RESEARCH ARTICLE

The protective effort of GPCR kinase 2–interacting protein-1 in neurons via promoting Beclin1-Parkin induced mitophagy at the early stage of spinal cord ischemia-reperfusion injury

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Abstract

In spinal cord ischemia-reperfusion (I/R) injury, large amounts of reactive oxygen species can cause mitochondrial damage. Therefore, mitophagy acts as the main mechanism for removing damaged mitochondria and protects nerve cells. This study aimed to illustrate the important role of GPCR kinase 2–interacting protein-1 (GIT1) in mitophagy in vivo and in vitro. The level of mitophagy in the neurons of Git1 knockout mice was significantly reduced after ischemia-reperfusion. However, the overexpression of adeno-associated virus with Git1 promoted mitophagy and inhibited the apoptosis of neurons. GIT1 regulated the phosphorylation of Beclin-1 in Thr119, which could promote the translocation of Parkin to the mitochondrial outer membrane. This process was independent of PTEN-induced kinase 1 (PINK1), but it could not rescue the role in the absence of PINK1. Overall, GIT1 enhanced mitophagy and protected neurons against ischemia-reperfusion injury and, hence, might serve as a new research site for the protection of ischemia-reperfusion injury.

KEYWORDS

GIT1, mitophagy, neuron, spinal cord injury

Abbreviations: BECN1, Beclin 1; BMS, Basso mouse scale; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; GIT1, GPCR kinase 2–interacting protein-1; I/R, ischemia-reperfusion; IgG, Immunoglobulin G; MAP2, microtubule-associated protein 2; NDP52, calcium binding and coiled-coil domain 2; NeuN, neuronal nuclei; OGD/R, oxygen-glucose deprivation/reperfusion; OPTN, optineurin; PBS, phosphate-buffered saline; PINK1, PTEN-induced kinase 1; ROS, reactive oxygen species; VDAC, anti-voltage-dependent anion channel.

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INTRODUCTION

Ischemia-reperfusion injury of the spinal cord, the most common secondary injury of the spinal cord, occurs after decompression surgery for spinal stenosis or aortic surgeries and leads to the derision of neurological function. It generates reactive oxygen species (ROS) causing oxidative stress, which may lead to the damage of mitochondria. The clearance of damaged mitochondria plays an important role in the early stage after spinal ischemia-reperfusion injury.

Mitophagy acts as mitochondria-specific autophagy that specifically phagocytoses mitochondria during mitochondrial damage and has been reported to protect against ischemia-reperfusion injury in many organs. ROS are produced during mitochondrial metabolism. They act as a regulator of signaling molecules in autophagy and regulates cell apoptosis and death. The process of mitophagy is divided into two parts. First, damaged mitochondria and autophagy-related proteins aggregate at the site of autophagosome formation. This is followed by autophagosome formation and phagocytosis of damaged mitochondria. The most important regulatory mechanism in mitophagy is the ROS/PTEN-induced kinase 1 (PINK1)/Parkin pathway. Under normal conditions, PINK1 is localized in the mitochondrial inner membrane and, with the assistance of presenilin-associated rhomboid-like protein, enters the cytoplasm and is degraded to a low level. However, when the mitochondrial membrane potential is lowered, PINK1 is not able to enter the mitochondria; it accumulates in the mitochondrial outer membrane and promotes Parkin aggregation on the outer membrane of mitochondria. Parkin is phosphorylated at ser65 and promotes the phosphorylation of ubiquitin to form ubiquitin chains in mitochondria. The ubiquitin chains bind to the autophagy receptor proteins such as, P62, OPTN, and NDP52. The autophagy receptor proteins bind to LC3 and eventually cause mitophagy. On the PINK1/Parkin pathway, Beclin-1 interacts with both PINK1 and Parkin and has a crucial impact. Beclin1 interacts with Parkin and promotes the translocation of Parkin to the mitochondrial membrane. PINK1 and Beclin-1 co-localize and interact in the mitochondria-associated membrane to promote autophagosomal formation. However, the specific way of how BECN1 affects mitophagy via the PINK1/Parkin pathway is still controversial. Overall, mitophagy is the main mechanism of mitochondrial renewal and may protect against ischemia-reperfusion injury in the early stage. In the late stage of reperfusion, the homeostasis of nerve cells undergoes complete collapse, leading to severe function disorder and overall synergistic dysfunction. At this time, mitophagy is over-activated and evolves into autophagic cell death.

GPCR kinase 2 interacting protein-1 (GIT1) is a GTPase-activating protein for the ADP-ribosylation factor family of small GTP-binding proteins. GIT1 is known to interact with many proteins and regulates their function and localization. Global Git1 knockout in mice inhibits mitochondrial formation during heart development but has no significant effect on mitochondria in other organs. GIT1 also plays a critical role in spinal cord injury. Recent studies have shown that GIT1 deficiency enhances JNK/p38 signaling and increases neuronal apoptosis in the middle stage of ischemia-reperfusion. A recent study also found that GIT1 interacted with BECN1 and regulated autophagy in osteoclasts.

