Original Paper

Lentiviral Vector-Mediated SHC3 Silencing Exacerbates Oxidative Stress Injury in Nigral Dopamine Neurons by Regulating the PI3K-AKT-FoxO Signaling Pathway in Rats with Parkinson's Disease

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Key Words

SHC3 • Gene silencing • Parkinson's disease • Oxidative stress injury • PI3K-AKT-FoxO signaling pathway

Abstract

Background/Aims: Parkinson's disease (PD) is a prevalent disease that leads to motor and cognitive disabilities, and oxidative stress (OS) injury was found to be related to the etiology of PD. Increasing evidence has shown that SHC3 is aberrantly expressed in neurons. The current study explored the involvement of SHC3 silencing in OS injury in the nigral dopamine neurons in rats with PD on the PI3K-AKT-FoxO signaling pathway. **Methods:** To study the mechanism and effects of SHC3 silencing in PD at the tissue level, 170 rats were selected, and a lentivirus-based packaging system was designed to silence SHC3 expression in rats. Furthermore, PC12 cells were selected for *in vitro* experimentation. To evaluate the effect of SHC3 silencing in nigral dopamine neuronal growth, an MTT assay, propidium iodide (PI) staining, and Annexin V-PI double staining were performed to detect cell viability, cell cycle progression and cell apoptosis, respectively. **Results:** SHC3 shRNA led to decreased ROS and MDA levels and enhanced GSH activity, indicating that SHC3 silencing leads to oxidative retardation. SHC3 silencing repressed the extent of Akt and FoxO phosphorylation, thereby inhibiting the PI3K-AKT-FoxO signaling pathway. Furthermore, in cell experiments, SHC3 silencing suppressed PC12 cell proliferation and cell cycle progression, whereas it enhanced cell apoptosis. **Conclusion:** The current study provides evidence suggesting that SHC3 silencing may aggravate OS injury in nigral dopamine neurons via downregulation of the PI3K-AKT-FoxO signaling pathway in PD rats.

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Introduction

Parkinson's disease (PD) is characterized by gradual degeneration of the central nervous system due to loss of dopamine in brain cells and is commonly manifested as a movement disorder [1, 2]. PD is the second most widespread neurodegenerative disease and impacts approximately 2% of the population over 65 years old worldwide [3]. Chronic increased levels of reactive oxygen species (ROS) or a decreased capacity for the elimination of ROS has been called oxidative stress (OS) [4]. In addition, OS plays a significant role in the degeneration of dopamine neurons in PD [5] and is one of the mechanisms proposed to be responsible for dopamine neuron death in PD [6]. At present, no effective restorative treatments are available to delay the neurodegenerative process of PD and only symptomatic treatments are available on the market [7]. Therefore, modifying the disease course through neuroprotective therapy is a significant clinical need, and studies regarding the cellular mechanisms involved in the particularly vulnerable nigral neurons have been the object of intensive investigation [8, 9].

Src homology and collagen (SHC) proteins, including SHCA, SHCB, SHCC (SHC3), SHCD, and various splice isoforms, are prototypical signaling molecules existing in mammalian cells [10]. Among these, SHC3 has been shown to be linked to neuronal cells [11] and is typically expressed in mature neurons at high levels [12]. In a signal transduction cascade, the phosphoinositide 3-kinase (PI3K) signaling pathway is involved in physiological functions such as cell survival and cell cycle, protein synthesis and growth, motility and metabolism [13]. Evidently, as a threonine/serine protein kinase, protein kinase B (AKT) was found to mediate the PI3K signaling pathway [14]. The forkhead box O (FoxO) transcription factors are some of the most conserved targets of AKT and play important roles in a number of physiological processes, such as metabolism, regulation of cell cycle and response to stress factors [15]. Previous evidence demonstrated that activation of the PI3K-AKT signaling pathway is correlated with rat pheochromocytoma 12 (PC12) cells hypoxia-dependent protection [16]. A previous study demonstrated that fasudil antagonized dopamine neuronal loss and inhibited inflammation in PD by activating the PI3K/p-AKT signaling pathway [17]. Interestingly, it was demonstrated that treatment with γ T3 and δ T3 in a cellular PD model led to obvious activation of the PI3K-AKT signaling pathway [18]. Moreover, SHC3 has been shown to be involved in the inactivation of the PI3K-AKT signaling pathway [12], and via interaction with AKT, SHC proteins can contribute to OS [10, 19]. Currently, the association between SHC3 and the PI3K-AKT-FoxO signaling pathway in PD remains to be explored. Therefore, the current study aims to explore the effects of SHC3 on OS injury in nigral dopamine neurons through the PI3K-AKT-FoxO signaling pathway in PD rats.

Materials and Methods

Ethical statement

The present study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals issued by the US National Institutes of Health (NIH). All efforts were made to minimize the suffering of the animals included in the study.

