The neuroprotective effects of Galectin-1 on Parkinson's Disease *via* regulation of Nrf2 expression

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Abstract. – **OBJECTIVE:** Parkinson's disease (PD) is one of the neurodegenerative diseases. Galectin-1 (Gal-1) is expressed in the central nervous system. Our study sought to explore the neuroprotective effect of Gal-1 in 1-methyl-4-phenyl pyridine ion (MPP+)-induced cytotoxicity on SH-SY5Y cells.

MATERIALS AND METHODS: SH-SY5Y cells were cultured *in vitro*, pretreated with Gal-1, and then exposed to MPP+. Thereafter, the generation of reactive oxygen species (ROS) in SH-SY5Y cells was investigated. The effects of Gal-1 on DNA breakage, cell damage (release of lactate dehydrogenase (LDH)), viability, and apoptosis in SH-SY5Y cells were examined by comet assay, LDH assay, WST-1 assay, and flow cytometry, respectively. Additionally, the regulatory effect of Gal-1 on Nrf2 expression was examined by western blot. Zebrafish embryos were pretreated with Gal-1 and then exposed to MPP+. The locomotor ability of zebrafish larvae was then investigated.

RESULTS: MPP+ induced the production of ROS in cells, which can be alleviated by pretreatment with Gal-1. Gal-1 protected cells from MPP+-induced cytotoxicity by preventing DNA breakage and cell injury. Gal-1 inhibited apoptosis in SH-SY5Y cells. The neuroprotective effect of Gal-1 could be abolished when Nrf2 expression knockdown. Moreover, exposure to MPP+ decreased the locomotor activity of zebrafish, which was attenuated by pretreatment with Gal-1.

CONCLUSIONS: Our study demonstrated that the administration of Gal-1 could protect neurons from cellular stress by preventing apoptosis and eliminating ROS. Moreover, the neuroprotective effect of Gal-1 in neuronal cells could be related to the activation of Nrf2 expression. Therefore, Gal-1 could be a promising strategy for treating PD.

Key Words:
    Galectin-1, Parkinson's disease, Nrf2, Reactive oxygen species, Neuroprotective effects.

Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, with an estimated prevalence of 6.3 million globally[1]. PD exerts multiple clinical symptoms, including tremor, muscle rigidity, bradykinesia, and dyskinesia, in addition to non-motor symptoms, including fatigue, sleep disturbance, anxiety, depression, and gastrointestinal dysfunctions. However, the therapy strategies used to combat PD are limited. To date, most PD therapies have focused on relieving clinical symptoms, and there is a lack of medicine to delay PD progress or prevent PD occurrence, although the research on PD pathology has made great progress in recent years. PD pathogenesis is related to oxidative stress, mitochondrial dysfunction, lysosomal dysfunction, and neuroinflammatory changes, which are characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compacta[2]. Therefore, protecting dopaminergic neurons against oxidative stress and associated deleterious effects is a new therapeutic strategy for combating PD. However, although some new neuroprotective medicines have been developed, such as brain-derived neurotrophic factor (BDNF)[3] and glial cell line-derived neurotrophic factor (GDNF)[4], there is still a lack of efficient neuroprotective drugs in research and clinics. Unfortunately, in randomized clinical trials of recombinant human GDNF by intraputamenal infusion, no significant improved symptoms were observed in PD patients[5]. Therefore, more research is needed to explore PD pathogenesis, and more neuroprotective reagents must be developed.

Galectin-1 (Gal-1) was the first protein discovered in the family of β-galactoside-binding proteins; it is encoded by the LSGALS1 gene located on chromo-

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some 22q12[6]. Gal-1 is expressed in various human cells and is secreted into the microenvironment. Researchers have revealed that Gal-1 can participate in multiple cell bio-behaviors, including cell proliferation[7], apoptosis[8]. Moreover, Gal-1 is expressed in the central nervous system[9] and can promote nerve regeneration[10]. However, to date, limited data exist on the effect of Gal-1 on PD pathogenesis. Marques et al[11] found that the level of Gal-1 in the cerebrospinal fluid of PD patients was decreased compared to non-neurological control patients, while Li et al[12] suggested that Gal-1 can inhibit the activation of microglial cells and thereby play an anti-inflammatory role in PD animal models. Therefore, we speculate that Gal-1 could play a neuroprotective role in PD pathology. In the present study, we investigated the effect of Gal-1 in PD pathology.

Materials and Methods

Cell Culture

Human SH-SY5Y cells (a neuroblastoma cell line, CRL-2266) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 10% FBS (HyClone, Logan, UT, USA), penicillin, and streptomycin (Beyotime Institute of Biotechnology, Shanghai, China) at 37°C with 5% CO_2.

Recombinant human Gal-1 was purchased from Pepro Tech, and 1-methyl-4-phenyl-pyridinium ion (MPP+) was purchased from Sigma Aldrich. Both Gal-1 and MPP+ were dissolved in phosphate-buffered saline (PBS). Cells were treated with Gal-1 at a concentration of 1–4 µg/ml[13] or MPP+ at a concentration of 500 µM[14].

Cell Viability Assay

The cells (5000 cells/well) were seeded in 96-well plates. Thereafter, the cells were pretreated with different concentrations of Gal-1 for 2 h, after which MPP+ was added to pretreated cells for 12 h, 24 h, and 48 h. The cells were stained with WST-1 (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's protocol. Cell viability was evaluated by measuring the absorbance of each well using a multimode plate reader (440 nm).

Caspase-3/8 Activity

The kits used to measure the activity of Caspase-3/8 were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). This measurement followed the manufacturer's instructions. In brief, total proteins were extracted from the cells using the lysis buffer from the kits and were then mixed with 85 µl of reaction buffer. Thereafter, the samples were mixed with 5 µl of Leu-Glu-His-Asp-p-nitroanilide and incubated at 37°C for 2 h. The activity of Caspase-3 or Caspase-8 was examined by measuring absorbance using a multiplate reader at 450 nm.

Comet Assay

To prepare the comet assay, 0.75% agarose was heated and coated on the slides at 37°C and then immediately maintained at 4°C for 1 h. The cells were collected and suspended in PBS buffer after treatment with Gal-1 and MPP+. The cell suspension was mixed with 0.5% agarose at 37°C, after which 30 µl of cell-agarose mix was immediately added to the slides and maintained in the dark for 10 min at 4°C. Next, the slides were transferred into a lysis solution and maintained overnight in the dark at 4°C before being immersed in an alkaline electrophoresis solution for 1 h in the dark at 4°C, followed by electrophoresis (25 V; 1 V/cm) for 30 min. The slides were neutralized with 0.4 M Tris-Hcl, fixed with 70% ethanol (5 min each step) at room temperature, and then dried at 37°C in the dark. Next, the slides were stained with propidium iodide (Beyotime Institute of Biotechnology, Shanghai, China) in the dark for 10 min at room temperature before being observed under a fluorescence microscope (Olympus, Shinjuku City, Tokyo, Japan). In each sample, 50 cells were observed under 400x magnification, and the DNA damage in olive tail moment (OTM) and tail intensity were evaluated for analysis. The tail intensity = percent of DNA in the comet tail. The OTM = tail intensity × (tail DNA mean-head DNA mean).