At present, no reports are available on the relationship between GIT1 protein and mitophagy. The present study found that Git1 knockdown inhibited Parkin translocation to mitochondria and inhibited mitophagy after ischemia-reperfusion injury. This occurred mainly because GIT1 promoted the phosphorylation of BECN1 at the Thr119 domain. Phosphorylated BECN1 promoted the translocation of Parkin, but not PINK1, to the mitochondria. In addition, GIT1 promoted the formation of autophagic flow and also played an important role in mitophagy. The overexpression of Git1 in vivo and in vitro increased mitophagy in ischemia-reperfusion injury and protected the neurons.

MATERIALS AND METHODS

Antibodies and reagents

The primary antibodies used were as follows: rabbit anti-LC3B (ab48394, Abcam, Cambridge, UK); mouse anti-NeuN (ab104224, Abcam); mouse anti-PINK1 (ab186303, Abcam); rabbit anti-Beclin 1 (ab207612, Abcam); rabbit anti-TOMM20 (ab186735, Abcam); rabbit anti-phospho-ubiquitin (ser65) (62802, CST, MA, USA); rabbit anti-PINK1 (BC100-494, Novus, CO, USA); mouse anti-GIT1 (NBP2-22423, Novus); mouse anti-Parkin (BC100-494, Novus); rabbit anti-phospho-ubiquitin (Ser65) Alexa Flour 488 conjugate (ABS1513-I-AF488, Merk Millipore, Darmstadt, Germany); rabbit anti-Parkin (phosphor-Ser65) (orb312554, Biorbyt, Cambridge, UK); rabbit anti-p-Beclin-1 (Thr119) (AP3765a, Abgent, CA, USA); rabbit anti-NeuN (26975-1-AP, Proteintech, IL, USA); rabbit anti-voltage-dependent anion channel (VDAC) (55259-1-AP, Proteintech); rabbit anti-cleaved caspase-9 (Cell Signaling Technology, MA, USA); and rat anti-LAMP2 (ab13524, Abcam). The secondary antibodies used were as follows: mouse anti-immunoglobulin G (IgG) (H + L) (115-035-003, Jackson ImmunoResearch, PA, USA); rabbit anti-IGG (H + L) (111-035-003, Jackson ImmunoResearch); rat anti-immunoglobulin G (IgG) (H + L) (112-035-003, Jackson ImmunoResearch, PA, USA); AMCA-conjugated Affinipure Goat Anti-Mouse IgG(H + L) (SA00010-1, Proteintech); anti-rabbit IgG light chain (ab99697, Abcam); and mouse anti-IgG light chain (A20512, Abbkine, CA, USA). CCCP was obtained from MCE, NJ, USA.
2.2 | Animals

As previously described,\textsuperscript{30} Git1\textsuperscript{−/−} mice (C57BL/6 background) were produced in the laboratory (Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester, NY, USA). Identification of specific knockout mice is shown in Figure S3. The Git1 WT littermates were used as controls. The animal experiments and operations were approved by the Animal Committee at the First Affiliated Hospital of Nanjing Medical University.

2.3 | Spinal cord ischemia-reperfusion injury model and tissue treatment

The mice anesthetized with ether were fixed on a constant-temperature pad in a supine position, and the body temperature was maintained at 37.5°C.\textsuperscript{31} Then, 5U heparin (150 IU/kg) was injected intravenously 5 minutes before the surgery. Using the abdominal median incision approach, the surgical site was prepared for skin disinfection and padding. After the left kidney was located through the peritoneum, the abdominal aorta was carefully searched and separated along the left renal artery. The abdominal aorta was clipped under the outlet of the left renal artery (unclipped in sham-operated mice). After 45 minutes, the aneurysm clip was removed, and the blood perfusion was restored. After 4-6 hours, the chest cavity was opened, the heart was exposed, the perfusion needle was punctured from the apex of the heart, and the right atrial appendage was cutoff and then fixed with 4% (w/v) paraformaldehyde after rapid perfusion with pre-cooled phosphate-buffered saline (PBS). The severe twitching of the limbs and tail of the mice showed that the perfusion was successful. After perfusion, the skin and muscles of the back were cut open, the laminae were carefully bitten off, and the spinal cord samples were taken out from between the L1 and L3 segments.

2.4 | Preparation of frozen sections

The spinal cord was fixed for 24 hours in 4% paraformaldehyde, dehydrated in 20% and 30% (w/v) sucrose solution, and then vertically embedded in OCT (optimal cutting temperature compound) (Sakura, CA, USA). The spinal cord tissue was cut into transverse sections with 10 μm thickness using a Leica slicer and stored in the refrigerator at −40°C for further processing.