Lentiviral vector construction and model establishment

The shRNA (5'-CACCGCTTGGCACATTCTGGAATTCAGAGATTCCAGAATGTGGC-3', 5'-AAGCAAAAGCTTGGCACATTCTGGAATCTCTTGAATTCAGAAATGTGGCAAGC-3') and negative control (NC) shRNA (5'-CACCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCCGAGAA-3', 5'-AAAATTCTCCGAACGTGTCACGTTCTCTTGAACGTGACACGTTCCGAGAAAC-3') sequences were designed and synthesized according to the confirmed effective siRNA target sequence and NC sequence. Target shRNA and NC shRNA were cloned into PLL3.7 vectors (Miaolingbio Inc., Wuhan, Hubei, China). Lentivirus-targeted SHC3 was obtained by cotransfection of recombinant plasmids and a packaging plasmid (pRSVrev, pMDLg-pRRE, and VSV-G) into PC12 cells.

A total of 170 clean male Sprague-Dawley (SD) rats (7 weeks old; weighing 220~250 g) (purchased from the Center for Animal Experiments at Wuhan University, Wuhan, Hubei, China) were included in the study. The rats were housed with free access to food and water in a quiet environment following a 12-h light-dark cycle. The rats were randomly divided and categorized into the blank group (20 rats), the model group (50 rats), the shRNA group (50 rats) and the NC group (50 rats). No operations were conducted on the rats in the blank group, and the same doses of phosphate-buffered saline (PBS), SHC3 shRNA lentivirus, and SHC3 NC lentivirus were injected into the substantia nigra of the rats in the model, shRNA and NC groups, respectively, using a stereotaxic technique. A week following the injections, the rats were injected with 100 µg of 6-hydroxydopamine (6-OHDA) (dissolved in normal saline containing 0.2% ascorbic acid at 4°C) (Sigma, St. Louis, MO, USA) for the establishment of the PD model. The rats were injected with 80 mg/kg ketamine in the abdominal cavity for anesthetization and fixed on a stereotaxic apparatus (51600 model; Wood Dale, IL, USA). The exposed skin was cleaned with alcohol swabs for disinfection. According to the Rat Brain in Stereotaxic Coordinates by Paxinos and Watson, 2 µL of lentivirus, PBS and 6-OHDA were injected at the location of A/P + 1.0, L/M + 2.7, and D/V - 4.0 at a rate of 1 µL/min [20]. The needle was gently extracted 10 min after injection. The rat scalps were then sutured and administered with analgesic measures. After revival and complete recovery to normal movement, rats were housed in the cages. Rats in each group were injected one time with apomorphine (0.5 mg/kg) (Sigma, St. Louis, MO, USA) into the abdominal cavity 4 weeks after administration of 6-OHDA injection. Following the 4 weeks, the rats were observed at fixed time intervals each week to record the frequency of spontaneous rotation per min over a 15-min period. The model was regarded as successfully established if the rats spontaneously spun counterclockwise at over 7 circles/min. The success rates of model establishment of the blank, shRNA and NC groups were 64% (32/50), 60% (30/50), and 62% (31/50), respectively.

Behavioral observation

After model establishment, the motor coordination of rats in each group was measured with a Rotarod experiment. A day prior to measurement, the rats were trained on a roller (diameter: 30 mm) three times at 1-h intervals. After 24 h, rats maintained balance and started moving on the roller, and the rotation rate was gradually increased from 4 rpm to 32 rpm within 1 min. After adaptation five times, time spent on the roller before falling down was recorded as the mean value and was recorded 5 times at 1-min intervals to obtain the mean value.

Rats included in the study underwent the pole-climbing test using the Ogawa method [21]. A straight wooden pole (60 cm in height, 18 mm in diameter) with a small wooden ball on the top was prepared. The pole was covered with gauze to ease any rats slipped down. The rats were placed on the top of the pole with their heads facing upward. The time of time that the rats needed to move from the top to the bottom of the pole was recorded five times at one-min intervals in order to obtain the mean value.

Tyrosine hydroxylase (TH) immunohistochemistry

Four weeks after successful model establishment, 10 rats in each group were randomly selected and injected with pentobarbital in the abdominal cavity for anesthetization. The hearts of the rats were perfused with 4% paraformaldehyde, and the midbrain tissue sections were extracted for TH staining. The tissue sections were dewaxed with xylene and dehydrated with gradient ethanol solutions, followed by antigen retrieval with sodium citrate and water-proof heating for 20 min. The sections were rinsed with PBS containing 0.1% Triton X-100 for 5 min, 3% H₂O₂ was added, and the sections were incubated at room temperature for 15 min. Next, the sections were rinsed with PBS three times, blocking serum was added, and the sections were incubated at room temperature for another 15 min. Subsequently, the sections were incubated at 4°C overnight with the TH antibody (dilution ratio of 1 : 1000, rabbit polyclonal, Abcam, Cambridge, MA, USA), and rinsed with PBS three times (3 min each). Next, biotin-labeled goat anti-rabbit immunoglobulin G (IgG) secondary antibody (dilution ratio of 1 : 200, ETI, USA) and streptomycin-labeled anti-biotin working solution (ETI, USA) were added to the sections to incubate for 30 min. Later, the sections were developed with diaminobenzidine (DAB) (Zsbio, Beijing, China), stained with hematoxylin, dehydrated with gradient ethanol, cleaned with xylene, and mounted with neutral balsam. Five sections of the substantia nigra in the midbrain near the injection position of each rat were selected in order to count the number of dark brown-stained TH-positive nigral cells under optical microscopy (magnified at 400×). The mean value was adopted for the final result.