Assay of Production of Reactive Oxygen Species (ROS) and Lactate Dehydrogenase (LDH) Leakage From Cells

The cells (5000 cells/well) were seeded in 96-well plates. Thereafter, the cells were pretreated with different concentrations of Gal-1 for 2 h, after which MPP+ was added to the pretreated cells for 48 h. Next, the culture medium was collected to examine the level of LDH. The assay kit for LDH measurement was purchased from Beyotime Biotechnology (Shanghai, China) and was used in accordance with the manufacturer's

protocol. The LDH level was examined by measuring absorbance using a multiplate reader at 490 nm. To evaluate the ROS level, the cells (5 x 10^6 cells/ml) were collected and resuspended in 2'7'-dichlorofluoroscein diacetate (10 mM), which was provided with the ROS assay kit (Beyotime Biotechnology, Shanghai, China), for 30 min at 37°C. The fluorescent activity was measured by a multiplate reader with an excitation at 502 nm and an emission at 525 nm.

Cell Apoptosis Assay

The cells were pretreated with different concentrations of Gal-1 for 2 h, after which MPP+ was added to the pretreated cells for 48 h. Thereafter, the cells were collected, washed with PBS, and resuspended in 100 μl of binding buffer at a concentration of 10^6 cells/ml. Then, 5 μl of annexin V-fluorescein isothiocyanate and 10 μl of propidium iodide (both were purchased from Beyotime Institute of Biotechnology, Shanghai, China) were added to the cell suspension, which was then incubated for 15 min at room temperature in the dark. Finally, the apoptosis of each sample was examined using a FACScan flow cytometer (BD Biosciences, East Rutherford, New Jersey, US), and the data were analyzed by FlowJo software (V10.6; BD Biosciences).

Zebrafish Experiments

The procedures and experiments were approved by the Committee on Ethics of Animal Experiments of Beijing Geriatric Hospital (2019134). AB-type zebrafish (*Danio rerio*) were obtained from the Animal Experimental Center of Hebei Medical University and were maintained at 28.5 ± 0.5°C, pH 7.5 ± 0.5, SaO2 > 80%, with a cycle of 14-h light/10-h dark. At 2 h post-fertilization (pf), the embryos were examined under a stereomicroscope to ascertain whether normal embryos had grown. Then, the normal embryos were randomly distributed into different groups (30 embryos per group): (1) exposed to Gal-1 (4 μ/ml) from 12 hpf to 5 dpf, with MPP+ (500 μM) added to the culture system from 2 dpf to 5 dpf; (2) exposed to MPP+ (500 μM) from 2 dpf to 5 dpf; (3) control group, exposed to PBS until 5 dpf. Then, the swimming behavior of zebrafish larvae in a 24-well plate was captured by a digital video tracking system and the total swimming distance was calculated for analysis[15].

Locomotor activity was recorded during alternating light and dark conditions (between 1:00 pm and 6:00 pm), presented in 30-min in-tervals. Following the transition cycles (light-to-dark or dark-to-light transition), the locomotor activity of zebrafish larvae was evaluated during five separate 30-min phases. The total distance swum during these five phases was recorded immediately after the transition to light conditions. The initiation of movement was defined as any instance of swim activity where the velocity exceeded a threshold of 2 mm/s. Cessation of movement was defined as occurring when the velocity dropped below a threshold of 1 mm/s. The cumulative time of movement (s) was defined as the sum of time when a larva was moving during the 30-min phases. The mean cumulative time of movement was represented as the mean accumulated time spent moving during the five separate phases.

After the experiments, ice water and sodium hypochlorite (6.15%) were added to the culture system for ≥ 20 min to euthanatize the zebrafish.

Western Blotting

The total protein was collected from the cells using the Radioimmunoprecipitation assay buffer (RIPA; Sigma-Aldrich, St. Louis, MO, USA) containing protease inhibitors, and the protein concentrations were determined by the Bicinchoninic acid method. Equal amounts of protein (20 μg/lane) were loaded and separated on SDS-polyacrylamide gels (8–10%) for electrophoresis. Thereafter, the protein bands on the gels were transferred to nitrocellulose membranes. Then, the membranes were blocked with 5% bovine serum albumin (BSA) (diluted in Tris-Cl-buffered saline-0.1% Tween-20, TBS-T) for 2 h at room temperature and were incubated with primary antibodies at 1:3000 overnight at 4°C. Next, the membranes were washed with TBS-T and then incubated with horseradish peroxidase-conjugated second bodies at 1:5000 (anti-mouse, sc-516102 or anti-rabbit, sc-2357; Santa Cruz Biotechnology, Dallas, Texas, US) for 1 h at room temperature. Next, the membranes were stained with enhanced chemiluminescence detection (Pierce; Thermo Fisher Scientific, Waltham, MA, US) to examine the protein bands by a chemiluminescence detection system (Bio-Rad Laboratories, Hercules, California, US). The primary antibodies were as follows: mouse Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, cat. no. sc-32233; Santa Cruz Biotechnology, Dallas, Texas, US), rabbit monoclonal nuclear factor erythroid-2-related factor 2 (Nrf2) antibody (cat. no. ab-62352; Abcam Biotechnology, Cambridge,

UK), rabbit monoclonal heme oxygenase 1(HO-1) antibody (cat. no. ab-52947; Abcam Biotechnology, Cambridge, UK).

siRNA Transfection

Cells were seeded (1×10^5 cells/well) into 12-well culture plates and transfected with 40 nM Nrf2 siRNA or scrambled negative control siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h at 37°C. Following 24 h of transfection, the transfection efficiency in cells was validated by Western blotting, and then the cells were used for subsequent experiments.

Measurement of Nrf2 Transcription Activity

To evaluate the nuclear activation of Nrf2 in SH-SY5Y cells, the nuclear extract lysates were obtained using the nuclear extraction kit (cat. no. ab-113474; Abcam Biotechnology, Cambridge, UK) following the manufacturer's instructions. The concentration of proteins was measured by the Bradford assay and then normalized on total protein content. Thereafter, each sample (20 µg) was added to the wells of a 96-well plate, after which the activity of Nrf2 was measured using the Nrf2 transcription factor assay kit (cat. no. ab-207223; Abcam Biotechnology, Cambridge, UK.) according to the manufacturer's protocols. The activity of Nrf2 was examined using a multiplate reader at 450 nm. The results were expressed as a percentage of the treatment group relative to the control group.

Statistical Analysis

Data are presented as the mean ± SEM. R software (version 3.6.2) was used to analyze the data. One-way ANOVA followed by Tukey's post-hoc test was used to compare multiple groups. A difference of $p < 0.05$ was considered to be statistically significant.

