidoor Hi-tech Biology (Xi'an, Shaanxi, China). ICA was diluted with normal saline and intragastrically administered to mice.

For the cell culture, ICA (purity >99%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). ICA was added to the cell culture medium after dilution with phosphate-buffered saline (PBS).

2.2 Animals

A53T Tg mice (B6. Tg (PDGF-h-α-synuclein A53T)-GC/ILAS, CSTR: 16397.09.0H01000945) were purchased from the Center for Experimental Animal Research, Chinese Academy of Medical Sciences (Beijing, China) [18]. Age-matched C57BL/6 (wild type, WT) mice were purchased from Beijing HFK Encapee (Beijing, China).

All mice were housed under a 12 h light/dark cycle with relative humidity of 55–60% and a temperature of 22 $\pm$ 2 °C and free access to water and food. Animal studies were approved by the Bioethics Committee of Xuanwu Hospital of the Capital Medical University (approval number: 20120912) and were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.3 Animal Treatment and Grouping

A53T Tg and WT mice of two different ages (5 and 10 months old) were allocated to two experimental groups. (1) 5-month-old A53T mice received a daily dose of ICA (either 50 or 100 µmol/kg) or saline (as the model group) over a period of 3 months; coetaneous WT mice were treated with normal saline or 100 µmol/kg ICA; n = 8–10 per group. (2) 10-month-old A53T Tg mice received a daily dose of ICA (either 50 or 100 µmol/kg) or saline (as the model group) over a period of 3 months; WT mice of the same age were treated with normal saline or 100 µmol/kg ICA; n = 10–12 per group. According to the molecular weight of ICA (676.65), the dose of 100 µmol/kg was converted to 67.7 mg/kg and 50 µmol/kg was converted to 33.8 mg/kg at the time of administration to the mice. All treatments were intragastrically administered.

2.4 Rotarod Test

The rotarod test (YLS-4C, Yanyi Life Science, Jinan, Shandong, China) was applied to evaluate the motor coordination and balance of the mice [21]. Mice were trained three times in 5-min trials before the test at a speed of 10 rotations per minute (rpm). The mice were then individually placed on the rotarod with a fixed speed (30 rpm) and cut-off time (180 s). The test was performed five times at intervals of at least 30 min, and the mean of the results was then calculated.

2.5 Pole Test

The pole test (XPS-2, Chinese Academy of Medical Sciences and Peking Union Medical, Beijing, China) was conducted to evaluate the coordination function of the mice [22]. The mice were placed head-up on the top of a pole with high rough-surface (height, 50 cm; diameter, 2 cm). The time taken for the 8-month-old mice to climb down the pole was recorded. The test was then performed in three trials at intervals of at least 30 min. Trials were excluded if the mouse jumped off or slid down the pole. The behaviors of the 13-month-old mice were observed as they descended from the top to the bottom of the pole and evaluated using a scoring method. Each mouse was allowed to descend the pole three times and the average score was then calculated. The scoring criteria for the pole test were set as follows: 5 points, use of all limbs to climb down the pole smoothly; 4 points, step-by-step downward spiral crawling, dragging the hind limbs; 3 points, pausing several times during the climb down but holding tightly to the pole; 2 points, sliding on the pole and falling off; and 1 point, inability to grab the pole, directly dropping.

2.6 Tissue Collection and Western Blotting

Four mice from each group were anesthetized using 2.5% Avertin (Sigma-Aldrich, St. Louis, MO, USA) and euthanized after behavioral testing. The brain was rapidly removed, and the striatum isolated and homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl, 0.5 mM ethylene glycol tetraacetic acid, 1 mM ethylenediaminetetraacetic acid, and a protease inhibitor cocktail (Cat. No. 04693116001; Sigma-Aldrich, St. Louis, MO, USA) [17,23]. Protein concentration was detected using an RC-DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA), and the protein samples were boiled for 5 min before storage.

Proteins were separated using sodium dodecyl sulfate (SDS)–Tris-glycine polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes. The following primary antibodies were used: mouse anti-

α-synuclein (Cat. No. ab1903; Abcam, Cambridge, UK), rabbit anti-p-α-syn (Ser129) (Cat. No. ab51253, Ser129-phosphorylated α-synuclein, Abcam), rabbit anti-parkin (Cat. No. P6248; Merck Millipore, Darmstadt, Germany), rabbit anti-PLK2 (snk, Cat. No. sc25421; Santa Cruz Biotechnoloy, Santa Cruz, CA, USA), and mouse anti-β-actin (Cat. No. A5316, Sigma-Aldrich). PVDF membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (1:2000). The immune complex was then visualized using enhanced chemiluminescence detection reagents (Cat. No. WBLUF0500, Merck Millipore). Images were captured using the FluorChem E System (Bio-Techne, Minneapolis, MN, USA) and analyzed using AlphaView software (Bio-Techne).