Measurement of OS indexes

Four weeks after successful model establishment, 10 rats in each group were randomly selected and decapitated, and their brains were removed immediately. The ventral brain tissues were separated on ice, dried with bibulous paper, and homogenized using a homogenizer. The homogenates were centrifuged at a low temperature and 2000 r/min for 6~8 min. The extracted tissue samples were divided into 4 subsamples and preserved at -80°C for further experimentation. The levels of superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) in the brain tissues were tested using an SOD reagent kit (Solarbio, Shanghai, China), an enzyme-linked immunosorbent assay (ELISA) reagent kit (Sangon Biotech, Shanghai, China), and a Reduced GSH assay kit (Sangon Biotech, Shanghai, China), respectively, in order to measure the extent of OS injury in the dopamine neuron of rats in each group. The experiment was repeated three times to obtain the mean value.

Cell culture

The rat neuronal PC12 cell line (Nanjing Hwatao BioPharm Co., Ltd., Nanjing, China) was cultured in a 5% CO₂ incubator at 37°C with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Hangzhou Sijiqing Bioengineering Material Co., Ltd., Hangzhou, China) and 5% horse serum with 1% double antibiotic (100 µg/mL streptomycin and 100 U/mL penicillin, both from Gibco, Grand Island, NY, USA). The medium was refreshed every 2~3 days. Cell passage was conducted as cells reached 80% confluence. Cells in the logarithmic phase of growth were selected for further experimentation.

Cell transfection and grouping

PC12 cells in the logarithmic phase of growth were seeded in a 6-well plate (1.8 mL in each well). The medium was replaced by antibiotic-free medium 24 h later and refreshed with serum-free medium as the cells reached 40 - 60% confluence. A pair of siRNA (siRNA1, siRNA2, and siRNA3) and one pair of NC siRNA (Table 1) were synthesized and purified by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) according to the rat SHC3 gene. Next, the cells were transfected with siRNA in accordance with the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight h after transfection, the inhibitory effect of siRNA on the expression of SHC3 was determined using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The siRNA exhibiting the highest transfection efficiency was selected for further experimentation. Subsequently, the cells were assigned to the following groups: the blank group (under regular culture), the model group (100 µL of 6-OHDA was added to a final concentration of 50 µg/L for PD model establishment), the siRNA 1~3 groups (100 µL of 6-OHDA was added, and cells were transfected with SHC3 siRNA/siRNA2/siRNA3) and the NC group (100 µL of 6-OHDA was added, and cells were transfected with NC siRNA).

(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

PC12 cells were seeded in a 96-well plate at a density of 1 × 10⁴ cells/well. Twelve h later, the medium was removed and the cells were cultured in serum-free medium for 24 h. In addition, 5 duplicates of each group were prepared. As the cells reached 50% confluence, 20 µL of MTT solution (5 g/L, Sigma, St. Louis, MO, USA) was added into each well at 6 h, 12 h, and 24 h time intervals after transfection. After a 4-h incubation at 37°C, the medium was removed, and culture was terminated. Subsequently, 100 µL of formazan solution was added to the cells in each well, which were then incubated at 37°C for 4 h to sufficiently resolve the crystalline substance. The blank group was regarded as the control, and the optical density (OD) value (A490) of each well was determined using an ELISA reader (DG5031, Shanghai Kehua Instrumentation Co., Ltd., Shanghai, China). The results were recorded in order to calculate cell viability. The cell viability was expressed as (OD value of the control group - OD value of the experiment group) / OD value (A490) of the control group × 100%. The experiment was repeated three times in order to obtain the mean value.

Table 1. siRNA sequences for SHC3 silencing. Notes: NC, Negative control; SHC3, Src-homology collagen C

siRNA	Sequence (5'-3')
siRNA1	GCUUGCCACAUCUGGAA
siRNA2	GCACCAAGTACAACCG
siRNA3	GCGCTCAGGAGGTC

Propidium iodide (PI) single staining

PC12 cells in the logarithmic phase of growth were seeded in a 6-well plate at a density of 1.2×10^5 cells/well and 2 mL/well. After culturing for 24 h, the cells were transfected with different treatments upon reaching 70%~80% confluence. Six h after transfection, the medium was refreshed, and cells were digested (not excessively) with ethylene diamine tetraacetic acid (EDTA)-free trypsin and collected for further use. Next, the cells were centrifuged at 2500 rpm/min for 5 min, rinsed with PBS two times, recentrifuged and collected. Then, the cells were fixed with 3 mL of 70% ethanol and incubated at 4°C overnight. The following day, the cells were centrifuged, the supernatant was discarded, PI staining reagent was added to the cells, and the cells were filtered through a cell filter finally. Subsequently, stained cells were analyzed using a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA) at a wavelength > 575 nm for detection. Finally, the percentages of cells present in different phases of the cell cycle were calculated and recorded.