2.5 | Establishment of an OGD/R model

The primary neurons or HEK293T cells were washed with sugar-free Dulbecco's modified Eagle's medium (DMEM) three times, followed by replacement with sugar-free DMEM, and placed in a 37°C anoxic incubator (external 95% N\textsubscript{2} and 5% CO\textsubscript{2} mixed gas for 15 minutes to remove oxygen from the incubator). After 1 hour, the culture medium was replaced with the normal medium and then the neurons or cells were cultured in the normal incubator for 0-6 hours for further analysis.

2.6 | Western blot analysis

A whole-protein extraction kit and a mitochondrial protein extraction kit were used to extract protein according to the manufacturer's protocol. The Bradford method was used to determine the protein concentration, and equal amounts of protein were extracted for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After complete separation, the protein was transferred to the polyvinylidyene difluoride membrane (SEQ00010; EMD Millipore, MA, USA) and sealed with 5% (m/m) bovine serum albumin (BSA). The corresponding bands were cut out according to the molecular weight of the protein and incubated with the primary antibody overnight with shaking at 4°C. The bands were exposed to ECL reagents (Pierce Biotechnology, MA, USA) after incubating with the secondary antibody for 2 hours at room temperature. ImageJ (National Institutes of Health, MD, USA) was used to semi-quantify the density of protein bands.

2.7 | Co-IP

The cells were cleaned with pre-cooled PBS twice and then appropriate lysate was added. The protein was quickly scraped down and moved to an EP tube. The supernatant was centrifuged at 4°C. The protein concentration was measured by the Bradford method. The extracted protein (800 μg) was incubated with specific indicated antibodies at 4°C overnight. Subsequently, magnetic beads were added to incubate for 2 hours at 4°C. The magnetic beads-antibody-protein complexes were adsorbed on a magnetic frame. The magnetic beads were washed with the pyrolysis solution three times, and then sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as previously described.

2.8 | Extraction and culture of primary neurons

Embryonic (E16-E18) Sprague-Dawley rats were used to extracted primary neurons. After disinfection with 75% alcohol, fetal rats were killed by cervical dislocation, and the spinal cord was dissected out by cutting open the skin and cartilage along the back. The cord was washed with pre-cooled
DMEM/nutrient mixture F-12 (Thermo Fisher Scientific, MA, USA) and digested with 0.25% trypsin (Thermo Fisher Scientific) and 0.05% deoxyribonuclease I (Sigma-Aldrich, MO, USA) in a 37°C incubator for 20 minutes. Horse serum (Sigma-Aldrich) was added to stop the reaction, and the suspension was centrifuged at 1000 rpm for 5 minutes at 4°C to collect the cells, followed by mixing with DMEM/F-12 containing 10% horse serum, penicillin (100 IU/mL), streptomycin (100 mg/mL; Thermo Fisher Scientific), and glutamine (0.5 mm; Thermo Fisher Scientific). After gently blowing several times, the cells were filtered with 200-µm nylon mesh, and the cell filtrate was taken out. The neurons were seeded onto poly-D-lysine-coated plates (Corning, NY, USA) and incubated at 37°C after adjusting the cell concentration. Four to six hours later, the neuron culture medium (serum-free 96% Neurobasal medium + 2% B27 [2%, w/v; Thermo Fisher Scientific] +0.5% double antibody penicillin [100 IU/mL] and streptomycin [100 mg/mL] + 1% glutamine [0.5 mm; Thermo Fisher Scientific]) was replaced after the adherence of neurons. The growth of neurons was observed under an inverted microscope. One half of the medium was changed every other day. When the cells were cultured for about 7 days, the purity of the obtained neurons was identified using anti-microtubule-associated protein 2 (1:500, rabbit IgG; Abcam) and neuronal nuclei antigen (1:800, mouse IgG; Abcam) under a fluorescence inversion microscope.

2.9 | Immunofluorescence staining

The cells or tissue sections of mice were fixed with polyformaldehyde (4%, w/v), permeabilized with 0.05% Triton X-100 for 15 minutes, and then sealed with 5% (BSA for 1 hour). After overnight incubation in a 4°C refrigerator with primary antibodies, the cells or tissue sections were treated with Alexa Fluor 488–conjugated and Alexa Fluor 594–conjugated goat secondary antibodies (Jackson ImmunoResearch) for 1 hour at room temperature. Finally, DAPI (Thermo Fisher Science) was added to stain the nuclei after triple washing with PBS. Immunoreactivity was observed and photographed using an epifluorescence (AxioVertA1 and ImagerA2) or a confocal fluorescence microscope (LSM510; Carl Zeiss). Each sample was randomly acquired in at least three visual fields, and the red/green fluorescence data were analyzed.