2.7 Cell Culture

The human dopaminergic cell line SH-SY5Y was purchased from the Cell Resource Center of Peking Union Medical College (Beijing, China). The cell line was previously authenticated by STR and tested for Mycoplasma infection by the Cell Resource Center of Peking Union Medical College. The results indicated that the cell line was derived from human and showed no mycoplasma contamination. The cells transfected with Green fluorescent protein (GFP)-tagged WT α-synuclein or vector (gifted by Prof. Hong Ma, Beijing Institute of Technology, Beijing, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cat. No. A1896701, Thermo Fisher Scientific, Fair Lawn, NJ, USA) supplemented with 10% fetal bovine serum (FBS) and 0.3 g/L G418 (Cat. No. A1720, diluted in 0.1 M HEPES, Thermo Fisher Scientific, Fair Lawn, NJ, USA) at 37 °C in an incubator humidified with 5% CO_2. All experiments were conducted in triplicate.

2.8 Cell Viability Assay

Cell viability was detected using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells (1×10^4 cells/mL) were seeded and incubated with different doses of ICA (1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µM) for 24 or 48 h. After incubation, MTT was mixed with the medium at a final concentration of 0.5 mg/mL. The MTT solution was removed after 4 h incubation at 37 °C, and 200 µL dimethyl sulfoxide (DMSO) was added to completely dissolve the formazan crystals. The absorbance of each well was measured at 570 nm using a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific, Fair Lawn, NJ, USA). Cell viability was calculated as follows: absorbance (optical density, OD) of the drug-treated groups/absorbance of the vehicle-treated group.

For the western blot assay, 2×10^5 cells were seeded in a flask and treated with ICA for 48 h. Cultured cells were harvested and lysed to obtain the protein.

2.9 Immunocytochemistry

Cells were seeded in 24-well plate and treated with 40 µM ICA for 48 h. After removing the culture medium, cultured cells were fixed in by 4% paraformaldehyde and 0.1% Triton X-100 for 30 min and then washed three times in 0.01 M PBS. After blocking with serum, the cultured cells were incubated with mouse anti-α-synuclein (1:200, Cat. No. ab1903, Abcam, Cambridge, UK) at 4 °C overnight. After washing in PBS, the fixed cells were incubated with goat anti-mouse IgG (Alexa Fluor 594, Cat. No. A-11005, 1:200, Thermo Fisher Scientific) and Hoechst33342 (Cat. No. R37165, Thermo Fisher Scientific). The cells were covered with mounting medium before visualization using a Nikon 80i microscope (Nikon, Tokyo, Japan).

2.10 Statistical Analyses

All data were analyzed using SPSS software (version 20.0, IBM Corp, Armonk, NY, USA). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc comparisons to identify significant differences among groups. Numerical data are provided as the mean $\pm$ standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

3. Results

3.1 ICA Attenuated Behavioral Dysfunction in A53T α-Synuclein Transgenic Mice at 8 and 13 Months of Age

The rotarod test was applied to detect motor balance and coordination in A53T Tg mice after daily intragastric administration of ICA for 3 months. Both the 8- and 13-month-old A53T Tg mice fell off the rotarod at 30 rpm quicker than the WT control mice, with the 13-month-old A53T Tg group showing a statistically significant difference ($p < 0.05$, Fig. 1A,B). ICA (50 and 100 µmol/kg) treatment increased the time to fall off the rotarod in both age groups of A53T Tg mice, with the ICA (100 µmol/kg) treatment in the 8-month-old A53T Tg group showing a statistically significant difference ($p < 0.05$, Fig. 1A,B).

The pole test assesses the coordination ability of mice. The 8-month-old A53T Tg mice climbed down the pole quicker than the WT control mice, whereas ICA-treated A53T Tg mice took longer to climb down the pole than the vehicle-treated A53T Tg mice, albeit without statistically significant differences (Fig. 1C). For 13-month-old mice, we evaluated the pole test performance score. A53T Tg mice had lower scores than the WT group ($p < 0.01$), and ICA (50 and 100 µmol/kg) treatment significantly increased the scores of A53T Tg mice ($p < 0.05$, $p < 0.01$, Fig. 1D). These results indicate that ICA was able to alleviate impaired motor function and coordination in A53T Tg mice at 8 and 13 months of age.