Annexin V/PI double staining

Cell apoptosis in each group was evaluated according to the instructions of the Annexin V fluorescein isothiocyanate (FITC)/PI double staining reagent kit (556547, Shanghai Solja Technology Co., Ltd., Shanghai, China). The $10 \times$ binding buffer was diluted to $1 \times$ binding buffer with deionized water. PC12 cells in each group were centrifuged at 2000 rpm for 5 min, collected, resuspended with precooled PBS, centrifuged at 2000 rpm for 5~10 min, and washed. The cell suspension was added to 1 mL of $1 \times$ binding buffer, mixed with 5 μ L of Annexin V-FITC, and cultured at room temperature for 10 min in the dark. Next, 5 μ L of PI was added to the cells, bathed in ice for 5 min, and immediately analyzed by flow cytometry (Cube6, Partec, Germany) was carried out to evaluate cell apoptosis using 488 nm excitation and 530 nm bandpass filter for FITC detection and a wavelength over 575 nm for PI detection.

RT-qPCR

Total RNA was extracted from the PC12 cells in each group using Trizol Reagent (Takara, Kusatsu, Shiga, Japan). Total RNA was reverse transcribed into cDNA using reverse transcription reagent kit (Takara, Dalian, Liaoning). The primer sequences are shown in Table 2. RT-qPCR was performed using Premix Ex Taq II reagent kit (Takara, Kusatsu, Shiga, Japan) and an ABI 7500 qPCR instrument (Advanced Biochemicals, Inc., Suwanee, GA, USA). The reaction conditions were as follows: predenaturation at 94°C for 5 min, and 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The 20 μ L reaction system was composed of the following: 10 μ L of SYBR Premix Ex Taq™ II, 0.4 μ L of PCR forward primer (10 μ M), 0.4 μ L of PCR reverse primer (10 μ M), 0.4 μ L of 6-carboxy-X-rhodamine (ROX) reference dye, 0.4 μ L cDNA, and 6.0 μ L of sterilized distilled water. β -actin was used as the internal reference, and the relative expression of genes was determined using the $2^{-\Delta\Delta C_t}$ method. The experiment was repeated three times in order to obtain the mean value.

Western blot analysis

Protein was extracted from PC12 cells according to the instructions for the protease inhibitor-supplemented M-PER reagent (Pierce, Rockford, IL, USA). The concentration of the extracted proteins was measured using a protein assay reagent kit (Bio-Rad, Hercules, CA, USA). Protein separation was conducted using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to a nitrocellulose membrane, which was blocked with skim milk powder, for 1 h using the wet-transfer method. Membranes were incubated overnight at 4°C with the following primary antibodies:

Table 2. Primer sequences for RT-qPCR. Notes: RT-qPCR, reverse transcription quantitative polymerase chain reaction; SHC, Src-homology collagen; AKT, protein kinase B; FoxO, Forkhead box O; Bcl-2, B-cell lymphoma-2; F, forward; R, reverse

Gene	Sequence (5'-3')
SHC3	F: AAAAGCTTATGAGTGCCACGAGGAAGACCGG
	R: TTTGGATCCCGGGTTCTCTCCACTGGTT
AKT	F: CCACAGGTCGCTACTATGCC
	R: ACAGCCGAAGTCCGTTATC
FoxO	F: AATTGTGTAAGACCGAGGA
	R: AAGTCATCATTTGCTGTGGGA
Bcl-2	F: CGACTTTGCAGAGATGTCCA
	R: ATGCCGGTTCAGGTACTCAG
Bax	F: AGACAGGGGCTTTTGTGTAC
	R: GAGGACTCCAGCCACAAGAT
Caspase 3	F: GGACCTGTGGACCTGAAAAA
	R: GCATGCCATATCATCGTCAG
Cyclin D1	F: GCGTACCTTGACACCAATCT
	R: GCTCCAGAGACAAGAAACG
Cyclin E	F: CGTCAACGACACGGGAGAA
	R: AAGCAGCAGGAGACACATAAG
Cyclin A	F: TATCTTCTTCTTGGTAG
	R: TAATGAATAGCCGTAATAG
β -actin	F: GAACCTAAGGCCAAC
	R: TGTACGCACGATTTC