Results

The Effect of Gal-1 on the Cell Viability of SH-SY5Y Cells

It is known that MPP+ selectively destroys dopaminergic neurons and causes PD in mammals; accordingly, it is widely used in PD research[14]. In the present study, we used MPP+ to treat SH-SY5Y cells, and the data showed that the treatment of MPP+ inhibited cell viability. Moreover,
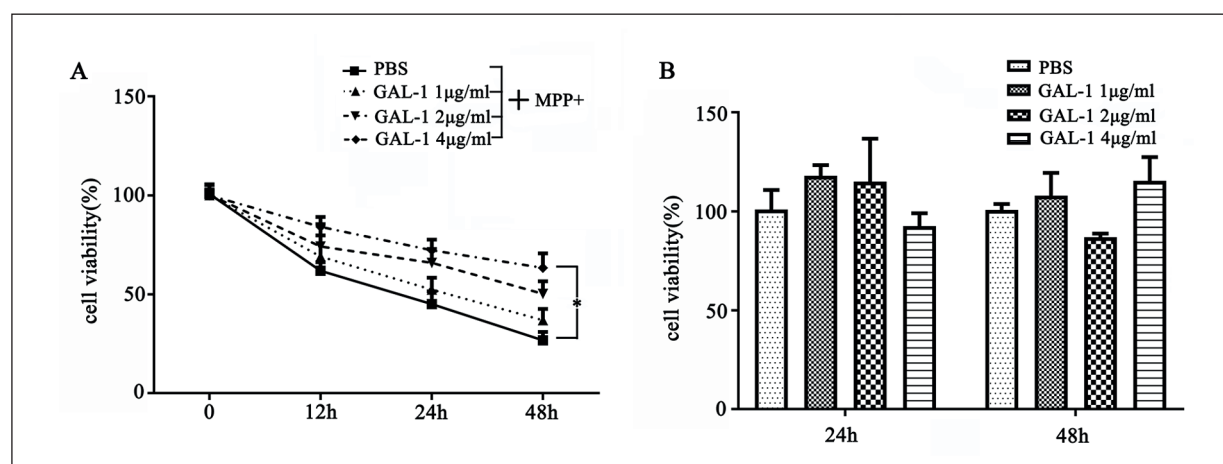
Figure 1. Effects of Gal-1 or a combination of Gal-1 and MPP+ on the viability of SH-SY5Y cells. **A**, Cell viability with a combination of MPP+ (500 µM) and increasing concentrations of Gal-1 (1–4 µg/ml). The cells were pretreated with Gal-1 for 2 h at a concentration of 1–4 µg/ml, after which MPP+ was added at a concentration of 500 µM for the indicated time; the control group was pretreated with PBS, after which MPP+ was added at a concentration of 500 µM for the indicated time. The viability of cells was determined by WST-1 assay. MPP+ significantly inhibited cell viability *in vitro* in the control group. However, the inhibitory effects of MPP+ on cell viability were alleviated by pretreatment with Gal-1 in a dose-dependent manner. Under the treatment of MPP+, the cells with a higher concentration of Gal-1 pretreatment had higher cell viability. **B**, Cell viability in the presence of increasing concentrations of Gal-1. The cells were treated with Gal-1 for 48 h at a concentration of 1–4 µg/ml, while cells in the control group were treated with PBS. The data showed no significant difference in cell viability among the Gal-1 treatment groups and the control group. All results were representative of three independent experiments performed in triplicate. The data are presented as mean ± SEM ($n = 3$). (*$p < 0.05$, #$p < 0.01$, one-way ANOVA followed by Tukey's post-hoc test).

our data showed that pretreatment of Gal-1 can alleviate cytotoxicity caused by MPP+. The cell pretreatment with Gal-1 had a higher viability rate at a concentration of 1 to 4 μg/ml compared to cells without Gal-1 pretreatment (Figure 1A). In addition, cell viability did not show a significant difference when the cells were treated with Gal-1 alone compared to the control group (treatment with PBS only) (Figure 1B).

The Effect of Gal-1 on Cell Injury Induced by MPP+

SH-SY5Y cells were exposed to MPP+ with/without pretreatment of Gal-1, after which cell injury was examined. The comet assay showed that MPP+ treatment-induced DNA breakage in SH-SY5Y cells, with increased OTMs and tail intensity. In contrast, cells pretreated with Gal-1 had lower OTM values and tail intensity dose-dependently, when subsequently treated with MPP+ (Figure 2).

Furthermore, we investigated the leakage of cell injury biomarker LDH from cells exposed to MPP+. The data showed that exposure to MPP+ increased LDH leakage from cells compared to the control group (treatment with PBS only), whereas cells pretreated with Gal-1 resulted in lower LDH leakage dose-dependently, compared to cells without Gal-1 pretreatment. These data suggest that Gal-1 can attenuate the increased permeability of the cell membrane by MPP+ (Figure 3A).

Gal-1 Alleviated MPP+-Induced Oxidative Stress in SH-SY5Y Cells

The overproduction of ROS plays a key role in the pathogenesis of PD, and the presence of MPP+ can promote the generation of ROS in cells, consequently inducing cell death[16]. Consistent with published data, we found that MPP+ treatment significantly increased the production of ROS in SH-SY5Y cells, whereas pretreatment with Gal-1 can attenuate the pro-ROS effect by MPP+ in cells in a dose-dependent manner (Figure 3B).
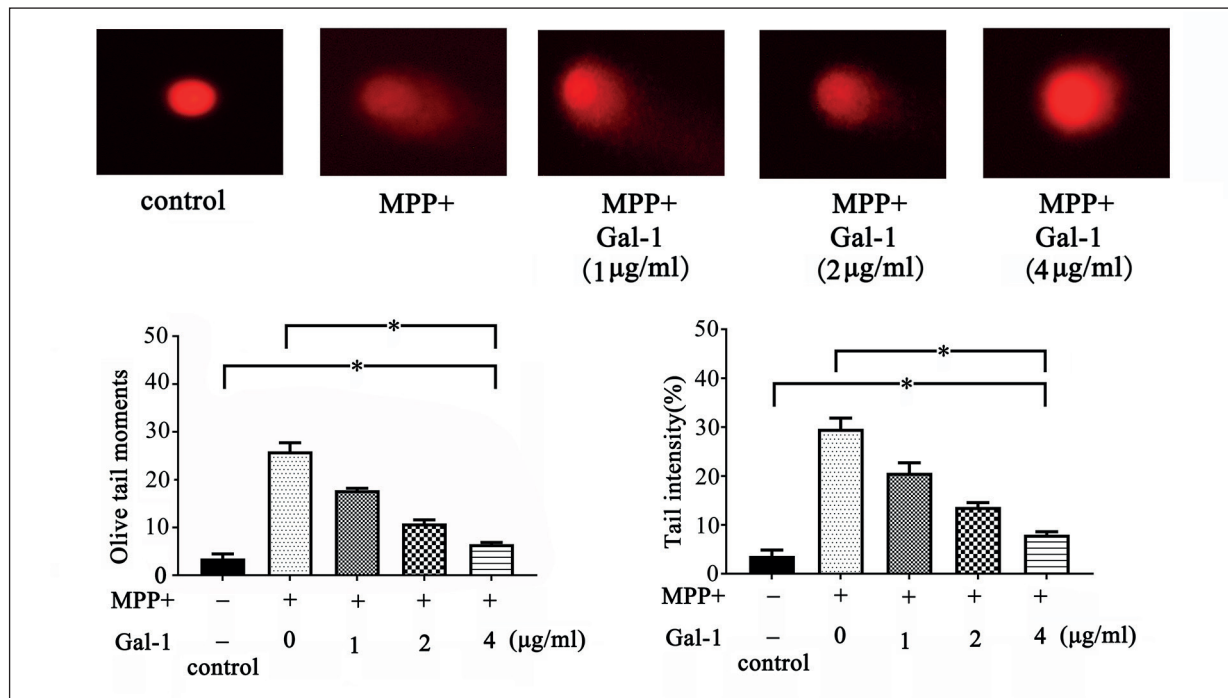
Figure 2. The effect of Gal-1 on DNA damage in SH-SY5Y cells exposed to MPP+. The SH-SY5Y cells were pretreated with Gal-1 for 2 h at a concentration of 0, 1, 2, 4 μg/ml, after which they were exposed to MPP+ at a concentration of 500 μM for 48 h; cells in the control group were treated with PBS only. Exposure to MPP+ induced DNA breakage in SY-SH5Y cells, and a significant increase in the OTM and the tail intensity were observed compared with the control group. However, pretreatment with Gal-1 can alleviate the DNA damage in SY-SH5Y cells induced by MPP+, in a dose-dependent manner. Under the pressure of MPP+, the higher concentration of Gal-1 pretreatment resulted in a lower OTM and tail intensity. Representative images of SY-SH5Y cells are shown. Magnification 200×. All results were representative of three independent experiments performed in triplicate. The data are presented as mean ± SEM (n = 3). (*p < 0.05, #p < 0.01, one-way ANOVA followed by Tukey's post-hoc test).
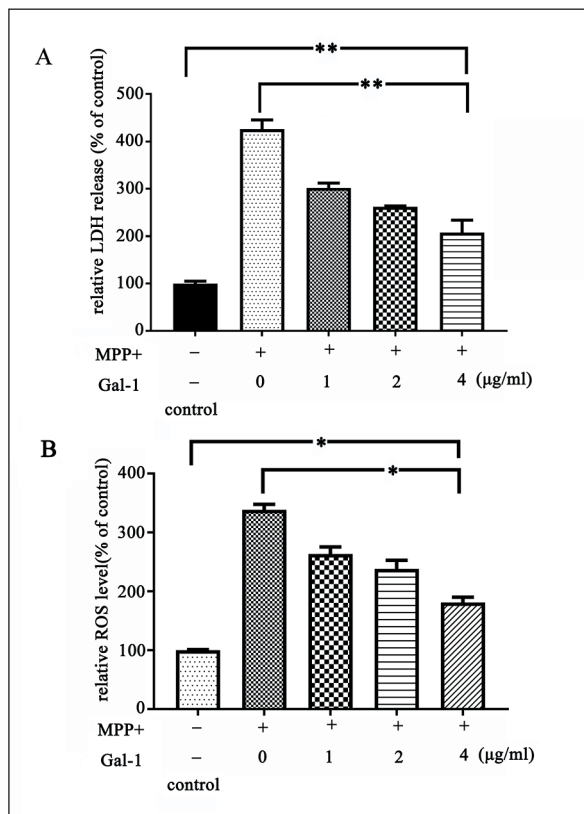
Figure 3. The effect of Gal-1 on LDH leakage and ROS production from SH-SY5Y cells exposed to MPP+. The SH-SY5Y cells were pretreated with Gal-1 for 2 h at a concentration of 0, 1, 2, 4 μg/ml, after which they were exposed to MPP+ at a concentration of 500 μM for 48 h; cells in the control group were pretreated with PBS only. **A**, Then, the cell culture medium was collected, for which LDH levels were measured, as described in Materials and Methods. Exposure to MPP+ induced LDH leakage from SH-SY5Y cells compared with that in the control group. However, pretreatment with Gal-1 can decrease the LDH leakage from SH-SY5Y cells induced by MPP+ in a dose-dependent manner. Under the pressure of MPP+, the higher concentration of Gal-1 pretreatment resulted in a lower level of LDH leakage. **B**, The ROS levels in SH-SY5Y cells were measured as described in Materials and Methods. Exposure to MPP+ significantly promoted the production of ROS in SH-SY5Y cells. However, pretreatment with Gal-1 decreased levels of ROS induced by MPP+ in SH-SY5Y cells in a dose-dependent manner. Under the pressure of MPP+, the higher concentration of Gal-1 pretreatment resulted in a lower ROS level. All results were representative of three independent experiments performed in triplicate. The data are presented as mean ± SEM ($n = 3$). ($*p < 0.05$, $\#p < 0.01$, one-way ANOVA followed by Tukey's post hoc test).