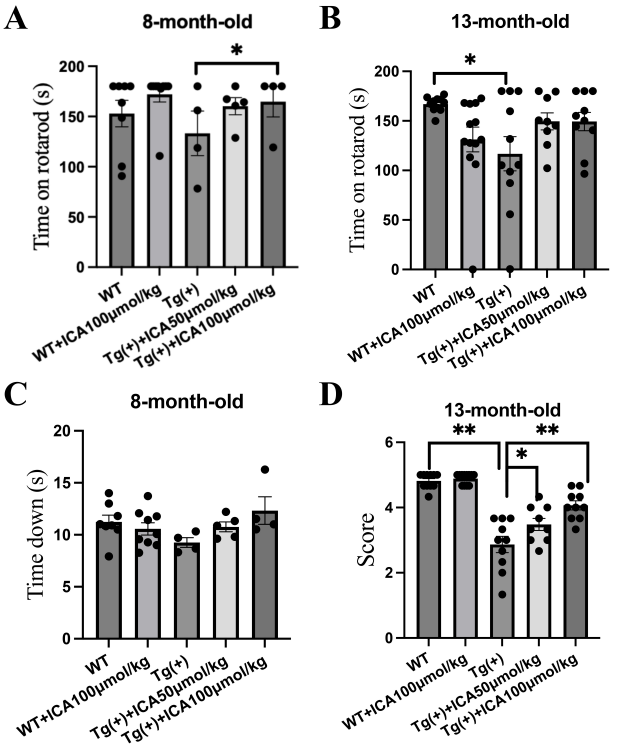

Fig. 1. ICA attenuates behavior dysfunction in young and aged A53T α-synuclein transgenic mice. The rotarod and pole tests were applied to assess the motor balance and coordination of the A53T Tg mice after intragastric administration of ICA for 3 months. (A) Time on the rotarod (latency to fall off the rotarod) of the 8-month-old mice in the rotarod test. (B) Time on the rotarod of the 13-month-old mice in the rotarod test. (C) Pole test; time spent climbing down the pole by the 8-month-old mice. (D) Pole test; performance score for climbing down the pole of mice at 13 months of age. Data are provided as the mean ± SEM, n = 4–9 per group (8-month-old mice), n = 9–12 per group (13-month-old mice). *$p < 0.05$, **$p < 0.01$, *vs.* model group. ICA, icariin; SEM, standard error of the mean; Tg (+), A53T α-synuclein transgenic mice; WT, wild type.

3.2 ICA Decreased the Expression Level and Aggregation of α-Synuclein in the Striatum of A53T Tg Mice

We used western blotting to detect the expression levels of α-synuclein in the striatum of 8-month-old and 13-month-old mice. The results showed that the levels of α-synuclein monomers and polymers were increased in the striatum of A53T Tg mice compared with WT mice at 8 months of age ($p < 0.05$, Fig. 2A,B). ICA treatment at doses of 50 and 100 μmol/kg significantly decreased the level of α-synuclein monomers ($p < 0.01$), with 100 μmol/kg ICA treatment significantly decreasing the level of α-synuclein polymers in the striatum of A53T Tg mice at 13 months of age ($p < 0.05$, Fig. 2C,D). These results indicate that ICA reduced the expression and aggregation of α-synuclein in the striatum of A53T mice.

3.3 ICA Decreased the Phosphorylation of α-Synuclein at Serine 129 in the Striatum of A53T Tg Mice

Phosphorylation of α-synuclein at Ser129 is an important marker of pathological forms of PD and related synucleinopathies. A53T Tg mice at 8 and 13 months of age showed elevated phosphorylation levels of α-synuclein at Ser129 in the striatum compared with WT mice ($p < 0.01$, Fig. 3). ICA treatment (100 μmol/kg) significantly decreased the phosphorylation level of α-synuclein at Ser129 in the striatum of A53T Tg mice at 8 months old ($p < 0.01$, Fig. 3A,B), but no significant difference was observed in A53T Tg mice at 13 months old (Fig. 3C,D). These results indicate that ICA decreased the phosphorylation of α-synuclein, which might inhibit the formation of pathological α-synuclein.

3.4 Effects of ICA on the Expression and Phosphorylation Levels of α-Synuclein in SH-SY5Y Cells Transfected with α-Synuclein

Wild-type α-synuclein-transfected SH-SY5Y cells were used to investigate the potential effects of ICA on α-synuclein expression and phosphorylation *in vitro*. α-Synuclein-transfected SH-SY5Y cells were treated with different dosages of ICA for 48 h. Cell viability after 24 h or 48 h of treatment with ICA was assessed using an MTT assay. The results indicated that ICA did not exhibit any toxic or inhibitory effects on SH-SY5Y cells at a dose range of 1.56–200 μM (Fig. 4A,B).

The expression and phosphorylation levels of α-synuclein were detected using western blotting. Compared with vehicle-treated cells transfected with GFP-tagged α-synuclein, ICA decreased the elevated expression level of GFP-tagged α-synuclein ($p < 0.05$, $p < 0.01$, Fig. 4C,D). Moreover, the level of Ser129-phosphorylated α-synuclein was significantly increased in vehicle-treated SH-SY5Y cells transfected with α-synuclein, and ICA treatment dose-dependently decreased the level of the Ser129-phosphorylated α-synuclein ($p < 0.01$, Fig. 4E,F).