rabbit polyclonal antibody SHC3 (dilution ratio of 1 : 1000, ab217755), rabbit monoclonal antibody AKT (dilution ratio of 1 : 1000), rabbit monoclonal antibody p-AKT (dilution ratio of 1 : 1000, Ser-473, 8200S), rabbit polyclonal antibody FoxO (dilution ratio of 1 : 2500, ab12162), rabbit polyclonal antibody p-FoxO (dilution ratio of 1 : 500~1 : 1000, Ser-253, ab47285), rabbit polyclonal antibody B-cell lymphoma-2 (Bcl-2) (dilution ratio of 1 : 500~1 : 1000, ab59348), rabbit monoclonal antibody Bax (Bcl-2 Associated X Protein) (dilution ratio of 1 : 1000~1 : 10000, ab32503), rabbit polyclonal antibody Caspase 3 (dilution ratio of 1 : 500, ab13847), rabbit monoclonal antibody Cyclin D1 (dilution ratio of 1 : 10000~1 : 50000, ab134175), rabbit polyclonal antibody Cyclin E (dilution ratio of 1 : 2000, ab71535), and rabbit monoclonal antibody Cyclin A (dilution ratio of 1 : 2000, ab181591). Subsequently, the membranes were rinsed with phosphate-buffered saline-Tween 20 (PBST) three times to remove the primary antibodies and further incubated with the horseradish peroxidase (HRP)-labeled secondary antibody (dilution ratio of 1 : 2000~1 : 5000, goat anti-rabbit antibody, ab6789) at room temperature for 1 h. Next, the membranes were rinsed three times with Tris-buffered saline with Tween (TBST), and proteins were detected with enhanced diaminobenzidine tetrahydrochloride (ECL) (Yeasten, Shanghai, China) and visualized in dark conditions. The rabbit monoclonal antibody β -actin (dilution ratio of 1 : 3000, ab8227) was used as the internal reference, and the gray value was analyzed using the ImageJ software. The primary antibodies Akt and p-Akt were purchased from Cell Signaling Technology (Danvers, MA, USA), and the remaining antibodies were purchased from Abcam Inc. (Cambridge, MA, USA). The experimental steps were also used to determine the related proteins in rat brain tissues.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All experimental procedures were repeated three times. Measurement data were shown as the mean $\pm$ standard deviation. Comparisons between two groups were analyzed using the *t*-test, and comparisons among multiple groups were analyzed with analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

Results

SHC3 silencing hampers rats' body movement

The results of the Rotarod experiment and pole-climbing test are shown in Fig. 1. In the Rotarod experiment, the duration time that rats spent on the roller before falling down was significantly longer in the blank group (100.91 ± 10.48 s) than in the model (45.03 ± 7.11 s) and NC groups (40.38 ± 5.12 s) ($p < 0.05$). The duration of time was significantly shorter in the shRNA group (22.37 ± 2.57 s) than in the model and NC groups ($p < 0.05$). No significant differences were observed between the model and NC groups ($p > 0.05$). In the pole-climbing test, the duration of time that the rats needed to move from the top to the bottom of the pole was significantly shorter in the blank group than in the model and NC groups ($p < 0.05$). The duration of time was significantly longer in the shRNA group than in the model and NC groups ($p < 0.05$). The NC and model groups exhibited no significant time duration differences ($p > 0.05$). Therefore, the aforementioned findings suggest that reduced expression of SHC3 causes motor retardation in rats.

SHC3 silencing reduces the numbers of dopamine neurons

The results of TH staining (Fig. 2) demonstrated that there were no evident morphological changes in the midbrain tissues of the blank group, in addition to

Fig. 1. SHC3 silencing hinders rats' body movement. Notes: Panel A, Rotarod experiment shows that the duration time of rats in the shRNA group was the shortest; Panel B, Pole-climbing test shows that the duration time of rats in the shRNA group was the longest; *, $p < 0.05$, compared with the blank group; &, $p < 0.05$, compared with the model group; #, $p < 0.05$, compared with the NC group; NC, negative control.

Fig. 2. SHC3 silencing induces obvious morphological changes. Notes: Panel A, TH staining results (400 ×) indicate that midbrain tissues in the shRNA group exhibit atrophy and degeneration of the remaining nerve cells with decreased dopamine neurons; B, Number of TH-positive cells decreased most in the shRNA group; *, $p < 0.05$, compared with the blank group; †, $p < 0.05$, compared with the model group; #, $p < 0.05$, compared with the NC group; NC, negative control; TH, Tyrosin hydroxylase.

Fig. 3. SHC3 silencing regulates the PI3K-AKT-FoxO signaling pathway in vivo. Notes: Panels A-B, Relevant protein level determination by western blot analysis indicates that shRNA reduces protein levels of two isoforms, p63SHC3 and p52SHC3, simultaneously; Panels C, D and E, The determination by RT-qPCR and western blot analysis indicates that mRNA expression and protein levels of PI3K-AKT-FoxO signaling pathway-related genes are downregulated by SHC3 silencing; *, $p < 0.05$, compared with the blank group; †, $p < 0.05$, compared with the model group; #, $p < 0.05$, compared with the NC group; SHC, Src homology and collagen; AKT, protein kinase B; FoxO, Forkhead box O; NC, negative control.

normally growing neurons. The TH-positive cell number was significantly higher in the blank group (266.00 ± 48.31) than in the model group (138.07 ± 35.12) and NC (123.44 ± 34.10) groups ($p < 0.05$). No significant differences were observed between the model and NC groups ($p > 0.05$). The midbrain tissues in the shRNA group displayed atrophy and degeneration of the remaining nerve cells. Compared with those in the model and NC groups, the number of dopamine neurons was found to be decreased significantly in the shRNA group ($p < 0.05$).