Gal-1 Alleviated MPP+-Induced Apoptosis in SH-SY5Y Cells

To investigate the neuroprotective effect of Gal-1 on cytotoxicity by MPP+, cells were pretreated with Gal-1 and then exposed to MPP+ *in*

vitro. The cell apoptosis rate was examined by flow cytometry. Compared to the control group (treatment with PBS only), treatment with MPP+ significantly increased the cell apoptosis rate, whereas the administration of Gal-1 significantly attenuated MPP+-induced apoptosis in SH-SY5Y cells in a dose-dependent manner (Figure 4A). Furthermore, consistent with the results of flow cytometry, treatment with MPP+ increased apoptotic protein activity (Caspase-3 and Caspase-8) in SH-SY5Y cells, which can be alleviated by the pretreatment of Gal-1 (Figure 4B).

## The Protective Effect of Gal-1 on MPP+-Induced Locomotor Deficiency in Zebrafish

The MPP+-induced PD model in zebrafish has been established and reported[17]. In the present study, normal embryos were exposed to MPP+, MPP+ combined with Gal-1 (4 μ/ml), and PBS only (control group). Exposure to MPP+ significantly decreased the locomotor abilities of zebrafish larvae, including swimming distance, initiation of movement, cumulative time of movements, and swimming velocity, compared to that in the control group (swimming distance: 986.12 ± 82.48 *vs.* 222.38 ± 34.04 mm; initiation of movement: 50.97 ± 4.05 *vs.* 30.65 ± 4.02; cumulative time of movements: 274.95 ± 24.68 s *vs.* 181.47 ± 29.87 s; and swimming velocity: 0.56 ± 0.03 mm/s *vs.* 0.25 ± 0.02 mm/s, see in Figure 5). In contrast, pretreatment with Gal-1 increased locomotor abilities compared to that in the MPP+ group (swimming distance: 222.38 ± 34.04 to 473.27 ± 37.09 mm; initiation of movement: 30.65 ± 4.02 *vs.* 44.30 ± 3.87; cumulative time of movements: 181.47 ± 29.87 s *vs.* 255.69 ± 28.93 s, and swimming velocity: 0.25 ± 0.02 mm/s *vs.* 0.40 ± 0.03 mm/s, see in Figure 5).