In the western blot assay, ICA at a dose of 40 μM decreased the levels of Ser129-phosphorylated α-synuclein without decreasing the elevated expression level of GFP-tagged α-synuclein. Since Ser129-phosphorylated α-synuclein tends to aggregate into its pathological form, we assessed α-synuclein aggregation in SH-SY5Y cells using immunocytochemistry. Morphologically, GFP staining was similar in the two groups of cells, indicating comparable expression levels of transfected α-synuclein (shown in green, Fig. 4G). However, detection with the α-synuclein antibody revealed that ICA treatment (40 μM) of SH-SY5Y cells transfected with α-synuclein reduced the deposition of aberrant α-synuclein compared with the vehicle-treated cells (shown in red, Fig. 4G).

These results suggest that ICA may inhibit the overexpression and aggregation of α-synuclein in SH-SY5Y cells.

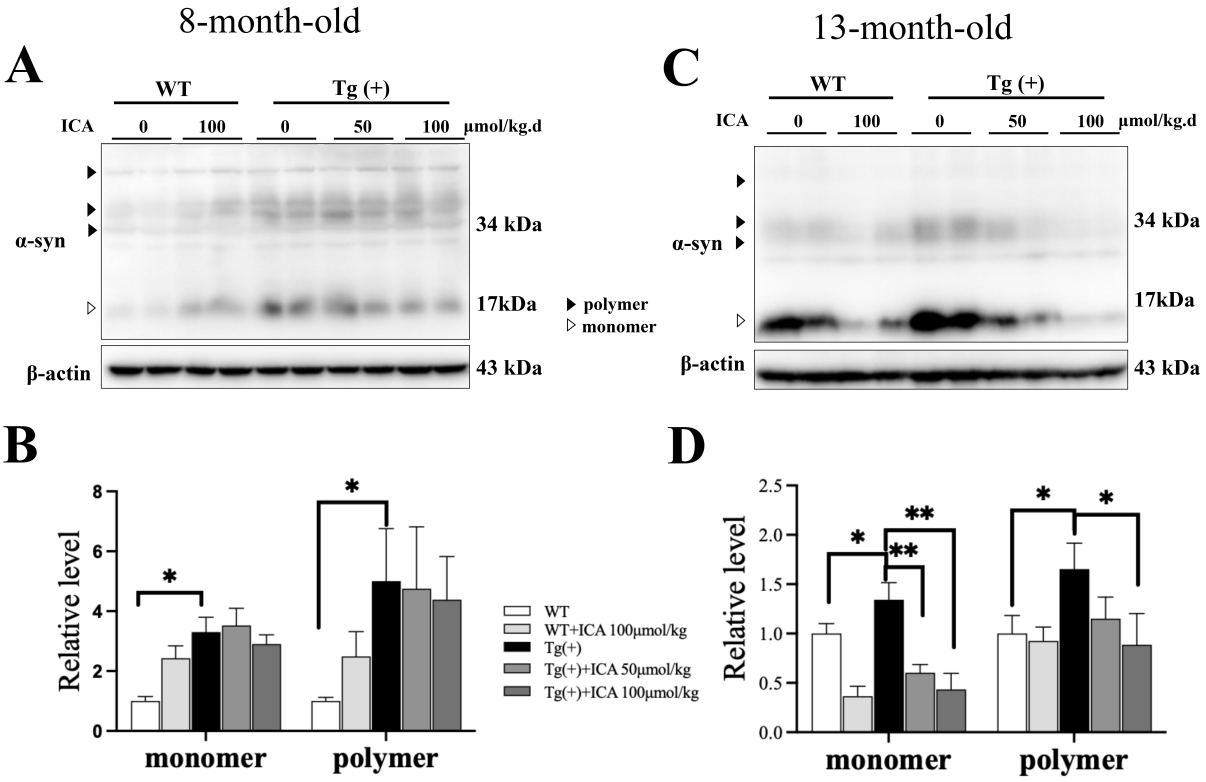

Fig. 2. Effects of ICA on the expression level and aggregation of α-synuclein (α-syn) in the striatum of A53T Tg mice (western blotting). (A) Representative blot images of α-synuclein in 8-month-old mice. (B) Quantitative analysis of the α-synuclein monomer and polymer forms in 8-month-old mice. (C) Representative blot images of α-synuclein in 13-month-old mice. (D) Quantitative analysis of the α-synuclein monomer and polymer forms in 13-month-old mice. The ratio of α-synuclein to β-actin was taken as 1. Data are provided as the mean ± SEM, n = 4. *$p < 0.05$, **$p < 0.01$, *vs.* Tg (+) model group. ICA, icariin; SEM, standard error of the mean; Tg (+), A53T α-synuclein transgenic mice; WT, wild type.