SHC3 silencing exacerbates the OS injury

The levels of the OS indexes SOD, MDA, and GSH are shown in Table 3. Compared with those in the blank group, the levels of SOD and GSH were significantly decreased, and the MDA level was significantly enhanced in the model, NC and shRNA groups ($p < 0.05$). No significant differences were observed between the model and NC groups ($p > 0.05$). Compared with those in the model and NC groups, the shRNA group showed significantly decreased levels of SOD and GSH, and significantly increased levels of MDA ($p < 0.05$). Therefore, SHC3 was implied to play a protective role in OS injury in PD rats.

Table 3. The level of SOD, MDA, and GSH in each group. Notes: SOD, superoxide dismutase; MDA, malondialdehyde; GSH, glutathione; NC, negative control

Groups	Number	SOD (NU/mg)	MDA (nmol/mg)	GSH (mg/g)
Blank	10	211.32 ± 15.28	4.76 ± 0.68	170.61 ± 12.35
Model	10	177.61 ± 11.74*	7.86 ± 1.27*	88.97 ± 7.66*
shRNA	10	124.29 ± 9.67*#	11.75 ± 1.43*#	57.43 ± 5.51*#
NC	10	170.54 ± 12.46*	8.06 ± 1.13*	85.34 ± 7.52*

SHC3 silencing represses the PI3K-AKT-FoxO signaling pathway in an in vivo experiment

To validate whether shRNA exerts an effect on two isoforms such as p63SHC3 and

Fig. 4. siRNA1 has the highest inhibitory efficiency compared with siRNA2 and siRNA3. Notes: Panel A, The mRNA expression of SHC3 is significantly decreased in the siRNA1 group detected by RT-qPCR; Panel B, Inhibition rate of SHC3 is significantly increased in the siRNA1 group presented as a bar graph; *, $p < 0.05$, compared with the blank group; &, $p < 0.05$, compared with the siRNA1 group; #, $p < 0.05$, compared with the siRNA2 group; SHC3, Src homology and collagen

p52SHC3 simultaneously, western blotting analysis was conducted to evaluate the protein levels of isoforms of SHC3 in rat brain tissues. The results indicate that the protein levels of both p63SHC3 and p52SHC3 were significantly reduced in response to shRNA ($p < 0.05$) (Fig. 3A-B). Furthermore, in order to verify whether SHC3 regulates PD through the PI3K-Akt-FoxO signaling pathway, the mRNA expression and protein levels of PI3K-Akt-FoxO-related genes in rat brain tissues were evaluated using RT-qPCR and western blot analysis (Fig. 3C-E). SHC3 mRNA expression was found to be downregulated in the shRNA group ($p < 0.05$), while SHC1, SHC2, and SHC4 mRNA expression was not significantly different with the other three groups ($p > 0.05$). In contrast to the blank group, the extent of Akt and FoxO phosphorylation was found to be reduced in the other three groups. Compared with the levels observed in the model and NC groups, the shRNA group displayed a decrease in Akt and FoxO phosphorylation ($p < 0.05$). No significant differences were observed between the NC and model groups ($p > 0.05$). The results indicate that silenced expression of SHC3 could suppress the PI3K-Akt-FoxO signaling pathway, thereby modulating nigral dopamine neurons in PD rats.

siRNA1 has the highest inhibitory efficiency

After transfection of PC12 cells with liposomes for 48 h, the mRNA expression of SHC3 was detected using RT-qPCR. Compared with that in the blank group, the mRNA expression of SHC3 was significantly lower in the model, NC, siRNA1, siRNA2, and siRNA3 groups ($p < 0.05$). No significant differences were observed between the model and NC groups ($p > 0.05$) (Fig. 4A). The inhibition efficiency in the siRNA1 group was found to be significantly higher than in the siRNA2 and siRNA3 groups ($p < 0.05$) (Fig. 4B). Consequently, the siRNA1 group was selected as the siRNA group in subsequent experiments.

SHC3 silencing inhibits cell viability

The result of the MTT assay (Fig. 5) indicated that SHC3 silencing inhibited the growth of PC12 cells. At the 6-h time interval, cell viability was found to be significantly decreased in the model and NC groups compared to that in the blank group ($p < 0.05$). No significant differences were observed between the model and NC groups ($p > 0.05$). Compared with that in the model and NC groups, the siRNA group displayed decreased cell activity ($p < 0.05$). The inhibitory effect of siRNA increased as time went on and peaked at the 24-h time interval. This inhibitory effect demonstrated no significant differences between the model and NC

Fig. 5. SHC3 silencing suppresses cell viability detected by MTT assay. Notes: *, $p < 0.05$, compared with the blank group; &, $p < 0.05$, compared with the model group; NC, negative control.

Fig. 6. SHC3 silencing represses cell cycle progression.

Notes: Panels A and B, some neurons re-enter into the cell cycle, and cells were arrested at the G0/G1 phase, detected by flow cytometry; Panels C, D and E, mRNA expression and protein levels of cell cycle-related genes Cyclin D1, Cyclin E, and Cyclin A are remarkably decreased in the siRNA group; *, $p < 0.05$, compared with the blank group; &, $p < 0.05$, compared with the model group; #, $p < 0.05$, compared with the NC group; SHC3, Src homology and collagen C; NC, negative control.