Gal-1 Regulated the Nrf2 Pathway in SH-SY5Y Cells

It is known that the activation of the Nrf2 pathway contributes to PD pathogenesis[18] and that the expression of HO-1 is a protein downstream of the Nrf2 pathway[19]. Thus, we investigated whether Gal-1 can regulate the Nrf2 pathway in SH-SY5Y cells. The data showed that treatment with MPP+ decreased the expression of Nrf2 and HO-1 expression in SH-SY5Y cells, which can be significantly reversed by pretreatment with Gal-1 (Figure 6A). The expression level of both Nrf2 and HO-1 was significantly higher in cells treated with MPP+ combined with Gal-1 compared to that in cells treated with only
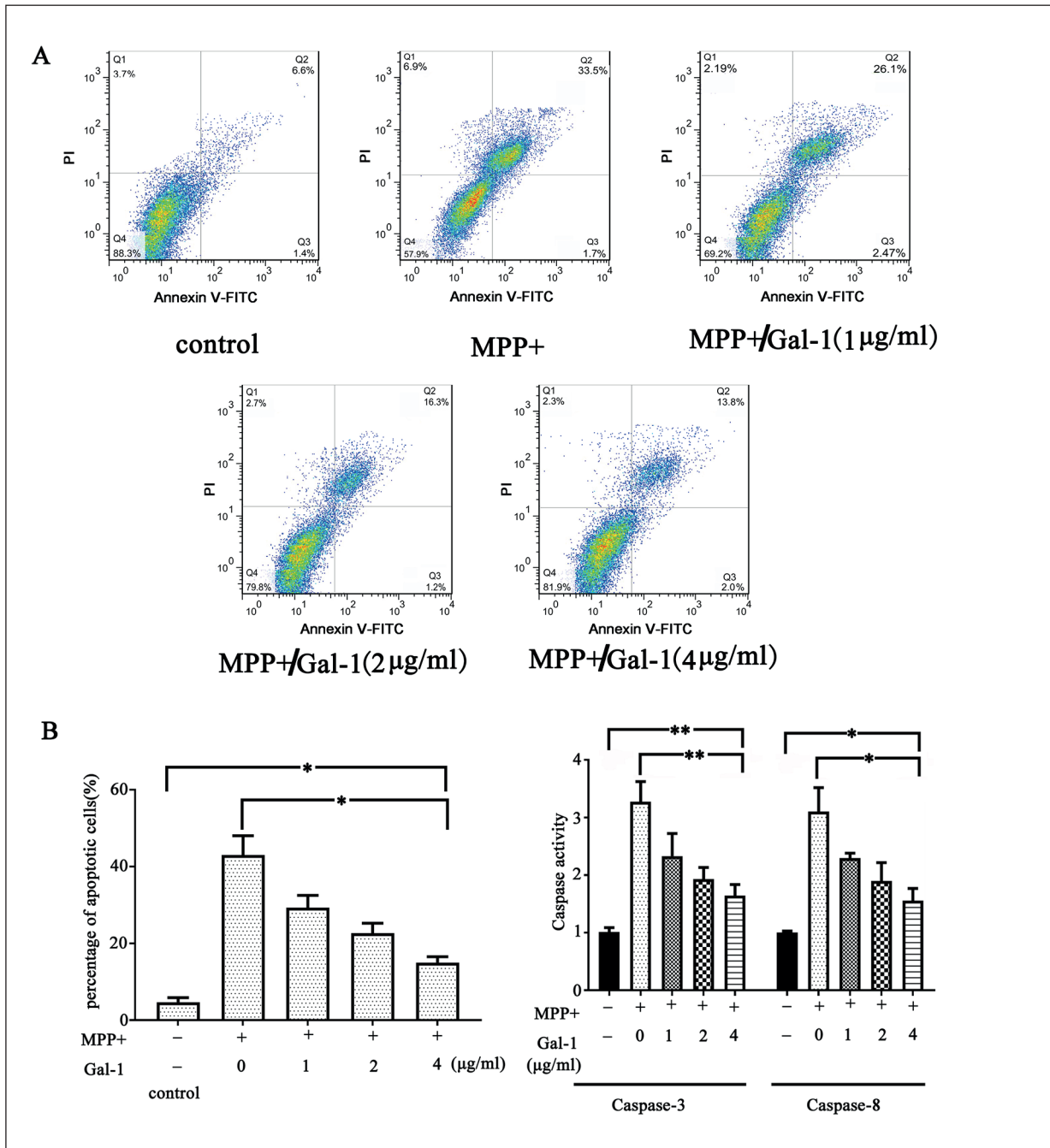
Figure 4. Pretreatment with Gal-1 alleviated apoptosis in SH-SY5Y cells induced by MPP+. The SH-SY5Y cells were pretreated with Gal-1 for 2 h at a concentration of 0, 1, 2, 4 μg/ml, after which they were exposed to MPP+ at a concentration of 500 μM for 48 h; cells in the control group were treated with PBS only. Thereafter, (**A**) the apoptotic cells were measured by FACScan, after staining with annexin V and PI. The sum of annexin V-positive cells and annexin V- + PI-positive cells indicates the total percentage of apoptotic cells. Exposure to MPP+ promoted cell apoptosis in SH-SY5Y cells, which was significantly higher than that in the control group. However, the pro-apoptosis effect of MPP+ could be alleviated by pretreatment with Gal-1 in a dose-dependent manner. The pretreatment with a higher concentration of Gal-1 had a lower percentage of apoptosis. Representative images and relative quantifications are shown. **B**, The data on apoptotic protein activities (Caspase-3 and Caspase-8) were consistent with the results of flow cytometry. Treatment of MPP+ promoted the activity of Caspase-3 and Caspase-8 in cells, which can be alleviated by pretreatment with Gal-1. Under the pressure of MPP+, the higher concentration of Gal-1 pretreatment resulted in lower activity of Caspase-3 and Caspase-8 in cells. All results were representative of three independent experiments performed in triplicate. The data are presented as the mean ± SEM. (*$p < 0.05$, #$p < 0.01$, one-way ANOVA followed by Tukey's post hoc test).
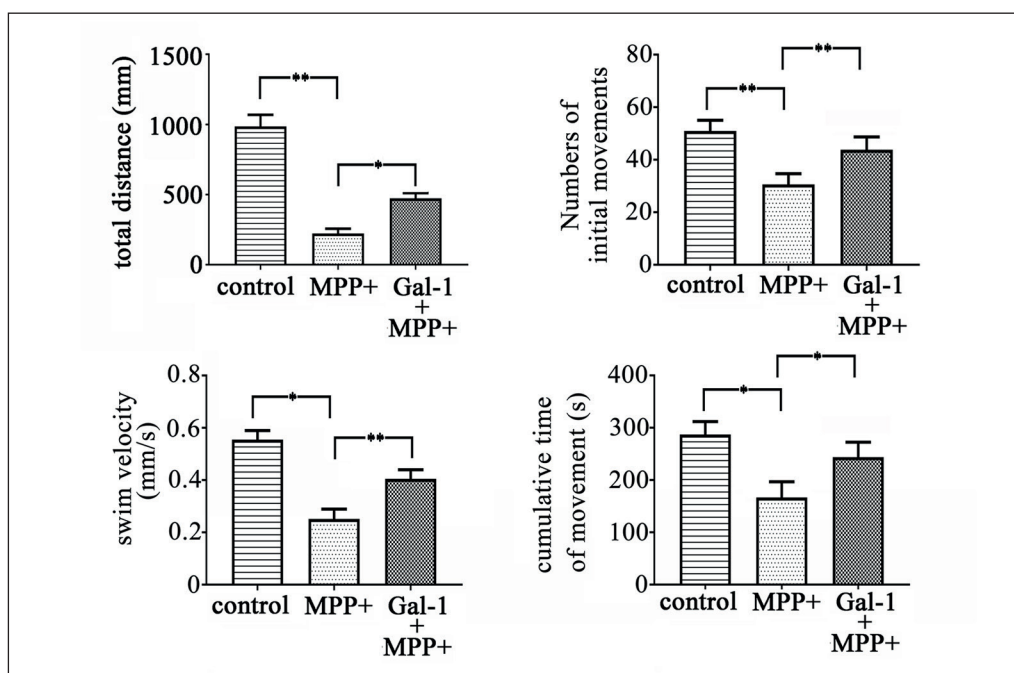
Figure 5. The effect of Gal-1 on locomotor activity of zebrafish larvae following neurotoxin MPP+ exposure. Zebrafish larvae (30 larvae per group were exposed to MPP+ (500 μM) from 2 dpf to 5 dpf; MPP+ (500 μM) from 2 dpf to 5 dpf plus Gal-1 (4 μ/ml) from 12 hpf to 5 dpf, or PBS until 5 dpf (control group). Then, the locomotor activity of zebrafish larvae in a 24-well plate was captured by a digital video tracking system, and the total swimming distance, initiation of movement, swim velocity and cumulative time of movement were calculated for analysis. Exposure to MPP+ significantly impaired the locomotor activity of zebrafish larvae, with decreasing total swimming distance, initiation of movement, swim velocity and cumulative time of movement, compared with the control group. However, pretreatment with Gal-1 can alleviate the impairment of MPP+ on the locomotor activity, including total swimming distance, initiation of movement and cessation of movement. All results were representative of three independent experiments (30 larvae per group). The data are presented as the mean ± SEM. (*$p <$ 0.05, #$p <$ 0.01, one-way ANOVA followed by Tukey's post-hoc test).