2.10 | BMS score and footprint analysis

BMS scores were measured 6, 12, 24, 96 hours, and 7 days after spinal cord ischemia-reperfusion injury. The BMS score was designed for mice by observing the changes in motor function of mice, including the main and secondary scoring system. The range of motion of the hind ankle joint, touch of sole and instep, trunk stability, and tail position were mainly observed. Nine points indicated the motor function of normal mice, while zero points represented complete paralysis. After understanding the scoring criteria, the experimenters scored using the double-blind method. The average of the results was taken for statistical analysis. A footprint analysis was performed in sham-operated, WT, and Git1 −/− mice 12 hours after the surgery to evaluate the motor function of mice. The mice were placed in a narrow passage padded with a white paper with blue ink on their forepaws and red ink on their hind paws. The obtained patterns were photographed with digital cameras and analyzed instead of surface footprints.

2.11 | Nissl staining

The frozen sections of the spinal cord 4-6 hours after the surgery were taken for Nissl staining to evaluate the motor neurons in the anterior horn of the spinal cord. Nissl body is a special structure of nerve cells, which is dyed by alkaline dyes such as tar violet due to its basophilic characteristics. Under physiological conditions, the Nissl bodies are large and numerous. When neurons are damaged, the number of Nissl bodies decreases or even disappears. Simply, the frozen sections were washed with distilled water, chloroform, anhydrous alcohol, 95% alcohol, and 70% alcohol for 1 minute each and then dyed with cresyl violet (FD Neuro Technologies, MD, USA) at 37°C for 10 minutes. After rinsing with distilled water, the sections were decolorized in 95% alcohol, followed by conventional ethanol dehydration and sealing with xylene and neutral gum. At least five specimens were randomly taken from each group for observation, and the Nissl bodies preserved in the anterior horn of the spinal cord were counted and averaged.

2.12 | TUNEL staining

The spinal cord sections were fixed, permeabilized, and blocked as previously described, and then incubated overnight with anti-NeuN (1:800, rabbit IgG; ab104224, Abcam) at 4°C. After three times washing with PBS, they were incubated with Alexa Fluor 488–conjugated goat anti-rabbit IgG (1:200, Jackson Immuno Research) at room temperature for 2 hours. The DeadEnd fluorometric TUNEL system (Promega, WI, USA) was used to identify and quantify the apoptotic cells in the spinal cord after ischemia-reperfusion injury, following the manufacturer’s protocol. The nuclei were counterstained with DAPI. Under a fluorescence microscope, the green fluorescence and red fluorescence co-localization indicated neurons that underwent apoptosis, and co-localization of blue fluorescence and red fluorescence indicated normal neurons.
2.13 | Double-labeled adenovirus mRFP-GFP-LC3

The double-labeled adenovirus mRFP-GFP-LC3 was purchased from Han Heng Biology, China, with determined titers (1 × 10^8). Sh-Git1 and sh-ScrHEK293T cells were distributed in confocal dishes. The mRFP-GFP-LC3 virus was transfected according to the manufacturer’s protocol and deprived of oxygen and agar 48 hours later. The nuclei were stained with Hoechst (Sigma-Aldrich), observed, and photographed under a confocal microscope (Zeiss, Oberkochen, Germany, LSM510). Yellow (GFP-positive/mRFP-positive) puncta represented autophagy, which became red (GFP-negative/mRFP-positive) after fusion with the lysosome. Yellow and red dots were counted to evaluate autophagy formation and autophagy flow.

2.14 | Detection of cell apoptosis by Annexin V-FITC/PI double staining

The cells digested with trypsin were collected and washed twice with pre-cooled PBS. After resuscitation in 100 µL of Annexin V binding buffer, 5 µL Annexin V-FITC solution and 10 µL of PI staining solution were added into the cells following which they were placed away from light at room temperature for 15 minutes. Finally, 400 µL of water was added for resuspension. The data were assessed by flow cytometry, and the results were analyzed using FlowJo software. The experiment was repeated three times.

2.15 | Lentivirus transfection

Git1 short hairpin RNA (shRNA) target sequences are follows. Mouse Git1-shRNAs (shGit11, 2, and 3) or NC-shRNA (shScr) were packaged with the pHB vector using the GV112 vector. Neuron cells were planted in a six-well plate and grown for 4-5 days. 293T cells were seeded on the six-well plate with 4 × 10^5 cells/mL in the logarithmic growth phase. The amount of virus was calculated according to the optimum MOI. The serum-free naked medium containing viral fluid (corresponding species) and polybrene was added. The fresh culture medium was added after 6 hours, and the cell protein was extracted after 48 hours. The neuron cells were treated directly, some of the 293T cells were preserved after passage, and the rest were screened with puromycin. The Git1 knockdown was detected by Western blot analysis, and the shRNA was determined to be the best knockdown group. The successfully transfected cells were named as shRNA-Git1 and negative control cells as NC-Git1.