3.5 ICA Increased the Expression Level of Parkin and Decreased the Expression Level of PLK2

To explain the possible mechanisms through which ICA affects protein expression and phosphorylation, we applied western blotting to detect the expression levels of parkin and PLK2. The expression of parkin was decreased in the brain of A53T Tg mice compared with WT mice at 13 months of age ($p < 0.05$) and ICA treatment significantly increased the expression level of parkin ($p < 0.05$, $p < 0.01$, Fig. 5A,B). This result suggests that ICA may activate parkin-related pathways, including the UPS-related pathways.

In SH-SY5Y cells transfected with α-synuclein, the expression level of PLK2 was significantly increased ($p < 0.05$), while ICA treatment decreased its expression ($p < 0.05$, $p < 0.01$, Fig. 5C,D). This result suggests that ICA inhibits the phosphorylation of α-synuclein by regulating PLK2.

4. Discussion

In the present study, A53T mutant α-synuclein transgenic mice and SH-SY5Y cells transfected with wild-type α-synuclein were used to examine the potential effect of ICA on pathological α-synuclein in PD and synucleinopathies. The results indicated that intragastric treatment of ICA for 3 months improved the impaired motor function and coordination in A53T Tg (+) mice at 8 and 13 months of age. ICA decreased the expression, Ser129 phosphorylation, and aggregation of α-synuclein in the striatum of A53T mice and α-synuclein-transfected cells. Moreover, ICA increased parkin expression and decreased PLK2 expression.

Usually, the pathology of neurodegenerative diseases, including PD, is progressive. Initially, abnormal neuronal activity and pathology are not evident owing to the capacity of the cell or neighboring cells to compensate. Eventually, clinical and pathological prodromes emerge with the failure of compensatory effects and cell degeneration [24]. To observe the effects of ICA on different PD stages, we used two groups of A53T Tg mice at the ages of 8 and 13 months. It has been previously reported that the motor function of 5-month-old A53T Tg mice is not impaired [18,25]. However, A53T Tg mice show obvious motor impairments at 8 months of age due to progressive pathological changes in α-synuclein [19,20]. In the present study, A53T Tg mice