MPP+. In addition, we evaluated the activity of Nrf2 in SH-SY5Y cells, and the data showed that MPP+ treatment decreased Nrf2 activity significantly. The inhibitory effect of MPP+ on Nrf2 activity can be attenuated by pretreatment with Gal-1 (Figure 6B). Moreover, our data showed that HO-1 expression was significantly decreased when Nrf2 expression was knocked down by SiRNA, and treatment with Gal-1 failed to regulate HO-1 expression when Nrf2 expression was knocked down (Figure 6C).

In addition, the multiple effects of Gal-1 on SH-SY5Y cells, including pro-viability, anti-apoptosis, and anti-ROS, can be abolished by knockdown of the expression of Nrf2 in cells (Figures 7 and 8).

Discussion

In the present study, we found that treatment with Gal-1 can facilitate neuronal survival by the recovery of growth inhibition and can prevent apoptosis. Additionally, treatment with Gal-1 had a recovery effect on zebrafish damaged by MPP+. Therefore, Gal-1 supplementation could be a potential therapeutic strategy for combating PD.

Gal-1, as the first identified bio-activated protein in the galectin's family, has been found to play various roles in cell bio-behaviors, including promoting cell proliferation, migration, and motility. In embryogenesis, endogenously generated Gal-1 contributes to the development of primary sensory neurons and the synaptic connections in the spinal cord[20]. Also, Gal-1 is expressed in the adult central nervous system, including the spinal cord, anterior brain, cerebellum, subventricular zone, and the olfactory bulb[6,21], and the expression of Gal-1 could regulate the proliferation and differentiation of neuronal stem cells[22] and modulate the activity of astrocytes[23]. Previous studies have revealed the dysregulated level of Gal-1 in some neuropathological diseases, including
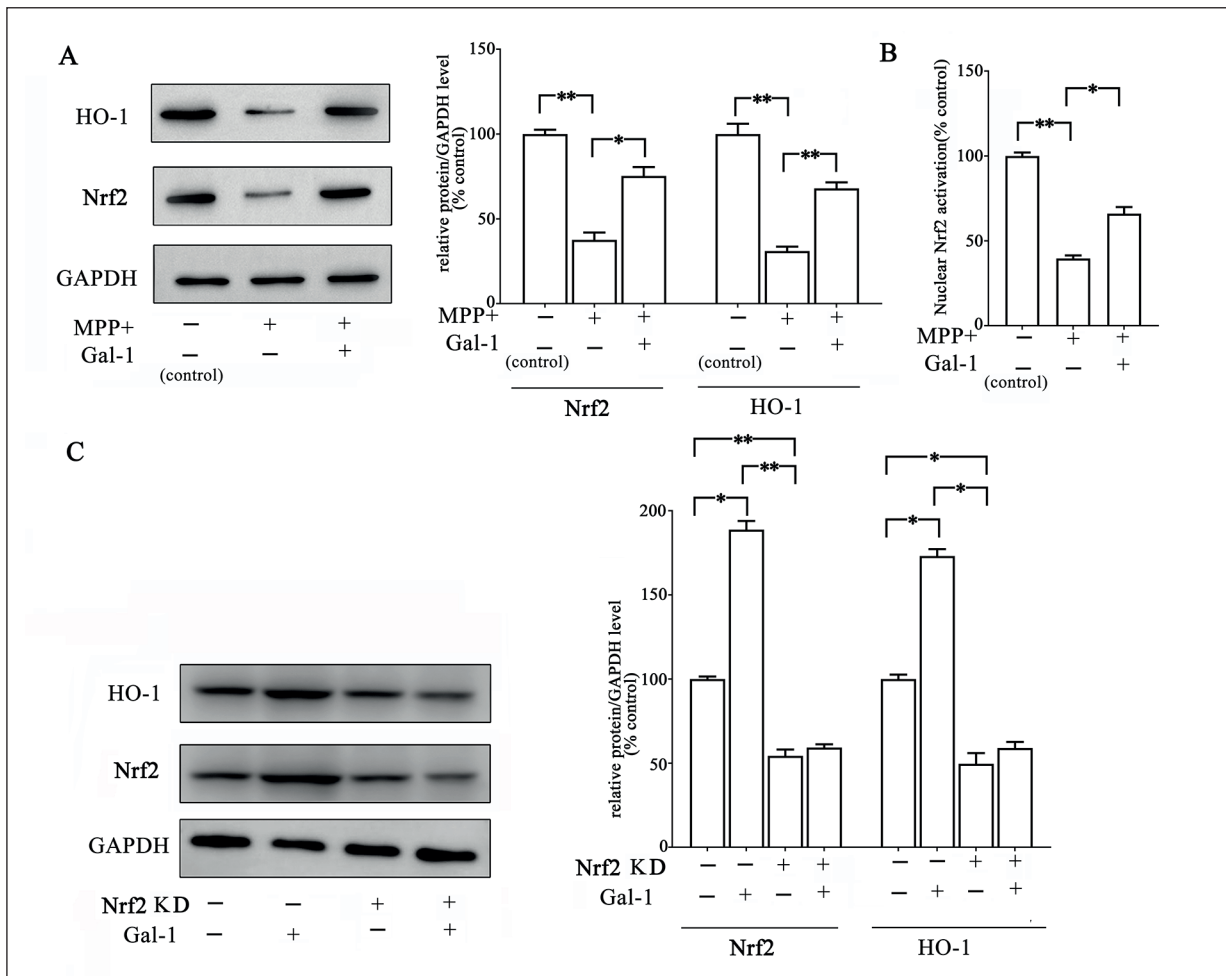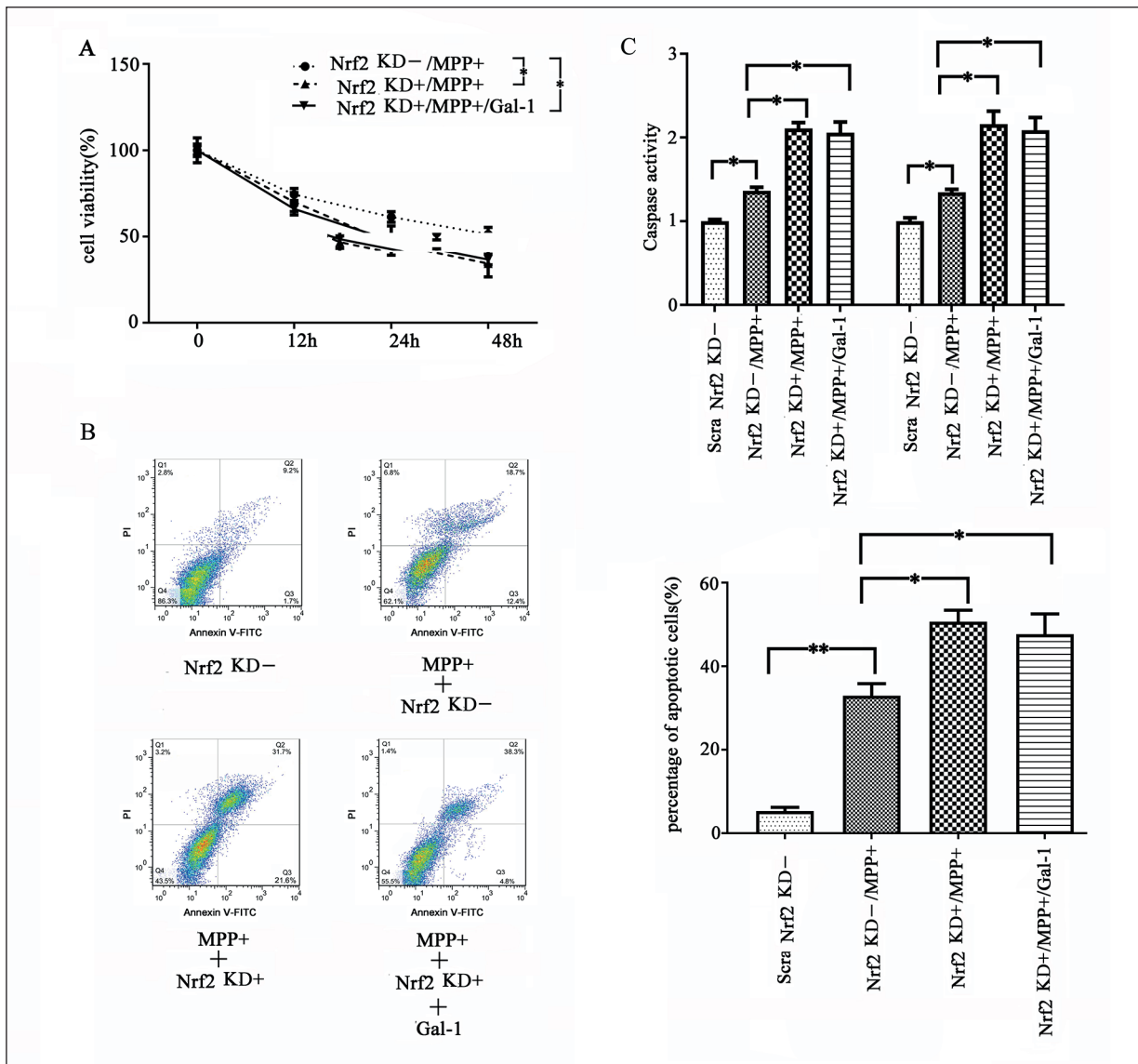
Figure 6. Gal-1 regulated the Nrf2 pathway in SY-SH5Y cells. **A**, SH-SY5Y cells were treated with MPP+ (500 µM) for 48 h with/without pretreatment with Gal-1 (4 µg/ml, 2 h) *in vitro*; cells in the control group were treated with PBS only. Nrf2 and HO-1 expression were measured. The western blot results showed that MPP+ can decrease the expression of both Nrf2 and HO-1. However, the inhibitory effect of MPP+ on the expression of Nrf2 and HO-1 can be alleviated by pretreatment with Gal-1. Under the pressure of MPP+, the cells with Gal-1 pretreatment had a higher level of both Nrf2 and HO-1 expression compared to that in cells without Gal-1 pretreatment. Representative images and quantitative results are shown. **B**, The transcriptional activity of Nrf2 was measured by enzyme-linked immunosorbent assay as described in the Materials and Methods section, and then the OD values were evaluated by a multimode plate reader. The data were normalized to a percentage relative to cells of the control group (PBS). MPP+ treatment significantly decreased Nrf2 activity compared to that in the control group. The cells pretreated with Gal-1 had higher Nrf2 activity compared to cells without Gal-1 pretreatment. **C**, The expression of Nrf2 was knocked down (KD) by transfection of SiRNA and then treated with Gal-1 (4 µg/ml) for 48 h. Thereafter, the expression of Nrf2 and HO-1 were examined by western blot, while the cells transfected with negative SiRNA served as controls. The results showed that Gal-1 treatment can increase the expression of both Nrf2 and HO-1, while the knockdown of the expression of Nrf2 in cells decreased the expression of HO-1. However, the knockdown expression of Nrf2 abolished the pro-effect of Gal-1 on HO-1 expression, for which no significant difference in the expression of HO-1 between Gal-1 treatment and no treatment in cells with Nrf2 knockdown was found. All results were representative of three independent experiments performed in triplicate. Data are presented as the mean ± SEM. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. (*$p < 0.05$ and ** $p < 0.01$). (Nrf2 KD +: Nrf2 knockdown with SiRNA; Nrf2 KD -: negative SiRNA).