Fig. 7. SHC3 silencing enhances cell apoptosis.

Notes: Panel A, PI staining results indicate that there were apoptotic cells in the siRNA group; Panel B, The siRNA group exhibits the most apoptotic cells.

Panel C, PI staining results indicate that there were apoptotic cells in the siRNA group; Panel D, The siRNA group exhibits the most apoptotic cells. Panels C, D and E, The mRNA expression and protein levels of Bcl-2 are decreased, and the mRNA expression and protein levels of Bax and Caspase 3 are increased in the siRNA group; *, $p < 0.05$, compared with the blank group; &, $p < 0.05$, compared with the model group; #, $p < 0.05$, compared with the NC group; NC, negative control; SHC3, Src homology and collagen C; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X Protein.

groups ($p > 0.05$). The aforementioned findings suggested that silenced expression of SHC3 repressed cell proliferation, which may exacerbate OS injury in nigral dopamine neurons in PD.

G0/G1 phase cells increased, and S/G2 phase cells decreased in the siRNA group

The results of flow cytometry are shown in Fig. 6. The number of cells in the G0/G1 phase was increased, and the number of cells in the S/G2 phase was decreased in the model, NC and siRNA groups compared to those the blank group ($p < 0.05$). No significant differences were observed between the model and NC groups ($p > 0.05$). Compared with those the model and NC groups, the siRNA group displayed a significantly increased number

Fig. 8. SHC3 silencing regulates the PI3K-AKT-FoxO signaling pathway in vitro. Notes: Panels A-B, Relevant protein level determinations by western blot analysis indicate that siRNA reduces the protein levels of two isoforms of SHC3, p63SHC3

and p52SHC3, most among all SHC family genes. A represents the p52SHC3 isoform; B represents the p63SHC3; Panels C, D and E, The determinations by RT-qPCR and western blot analysis indicate that mRNA expression and protein levels of the PI3K-AKT-FoxO signaling pathway-related genes are downregulated by SHC3 silencing; *, $p < 0.05$, compared with the blank group; &, $p < 0.05$, compared with the model group; #, $p < 0.05$, compared with the NC group; SHC, Src homology and collagen; AKT, protein kinase B; FoxO, Forkhead box O; NC, negative control.

of cells in the G0/G1 phase and a significantly increased number of cells in the S/G2 phase ($p < 0.05$), suggesting that some neurons re-entered to the cell cycle and that cells were arrested in the G0/G1 phase by SHC3 silencing. RT-qPCR and western blot analysis showed that the levels of cell cycle-related genes Cyclin D1, Cyclin E, and Cyclin A were lower in the model, siRNA and NC groups compared with the blank group ($p < 0.05$). Compared with the model and NC groups, the siRNA group exhibited lower levels of Cyclin D1, Cyclin E, and Cyclin A ($p < 0.05$). The model and NC groups displayed no significant differences ($p > 0.05$). These findings indicated that silenced expression of SHC3 suppressed cell cycle progression, which may exacerbate OS injury in nigral dopamine neurons in PD.

SHC3 silencing increases cell apoptosis

The results of TUNEL staining (Fig. 7) demonstrate that cell apoptosis was elevated in the siRNA group compared to that in the blank and model groups ($p < 0.05$), indicating that SHC3 inhibits apoptosis in dopaminergic neurons in PD. RT-qPCR and western blot analysis displayed that levels of Bcl-2 were reduced, and levels of Bax and Caspase 3 were increased in the model, NC, and siRNA groups compared to those of the blank group ($p < 0.05$). No significant differences were found between the model and NC groups ($p > 0.05$). Compared with the model and NC groups, the siRNA group exhibited decreased levels of Bcl-2, in addition to increased levels of Bax and Caspase 3, suggesting that SHC3 silencing promoted cell apoptosis in dopaminergic neurons via regulation of Bcl-2, Bax, and Caspase 3. The aforementioned findings suggested that lower expression of SHC3 induced cell apoptosis and consequently aggravated OS injury in nigral dopamine neurons in PD.

SHC3 silencing represses the PI3K-AKT-FoxO signaling pathway

The results of RT-qPCR and western blot analysis are shown in Fig. 8. A polyclonal antibody was used to detect the two isoforms, p63SHC3 and p52SHC3, through western blot analysis. The protein levels of p63SHC3 and p52SHC3 were found to be reduced in the siRNA group compared with those in the model, NC, and blank groups ($p < 0.05$), suggesting that the protein levels of the two isoforms of SHC3, p63SHC3 and p52SHC3, were suppressed in response to siRNA. There were no significant differences in levels of AKT and FoxO

among the four groups, whereas the extent of AKT and FoxO phosphorylation was found to be significantly lower in the model, NC, and siRNA groups than in the blank group ($p < 0.05$). Compared with that in the model and NC groups, the siRNA group displayed a reduced level of AKT and FoxO phosphorylation ($p < 0.05$). The model and NC groups displayed no significant difference ($p > 0.05$). The above results revealed that SHC3 silencing may repress the PI3K-AKT-FoxO signaling pathway and protect nigral dopamine neurons from degenerative changes in PD rats.