Mouse:
shRNA 1: 5’-CGATCAACTCAGGTCTAGTTAACAAGTCCGTCT-3’
shRNA 2: 5’-CCCTCTAGGTGTTTAACGTCCGTCT-3’
shRNA 3: 5’-CGGTTCGTTCTACCTACGTTT-3’
Scrambled sequence: 5’-TTCTCGAAGCTGTCACGT-3’

Human:
shRNA 1: 5’-GATCCAAGAATGGGCACTTC-3’
shRNA 2: 5’-CACCCTGGACTACGATT-3’
shRNA 3: 5’-TGCTCAGAAAGCTGTACCT-3’
Scrambled sequence: 5’-TTCTCGAAGCTGTCACGT-3’

2.16 | PINK1-siRNA transfection

The sequence of human PINK1 small interfering RNA (siRNA) was used: 5’-GAAAUCCGACAAUCCUUCUUU-3’. The required dose of siRNA and the dose of Lipofectamine2000 were calculated. The small interference sequence (5 µL) and 5 µL of Lipofectamine2000 were added to 125 µL of the Opti-MEM medium individually. Five minutes later, the Opti-MEM medium containing small interference sequence was mixed with the Opti-MEM medium containing Lipofectamine2000 for 15 minutes. The mixture (250 µL) was added to the cell pore of the six-well culture plate, followed by the replacement of the normal medium 6 hours later. After transfection for 36 hours, the cells were treated with OGD/R as previously described, and the functions of cells were tested.

2.17 | Git1-overexpression plasmid transfection

The plasmid containing Git1-OE and a negative control plasmid were obtained from FulenGen Ltd. Co. (Guangzhou, China). 293T cells were seeded in six-well plates and washed twice before transfection. The plasmid (0.8 g) was mixed gently with 50 µL of the Opti-MEM medium containing 2 µL of Lipofectamine2000, and incubated at room temperature for 20 minutes. Then plasmid-Lipofectamine2000 complex (100 µL) was added to the culture plate of the six-well culture plate, followed by the replacement of the normal medium 6 hours later. After transfection for 48 hours, the cells were treated with OGD/R as previously described, and the functions of cells were tested.

2.18 | Adeno-associated virus

Mouse Git1-Overexpress (AAV) 2/9-Syn-MCS-Git1-3xFlag was purchased from Hanheng (Shanghai, China). The lumbar vertebrae of mice were carefully opened. After spinal cord exposure, a 10-µL microinjector was used to inject 2 µL virus per mouse between T12-L2 at the rate of
100 nL/min. After the injection, the needle was retained for 5 minutes and then withdrawn slowly. After 4-6 weeks, the spinal cord specimens were taken and identified using fluorescence.

2.19 Transmission electron microscopy

As described earlier, after 4-6 hours of ischemia-reperfusion, the mice were perfused with pre-cooled PBS, 4% paraformaldehyde, and 0.25% glutaraldehyde, and the spinal cord tissue was taken out. The tissue was fixed in a PBS mixture of 2% paraformaldehyde and 2.5% glutaraldehyde overnight at 4°C. The spinal cord tissue of the ischemic area was cut into 50-µm cubes, fixed with osmium tetroxide for 1 hour, dehydrated with a gradient of ethanol, and finally placed in an epoxy resin. After treatment at 80°C for 24 hours, it was cut into an ultrathin portion of 100 nm, stained with uranyl acetate and lead citrate, observed under a TEM (Tecnai G2 Spirit Bio TWIN, FEI, USA), and photographed.

2.20 JC-1 staining and ROS determination

JC-1 staining was used to detect the changes in mitochondrial membrane potential. sh-Git1 and sh-Scr HEK293T cells were plated in 24-well plates and stained according to the manufacturer’s protocol. The JC-1 staining kit was purchased from Beyotime Biotechnology (Shanghai, China). Briefly, JC-1 staining working solution was prepared according to the kit protocol, and 200 µL of it was added and incubated with cells in a 37°C incubator for 20 minutes. During the incubation period, the staining buffer was prepared in a 1:4 ratio with staining buffer (5×) and distilled water. The supernatant was aspirated, washed twice with pre-cooled JC-1 staining buffer, and changed to the conventional cell culture medium, observed under a fluorescence microscope, and photographed.

Primary neuronal cell cultured in the 24-well plate was used to perform ROS determination. The ROS detection kit was purchased from Beyotime Biotechnology. The cell culture medium was removed and incubated with DCFH-DA in the cell culture chamber for 20 minutes. The cells were washed three times with serum-free cell culture medium to fully remove the DCFH-DA that did not enter the cell and then observed under the fluorescence microscope.