Alzheimer's disease, PD, and amyotrophic lateral sclerosis. Wada et al[24] reported an increased Gal-1 level in patients with amyotrophic lateral sclerosis, while Pineda et al[25] suggested that the Gal-1 receptor participated in the activation of microglia in the pathology of Alzheimer's disease. Moreover, in PD, researchers have found a decreased Gal-1 level in the cerebrospinal fluid of PD patients compared to non-neurological control patients[11]. Another study on PD by proteome analysis revealed the positive expression of Gal-1 in the human substantia nigra[26], while sup-
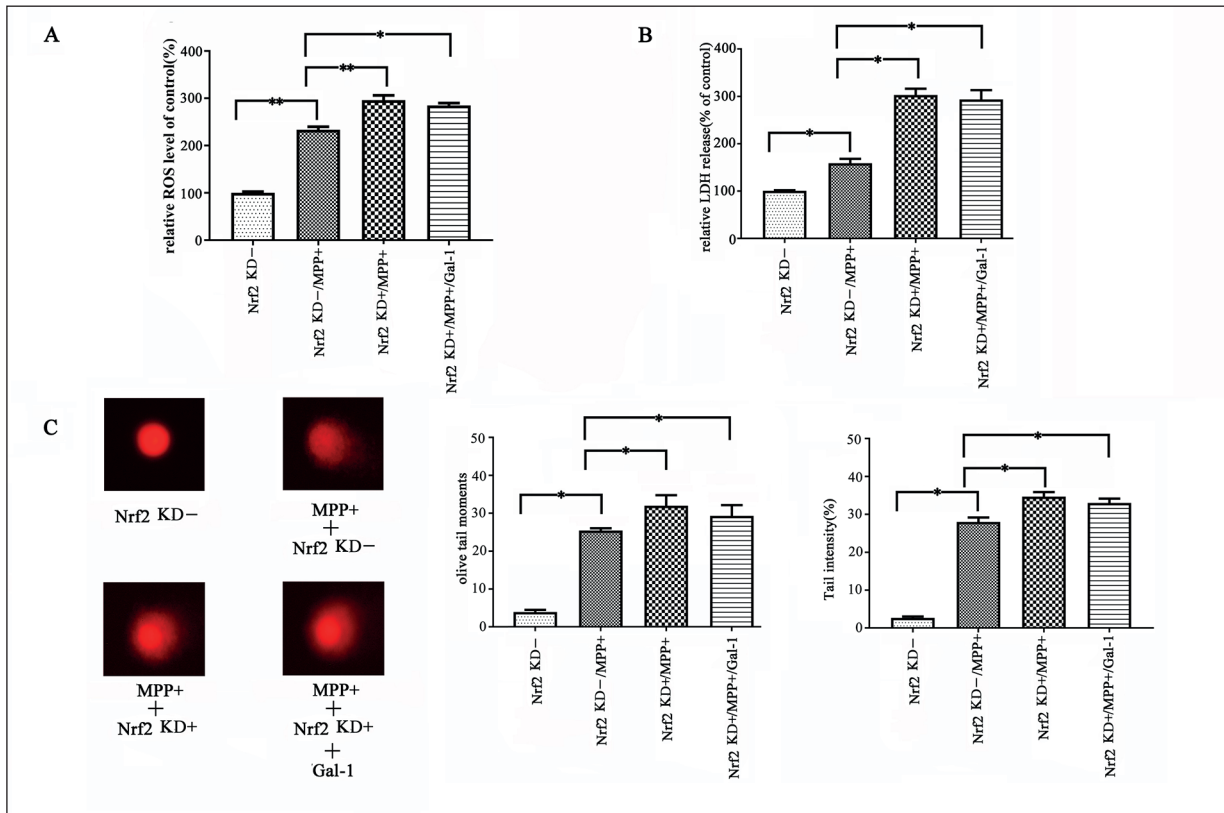
Figure 7. Gal-1 regulated cell viability and apoptosis through the Nrf2 pathway in SH-SY5Y cells. The cells with Nrf2 knockdown were pretreated with Gal-1 (4 μg/ml) for 2 h, after which they were exposed to MPP+ for 48 h. Thereafter, cell viability, cell apoptosis, and Caspase-3/8 activity were examined as described in Materials and Methods. The results showed that the knockdown of Nrf2 expression facilitated cell injury by MPP+, with decreased cell viability (**A**) and increased cell apoptosis (**B**: apoptotic rates, and **C**: apoptotic protein Caspase-3 and Caspase-8 activities) compared to the cells transfected with negative SiRNA. Moreover, the knockdown expression of Nrf2 can abolish the protective effect of Gal-1 in cells treated with MPP+, for which there was no statistical difference in cell viability and apoptosis, between Gal-1 treatment and no treatment in cells with Nrf2 knockdown under the pressure of MPP+. All results were representative of three independent experiments performed in triplicate, and the data are shown as the mean ± SEM. (*$p < 0.05$; **$p < 0.01$, by one-way ANOVA followed by Tukey's post hoc test.). (Nrf2 KD +: Nrf2 knockdown with SiRNA; Nrf2 KD -: negative SiRNA).