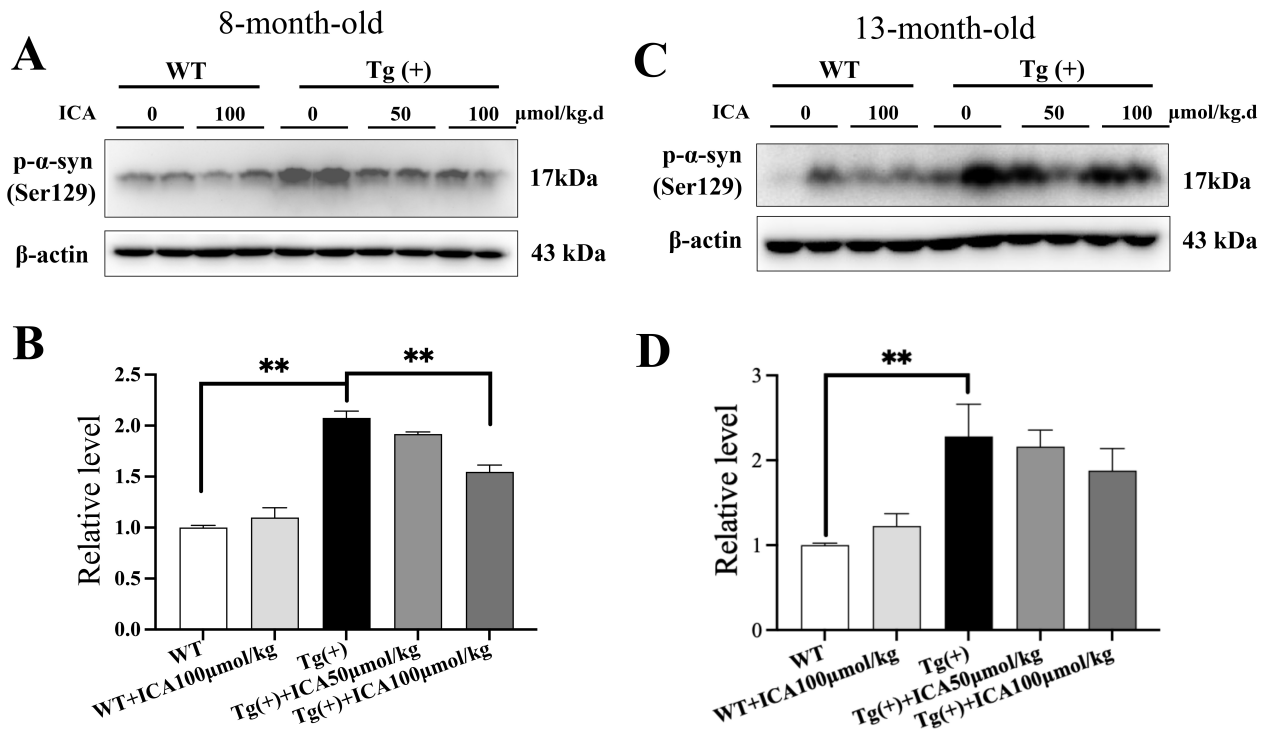

Fig. 3. Effects of ICA on the phosphorylation of α-synuclein at serine 129 in the striatum of the A53T Tg mice (western blotting). (A,B) Representative blots and quantitative analysis of p-α-syn (Ser129) in the striatum of mice at 8 months of age. (C,D) Representative blots and quantitative analysis of p-α-syn (Ser129) in the striatum of mice at 13 months of age. The ratio of p-α-syn (Ser129) to β-actin was taken as 1. Data are provided as the mean $\pm$ SEM, n = 4 per group. $^{**}p < 0.01$, *vs.* Tg (+) model group. p-α-syn (Ser129), α-synuclein phosphorylated at serine 129.

showed impaired motor function and coordination in the rotarod and pole tests. Treatment with ICA for 3 months improved the behavioral performance of A53T Tg mice at 8 and 13 months, indicating that both earlier intervention and therapeutic treatment with ICA had beneficial effects on α-synuclein-induced motor impairment.

High expression of α-synuclein has been found in the early stages of PD in both the brains of patients and animal models [25]. In the present study, ICA decreased the expression of α-synuclein in the striatum of 13-month-old A53T mice, which might partially explain the beneficial effects of ICA on PD. Besides A53T mutant α-synuclein, the over-expression of wild-type α-synuclein is also a risk factor for PD [26,27]. Thus, we used SH-SY5Y cells, a human dopaminergic cell line, to overexpress wild-type α-synuclein through gene transfection. The results showed ICA has inhibitory effects on the overexpression of α-synuclein, which might be the underlying mechanism ameliorating motor deficits in PD mice.

The phosphorylation and abnormal aggregation of α-synuclein show important impacts on α-synuclein-related pathology in PD and synucleinopathies [28]. A previous study on aging monkey brains showed that oligomerization and phosphorylation of α-synuclein progressively increased with age in the striatum and hippocampus of the mice [29]. Oligomerization or the polymer form of α-synuclein indicate its aggregation, and the aggregation of α-synuclein appears to be a key predictor of neuronal loss and a pivotal event in the pathogenesis of synucleinopathies and PD [30,31]. In this study, ICA decreased the expression level of α-synuclein in 13-month-old A53T mice and the aggregation of overexpressed α-synuclein in SH-SY5Y cells, indicating that ICA inhibited the aggregation of α-synuclein.

Phosphorylation at Ser129 is one of the main pathological modifications of α-synuclein in sporadic and familial Lewy body disease [5]. Members of the PLK family have been reported to phosphorylate α-synuclein, with PLK2 phosphorylating α-synuclein at Ser129 in the central nervous system [6,7]. Moreover, PLK2 modulates α-synuclein aggregation in mammalian cells and yeast [6]. The inhibition of PLK2 represents a promising direction for developing novel therapeutics for synucleinopathies [32]. In the present study, ICA decreased the phosphorylation level of α-synuclein and the expression of PLK2, indicating the possible effects of ICA on α-synuclein accumulation and toxicity.

The UPS can eliminate unfolded or misfolded proteins through several enzymatic reactions involving ubiquitin (Ub) protein ligases (E3), Ub-activating enzymes (E1),