3 Statistical Analyses

Data were analyzed using GraphPad Prism 7 (GraphPad Software, CA, USA). Data were shown as the mean ± standard deviation (SD) for at least three independent experiments. Two-tailed Student’s t tests were used for comparison between two groups, while Kruskal-Wallis tests were used for comparison of more than two groups. Values of P less than .05 were considered statistically significant.

4 RESULTS

4.1 GIT1 played a critical role in mitophagy caused by spinal cord ischemia-reperfusion injury (I/R)

The protective effect of GIT1 on spinal cord injury was further studied by producing the neuron Git1 knockout (KO) mice (Figure S3). As previously reported, we utilize the co-localization of the lysosomal-associated membrane protein 2 (LAMP2, marker of lysosomal) and Mitochondrial Membrane 20 (TOMM20, marker of mitochondria) to detect the mitophagy in vivo. Increased co-localization indicates an increase in mitophagy and NeuN were used as the marker of neurons (Figure 1A). Statistics showed that the deletion of Git1 did not affect the mitophagy in neurons under normal conditions. However, mitophagy was inhibited in Git1 −/− mice after 4 hours ischemia-reperfusion injury (Figure 1B). Parkin, an E3 ubiquitin ligase, is phosphorylated in the Ser65 domain. It promotes the phosphorylation of ubiquitin and translocation to mitochondria. Therefore, the expression level of phospho-Parkin (Ser65) and phospho-ubiquitin (Ser65) can be used as the detection index of mitophagy. It is obvious that these expression levels are reduced in ischemia-reperfusion in the absence of GIT1 (Figure 1C-E).

4.2 GIT1 protected neurons against the spinal cord injury caused by ischemia-reperfusion

The TUNEL assay was performed to detect the apoptosis of neurons to further understand the impact of GIT1 deficiency on neurons undergoing ischemia-perfusion. The knockout of Git1 did not affect the neuronal apoptosis in the control group. However, 4 hours after ischemia-reperfusion, the deficiency of GIT1 increased the number of neuronal apoptosis (Figure 2A,D). Nissl staining 4 hours after OGD/R showed a significant reduction in the number of Nissl bodies in the Git1−/− group of neurons. Also, more neuronal cells in the Git1−/− group underwent chromatolysis, which is the manifestation of neuronal damage after hypoxia (Figure 2B,E). This suggested an increase in damage to neuronal cells in the Git1−/− group. The effect of GIT1 on ischemia-reperfusion injury was tested in vivo using the Basso mouse scale (BMS) and determining the scores after 0, 6, 12, 24,
96 hours, and 7 days\textsuperscript{37} (Figure 2F). The gait analysis also showed that the knockout of Git1 did not change the gait in mice. Also, the gait analysis was performed 3 days after the injury. Compared with the WT group, the Git1 \(-/-\) mice showed worse gait recovery and a decrease in motor coordination (Figure 2C). In summary, these results demonstrated that GIT1 protected neurons at the early stage after ischemia-reperfusion.

**4.3 | GIT1 had no significant effect on mitochondrial damage**

As previously reported, ROS induce the PINK1/Parkin pathway to initiate mitophagy.\textsuperscript{38} When the neurons are damaged, the cytoskeleton rearrange and the Nissl bodies temporarily disappear. In this study, spinal neuron cells were extracted from the WT and Git1\(-/-\) groups (Figure S4) to
FIGURE 2  Identification of apoptosis and morphology in Git1 WT/Git1 −/− mice. A. Representative images of immunofluorescence of TUNEL to label apoptosis (in green), with NeuN labeling in red and DAPI in blue. Tissues from the spinal cord of littermates of control and Git1 −/− mice undergoing the sham operation or ischemia-reperfusion for 4 hours. Scale bars = 200 μm. B. Representative Nissl staining of the spinal cord of littermates of control and Git1 −/− mice undergoing the sham operation or ischemia-reperfusion for 4 hours. Scale bars = 100 μm. C. Representative footprints of walking 7 days after ischemia-reperfusion injury. Blue represents front paw print, and red represents hind paw print. D and E, Statistical analysis of the TUNEL assay (D) and the density of Nissl bodies (values are the mean ± SD, *P < .05, ***P < .001, two-tailed Student’s t tests). F, The BMS scores of Git1 −/− (n = 5) and Git1 WT (n = 10) mice within 7 days (values are the mean ± SD, *P < .05, two-tailed Student’s t tests)
detect the changes in the mitochondrial membrane potential, which marked mitochondrial damage and early stage of apoptosis. When the mitochondrial membrane potential was high, JC1-aggregates and produced red fluorescence. Also, when the mitochondrial membrane potential was low, JC-1 could not accumulate in the matrix of mitochondria. At this time, JC-1 produced green fluorescence. After 4 hours reperfusion, the green/red fluorescence ratio in the Git1 −/− group did not increase significantly compared with the WT group (Figure 3A,D). No significant difference was found in the level of ROS in 293T cells between the Git1 −/− and control groups (Figure S1A,B). This indicated that GIT1 had no significant effect on mitochondrial damage. 293T cells are used to explore the process by which GIT1 affected mitophagy. Mt-Keima lentivirus was transfected into 293T cells in vitro to detect mitophagy in cells. The mt-Keima-labeled mitochondria emitted green fluorescence in a normal (pH = 7) environment. However, when mitophagy occurred, mitochondria were engulfed by lysosomes, and mt-Keima emitted red fluorescence in an acidic environment (pH = 4). A mitophagy agonist CCCP (Carbonyl cyanide 3-chlorophenylhydrazone) was used as a positive control. After 4 hours ischemia-reperfusion of 293T cells, mitophagy increased significantly. However, after interference with Git1, mitophagy decreased, indicating that the loss of GIT1 in 293T cells affected mitophagy. These trends were the same as in the positive control (Figure 3B,E). Transfection of mt-Keima in neurons from Git1 knockout mice also found that mitophagy were inhibited (Figure 3C,E).