Discussion

Parkinson's disease (PD), as the second most prevalent degenerative disorder of the central nervous system, currently affects more than 100,000 individuals worldwide [22–24]. Despite extensive preclinical studies in PD animal models, there is still a lack of effective neuroprotective drugs for PD available [25, 26]. Interestingly, the PI3K-AKT-FoxO signaling pathway has been suggested to be implicated in the treatment of PD [27]. The current study suggests that SHC3 silencing inhibits the PI3K-AKT-FoxO signaling pathway and consequently exacerbates OS injury in nigral dopamine neurons in PD rats.

The findings of the current study revealed that SHC3 silencing may decrease SOD and MDA and enhance the activity of GSH. SOD is verified to be expressed to antagonize the oxidative burden [28]. A previous study demonstrated that high levels of SOD1 decreased OS and further alleviated neuronal injury from cerebral ischemia [29]. MDA is considered an OS biomarker and is expressed in the process of oxidative degradation of polyunsaturated lipids [30]. MDA levels have also been found to be downregulated after treatment in patients with hypothyroidism, which is a state of enhanced OS [31]. It was previously demonstrated that reduced GSH status signifies an increased level of OS [32]. Another study demonstrated that primary cultures of cortical neurons in SHC3 null mice are more sensitive to hypoxia/oxidative-induced apoptosis owing to downregulation of the PI3K-AKT signaling pathway [33], and decreased levels of GSH contribute to the enhanced vulnerability of the aged brain to OS [34]. From the aforementioned evidence, SHC3 silencing was shown to be associated with increased OS injury in nigral dopamine neurons.

Additionally, the current study demonstrated that in PC12 cells, SHC3 siRNA treatment decelerated cell proliferation, accelerated G1/G0 phase cells, decreased S/G2 phase cells, and enhanced cell apoptosis. SHC3 is a phosphotyrosine-binding domain-containing adaptor protein that signals cellular differentiation and survival pathways, for example, the AKT signaling pathway [35]. It was previously revealed that SHC3 overexpression promotes neuronal cell survival and inhibits apoptosis by activating the PI3K-AKT signaling pathway [36]. A previous study showed that expression of p52SHC3, a truncated variant of SHC3, leads to increased AKT phosphorylation and enhanced apoptosis *in vitro* [37]. Previous studies have demonstrated that reduced levels of Cyclin D1 were correlated with decreased pAkt levels and decreased phosphorylation levels of PI3K-AKT and that inhibition of PI3K coordinately reduces Cyclin E protein levels [38, 39]. Moreover, Cyclin A1 was reported to be a crucial downstream target of the PI3K/AKT signaling pathway, which conveys survival signals in response to IL6 stimulation [40]. As a member of the Bcl-2 family, Bax is a type III nuclear-encoded protein that is expressed in higher eukaryotes and is able to pierce the outer membrane of mitochondria to mediate cell death through apoptosis [41]. Inhibition of the PI3K-AKT signaling pathway has been demonstrated to be correlated with reduced levels of anti-apoptotic Bcl-2 and increased levels of pro-apoptotic Bax, subsequently leading to cell apoptosis [42, 43]. Caspase 3, a frequently activated mediator of programmed apoptosis, is known to catalyze the cleavage of numerous key cellular proteins, such as TG2 and Atg7 KD [44]. A previous study demonstrated that downregulation of AKT phosphorylation following Caspase 3 activation has the ability to inhibit generalized cellular proliferation and induce apoptosis [45].

In addition, the current study demonstrated that cells transfected with SHC3 siRNA exhibited reduced levels of AKT and FoxO phosphorylation. The reduced expression of SHC3 could suppress the activation of the PI3K-AKT-FoxO signaling pathway, thereby modulating nigral dopamine neurons in PD rats. A previous study found that decreased AKT expression was associated with reduced dopaminergic neurons in PD, and furthermore, the activation of AKT is linked to the abundance of dopaminergic neurons, offering neuroprotection in PD [46]. In addition, another study found that FoxO proteins modulated gene expression involved in oxidative stress and were regulated by AKT [47]. Interestingly, the stress response factor FoxO3, a member of the FoxO family, was considered neuroprotective in PD by another study [48]. Moreover, SHC3 was found to positively affect neural cell survival through decreased Akt phosphorylation [37]. Therefore, the aforementioned findings demonstrated that the PI3K-AKT-FoxO signaling pathway was downregulated via SHC3 silencing in nigral dopamine neurons in PD rats.

In summary, the current study demonstrates that SHC3 silencing aggravated oxidative injury in nigral dopamine neurons via downregulation of the PI3K-AKT-FoxO signaling pathway in PD rats. These findings provide a potential mechanism for the further understanding and treatment of PD. However, further exploration of the regulation between SHC3 and the PI3K-AKT-FoxO signaling pathway is warranted in order to better understand the precise molecular regulation.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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