plementation with Gal-1 can inhibit microglial activation and thereby improve motor deficits in PD mice models[12].

Promoting neuron survival or proliferation is a promising approach to treat PD, one which has been the focus of many researchers in recent years. In the present study, we found that treatment with Gal-1 can facilitate neuronal survival. Consistently, Kajitani et al[27] suggested that Gal-1 can promote the proliferation of neural progenitors in the mouse hippocampus, while Wang et al[28] found that Gal-1-secreting neural stem cells

Figure 8. Gal-1 regulated ROS production and cell injury through the Nrf2 pathway in SH-SY5Y cells. The cells with Nrf2 knockdown were pretreated with Gal-1 (4 μg/ml) for 2 h, after which they were exposed to MPP+ for 48 h. Thereafter, ROS production, LDH leakage of cells, and DNA damage were examined as described in Materials in Methods. The results showed that the knockdown of Nrf2 expression facilitated cell injury by MPP+, with increased ROS production (**A**), LDH leakage (**B**), and DNA damage (**C**) in cells, compared to the cells transfected with negative SiRNA. Moreover, the knockdown expression of Nrf2 can abolish the protective effect of Gal-1 in cells treated with MPP+, for which there was no statistical difference in DNA damage, ROS production, and LDH leakage between Gal-1 treatment and no treatment in cells with Nrf2 knockdown under the pressure of MPP+. All experiments were performed in triplicate, and the data are shown as the mean ± SEM. (*$p <$ 0.05; **$p <$ 0.01, by one-way ANOVA). (Nrf2 KD +: Nrf2 knockdown with SiRNA; Nrf2 KD -: negative SiRNA).

were more resistant to brain injury. Besides, the anti-inflammatory role of Gal-1 could be another way to contribute to PD pathology. Li et al[12] observed that Gal-1 can inhibit the activation of microglia and its related inflammatory response through the MAPK/IκB/NFκB pathway. Further, Aalinkeel and Mahajan[29] suggested that endogenous Gal-1 inhibited the classical activation of microglial cells.

As byproducts of the normal metabolism of cells, ROS are regularly generated by the cells. Under physical conditions, ROS are eliminated by detoxifying systems in cells, such as glutathione and superoxide dismutase. However, some circumstances, such as aging, genetic change, or environmental factors, could lead to the overproduction of ROS, which overwhelms the abilities of detoxifying systems and consequent-

ly results in cell injury, including DNA breakage, cell membrane disturbance, and protein dysfunction. Neurons are especially sensitive to oxidative stress due to their high oxygen consumption. The overproduction of ROS induces neuronal death and thereby alters brain function, as observed in neurodegenerative disorders[30]. Moreover, the dysregulation of ROS generation and detoxifying systems is the classical pathological hallmark of PD[31]. In the pathology research on PD, researchers have found that MPP+ can induce the generation of ROS in cells, which then induces cell death. In our study, we found ROS generation in cells under the presence of MPP+, which could inhibit neuronal viability and induce cell injury and apoptosis. In addition, the toxicity effect of MPP+ eventually induced dopaminergic neuronal cell loss in ze-

brafish[32] and subsequently affected their locomotor ability. However, pretreatment with Gal-1 attenuated the increased level of ROS generation by MPP+ in cells. Thus, we suggest that Gal-1 could play a neuroprotective role through an anti-ROS system.

Furthermore, in this study, we investigated the potential multiple pathways that could be regulated by Gal-1, including PI3K, NF-κB, Nrf2, and PTEN-induced Kinase 1 (PINK1). Our data showed that Gal-1 failed to regulate PI3K or phosphorylated PI3K, p65 or phosphorylated P65 (molecule in NF-κB pathway), and PINK1 expression in SH-SY5Y cells (data not shown). In contrast, Gal-1 can promote the expression of Nrf2, which is a key player in PD pathology through the regulation of the metabolism of ROS in cells. Nrf2 is a transcription factor in the phase II antioxidant and xenobiotic response pathway and is quiescent in the cytosol in normal conditions[33]. When cell homeostasis is perturbed by redox imbalance, Nrf2 can translocate into the nucleus and regulate the expression of multiple genes related to antioxidants, such as HO-1, via the cis-acting antioxidant response element (ARE)[34]. In one study on lung injury, Huang et al[35] found that Gal-1 can regulate the expression of Nrf2 in cells. Moreover, the decline in activity of Nrf2 has been observed in PD patients[36]. Also, in PD animal models, researchers have observed that inactivation of Nrf2 could exacerbate neural damage and loss[34], whereas restoring the expression of Nrf2 can promote neuronal survival and function and thereby delay the progression of neurodegenerative disease[18]. Studies have demonstrated that the activation of Nrf2 could inhibit cell injury by MPP+[37]. Likewise, in the present study, knockdown expression of Nrf2 resulted in cell injury by ROS. Also, the knockout of Nrf2 in mice brains reduced the survival of dopaminergic neurons[38]. In contrast, we found that a supplement of Gal-1 could increase the expression of Nrf2, which alleviates the ROS production induced by MPP+ and eventually protects cells from cellular stress. Additionally, the activation of Nrf2 by Gal-1 increases the expression of HO-1. HO-1, downstream of the Nrf2-ARE pathway, is an enzyme that participates in cell defense against oxidative stress. The activated HO-1 can protect cells from ROS injury via multiple pathways, including maintenance of iron homeostasis, degradation of heme to antioxidant biliverdin[39], and upregulation of the expression of superoxide dismutase and catalase[40].

Conclusions

In summary, our study has demonstrated that the administration of Gal-1 could protect neurons from cellular stress by the recovery of growth inhibition, the prevention of apoptosis, and the elimination of ROS. Moreover, the neuroprotective effect of Gal-1 in neuronal cells could be related to the activation of Nrf2 expression. Therefore, Gal-1 could be a promising strategy for combating PD.

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