4.4 | GIT1 promoted mitophagy by promoting the phosphorylation of BECN1 at Thr119 and also promoted the translocation of Parkin to mitochondria

Since mitophagy showed an excessive degradation of Parkin after 6 hours and mitophagy gradually weakened, the protein expression level was examined within 6 hours. How GIT1 affected mitophagy was further investigated in which the protein expression levels in the Git1 knockdown and control groups were examined after 1, 2, 4, and 6 hours after OGD/R. The expression of Git1 did not change significantly with the increase in reperfusion time. However, the levels of autophagy-related proteins LC3 and Beclin-1 decreased and the level of P62 increased with time after Git1 knockdown, which marked the decrease in autophagy. Normalized relative ratio of LC3II/GAPDH is shown in Figure 4C. The levels of mitophagy-related proteins p-Parkin and PINK1 were also affected. They increased with time and reached a peak in 4 hours. However, the expression of total Parkin did not change significantly. All these proteins were suppressed while Git1 was knocked down (Figure 4A). Since the PINK1/Parkin pathway is the most classical pathway in mitophagy, co-immunoprecipitation (co-IP) was used to detect proteins that GIT1 protein interacted with. The co-IP also showed that GIT1 interacted with Parkin, and the interaction strengthened after OGD/R (Figure 4B). BECN1 plays a significant role in mitophagy. However, the relationship between BECN1 and PINK1/Parkin is still controversial. Previous studies confirmed that GIT1 interacted with BECN1 and promoted the phosphorylation of BECN1 in Thr119. The co-IP also showed that BECN1 interacted with GIT1, and the interaction strengthened after OGD/R (Figure S2A). However, the interaction between GIT1 and Parkin disappeared when Beclin-1 was disturbed (Figure S2B). Western blot analysis showed that the phosphorylated BECN1 at Thr119 positively correlated with reperfusion time and disappeared after Git1 was knocked down (Figure 4A). Overall, GIT1 interacted with Parkin via Beclin-1.

4.5 | GIT1 activated the interaction between BECN1 and Parkin and promoted Parkin translocation to mitochondria by phosphorylating BECN1

As shown in Figure 5A,B, Parkin's interaction with BECN1 significantly reduced when GIT1 was deficient after 4 hours reperfusion. Parkin was specifically translocated to the mitochondrial outer membrane during mitochondrial damage to promote mitophagy. TOMM20 acts as a marker protein for the mitochondrial outer membrane, and its co-localization with Parkin can reflect Parkin's translocation. As it is difficult to find out the transposition of endogenous Parkin, Parkin protein was overexpressed with red fluorescent protein. Parkin apparently translocated to the mitochondria after OGD/R, while knocking down Git1 significantly inhibited the translocation (Figure 5C,D). Specific extraction of mitochondrial proteins and western blot analysis were conducted to verify mitochondrial protein expression. The content of Parkin in mitochondria increased significantly after OGD/R, accompanied by the phosphorylation of Parkin and ubiquitin. However, in the absence of GIT1, the expression of these specific proteins decreased significantly (Figure 5E,F). The expression of cleaved caspase-9 was examined to detect apoptosis; apoptosis increased when Git1 was knocked down (Figure 5G). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining and flow cytometry also showed that the proportion of apoptotic cells increased (Figure 5H,I). This indicated that GIT1 reduced mitophagy by inhibiting Parkin translocation to mitochondria and inhibited its protective effect.
FIGURE 3  Detection of mitophagy in vivo with or without the deficiency of GIT1. A, Representative images of immunofluorescence of JC-1 to label mitochondrial membrane potential. Neurons separated from the spinal cord of littermates of control and Git1 −/− mice undergoing the sham operation or ischemia-reperfusion for 4 hours. Scale bars = 200 μm. B, Representative images of mt-Keima to detect the normal mitochondria (in green) and mitochondria in autophagosomes (in red) in 293T cells. The si-NC/si-Git1 293T cells were treated with DMSO, 10μM CCCP, or OGD/R for 4 hours. Scale bars = 20 μm. C, Representative images of mt-Keima in neurons from Git1 WT and Git1 −/− mice. D and E, Statistical analysis of the ratio of mitochondrial membrane potential (D) and the ratio of mitophagy in 293T cells and neurons (E) (values are the mean ± SD, *P < .05, ***P < .001, two-tailed Student’s t tests)
4.6 | PINK1 played a decisive role in mitophagy, and the overexpression of Git1 could not rescue the effect of PINK1’s inhibition