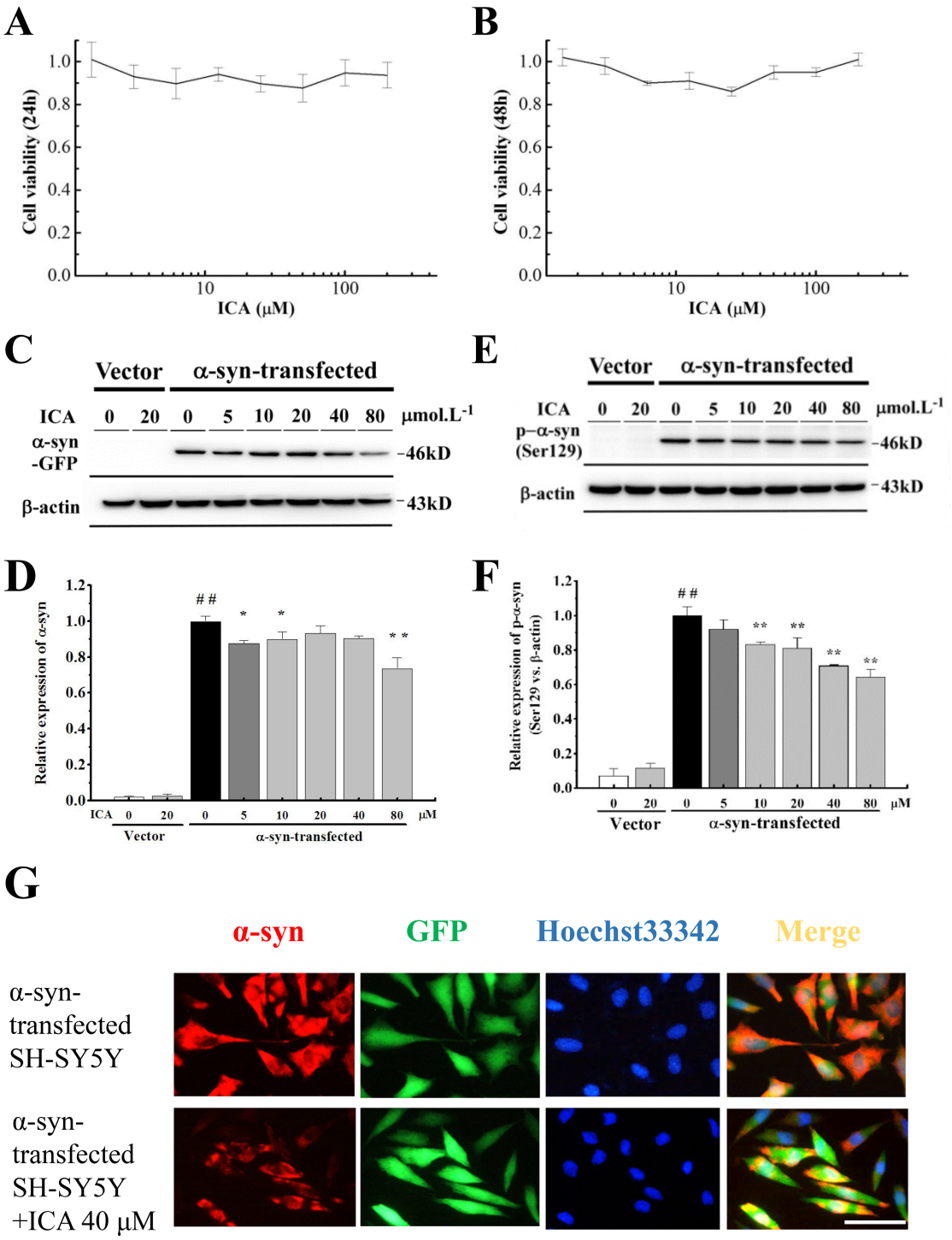

Fig. 4. Effects of ICA on the expression and phosphorylation of α-synuclein (α-syn) in SH-SY5Y cells. (A) Cell viability after a 24-h treatment with ICA at different doses. (B) Cell viability after a 48-h treatment with ICA at different doses; cell viability = optical density (OD) in ICA-treated group/OD in control group. (C,D) Representative western blot images and quantitative analysis of α-synuclein in SH-SY5Y cells. (E,F) Representative blots and quantitative analysis of Ser129-phosphorylated α-synuclein in SH-SY5Y cells. The ratio of α-syn and p-α-syn (Ser129) to β-actin in the vehicle-treated α-syn-transfected group was taken as 100%. (G) Representative images of immunocytochemistry staining for α-synuclein (α-syn, red), GFP (green), and nucleus (Hoechst33342, blue) as well as the merged images; scale bar = 50 μm. Data are provided as the mean ± SEM, n = 3. $^{\#\#}p < 0.01$, vehicle-treated α-syn-transfected group *vs.* vector control group; $^{*}p < 0.05$, $^{**}p < 0.01$, ICA-treated α-syn-transfected group *vs.* vehicle-treated α-syn-transfected group. GFP, green fluorescent protein; p-α-syn (Ser129), α-synuclein phosphorylated at serine 129.

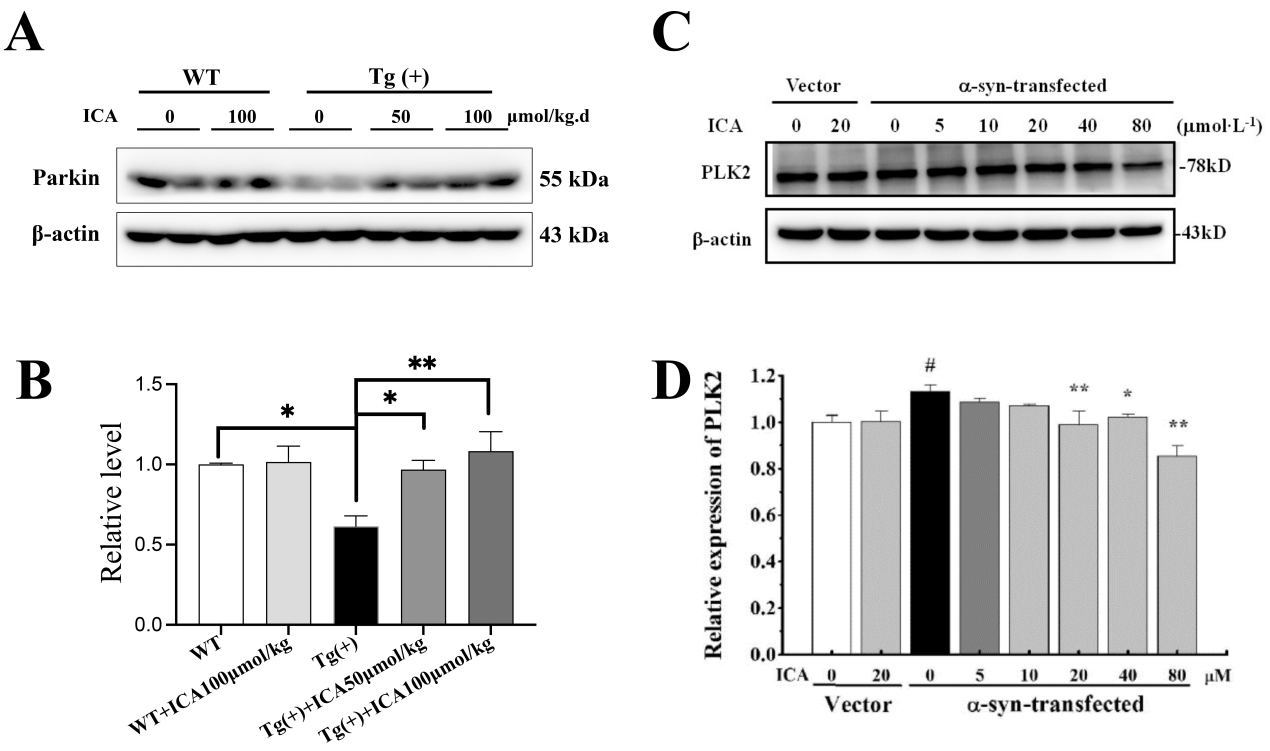

Fig. 5. Effects of ICA on the expression of Parkin and PLK2. (A,B) Representative blots and quantitative data of parkin in the brain of 13-month-old A53T Tg mice; n = 4; $*p < 0.05$, $**p < 0.01$, *vs.* Tg (+) model group. (C,D) Representative blots and quantitative data of PLK2 in the α-synuclein-transfected SH-SY5Y cells; n = 3; $^{\#}p < 0.05$, vehicle-treated α-syn-transfected group *vs.* vector control group; $*p < 0.05$, $**p < 0.01$, ICA-treated α-syn-transfected group *vs.* vehicle-treated α-syn-transfected group. The ratio of parkin and PLK2 to β-actin was taken as 1. Data are provided as the mean ± SEM. PLK2, polo-like kinase 2.

and Ub-conjugating enzymes (E2), which contribute to the degradation of α-synuclein [8,33]. Although the degradation mechanism of α-synuclein in neurons is unclear, agents targeting degradation are considered a promising strategy for synucleinopathy treatment [34,35]. Parkin is a well-known Ub E3 ligase that attaches a polyubiquitin chain to proteins to target them for UPS degradation and plays a key role in the degradation of α-synuclein [9]. The expression level of parkin is decreased in α-synuclein overexpression models and contributes to α-synuclein degradation, dysfunction, and neurotoxicity [10,11]. Thus, increasing parkin expression has been reported to show a protective effect by promoting the proteasomal clearance of α-synuclein [36,37]. In the present study, we found that ICA increased the expression level of parkin in A53T Tg mice, which might partially explain the beneficial effects of ICA on α-synuclein degradation in synucleinopathies.

5. Conclusions

In conclusion, we used A53T mutant α-synuclein transgenic mice and SH-SY5Y cells transfected with wild-type α-synuclein to examine the pharmacological effects of ICA on α-synuclein-related pathology in synucleinopathies, including PD, and the mechanisms involved. We found that the intragastric treatment of ICA for 3 months significantly improved motor function and coordination in A53T Tg mice at 8 and 13 months of age. ICA alleviated α-synuclein pathology by decreasing the expression, Ser129 phosphorylation, and aggregation of α-synuclein in the striatum of A53T Tg mice and α-synuclein-overexpressing cells. The underlying mechanisms include an ICA-induced decrease in the expression of PLK2 and an increase in the expression level of the UPS-associated protein parkin.

Availability of Data and Materials

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

Author Contributions

CS, LZ and LL designed the research study. CS, XZ and DM conducted experiments. CS and DM analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

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Ethics Approval and Consent to Participate

Animal studies were approved by the Bioethics Committee of Xuanwu Hospital of Capital Medical University (approval number: 20120912) and were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The study was conducted in compliance with the ARRIVE guidelines. All methods were performed in accordance with relevant guidelines and regulations.

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Conflict of Interest

The authors declare no conflict of interest.

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