Beclin-1 can also interact with PINK1. Hence, co-IP was performed in the absence of GIT1. PINK1 interacted with BECN1 regardless of the lack of GIT1, but the expression level of pull-down protein was not affected (Figure 6A,B). Therefore, GIT1 promoted mitophagy with no effect on PINK1. However, the ROS/PINK1/Parkin pathway is the classical pathway in mitophagy. The translocation of Parkin was observed after GIT1 expression and interference with PINK1. The overexpression of Git1 promoted Parkin mitochondrial translocation after OGD/R. Parkin’s translocation significantly reduced when Pink1 was knocked down, but could not be compensated when Git1 was overexpressed (Figure 6C,D). Mitophagy marked by mt-Keima reduced when Pink1 was knocked down. Conversely, mitophagy increased when Git1 was overexpressed. Simultaneous interference with Pink1 and overexpression of Git1 could not rescue the mitophagy compared with simply knocking down Pink1 (Figure 6E,F). With the decrease in mitophagy, the expression level of cleaved caspase-9 and the proportion of apoptotic cells increased (Figure 7A, E, and F). Western blot analysis confirmed the phosphorylation of BECN1 after OGD/R, and its expression slightly increased after the overexpression of Git1. The knockdown of Pink1 did not affect the phosphorylation of Beclin-1 (Figure 7A,B). The expression of mitochondrial proteins was in line with the occurrence of mitophagy (Figure 7C,D). In conclusion, GIT1 promoted Parkin translocation to mitochondria by phosphorylating BECN1 to promote mitophagy. However, PINK1 played a decisive role in the phosphorylation and translocation of Parkin.
FIGURE 5  GIT1 activated the interaction between BECN1 and Parkin and promoted Parkin translocation to mitochondria by phosphorylating BECN1. A, Immunoprecipitation between Beclin-1 and Parkin in 293T cells infected with Git1 shRNA or scrambled shRNA 4 hours after OGD/R. B, Statistical analysis of interactions between Parkin and Beclin-1 (values are the mean ± SD, *P < .05, two-tailed Student's t tests). C, Co-localization of overexpressed Parkin with mitochondria in si-NC/si-Git1 293T cells. Oxygen and glucose deprivation for 1 hour and reperfusion for 4 hours. D, Statistical analysis of Parkin/TOMM20 co-localization (values are the mean ± SD, **P < .01, two-tailed Student's t tests). E, Representative western blot of the expression of mitochondrial proteins (Parkin, p-Parkin, and p-Ub). Oxygen and glucose deprivation for 1 hour and reperfusion for 4 hours. VDAC was used as an internal reference. F, Statistical analysis of the ratio of phosphorylated Parkin (values are the mean ± SD, ***P < .001, two-tailed Student's t tests). G, Representative Western blot of cleaved caspase-9 in total protein. H, Representative flow cytometry to detect the proportion of apoptotic cells. Oxygen and glucose deprivation for 1 hour and reperfusion for 4 hours. I, Statistical analysis of the apoptotic rate (values are the mean ± SD, *P < .05, two-tailed Student's t tests).
4.7 | GIT1 also promoted the mitophagy by enhancing the autophagic flux

Parkin translocation to mitochondria and phosphorylation at Ser65 promoted ubiquitin phosphorylation at Ser65; it is associated with LC3 to promote mitophagy. Previous studies have shown that GIT1 interacts with BECN1 to affect the formation of autophagic flow. Western blot analysis also confirmed that the expression of LC3 increased with reperfusion time, while the expression of LC3 was inhibited in the absence of GIT1 (Figure 4A). LC3 double-labeled virus was transfected to detect the autophagic flow. Laser confocal microscopy showed that red and yellow dots increased after OGD/R, but decreased after interference with GIT1 (Figure 8A,B). An increased autophagic flow is also an important factor in enhancing mitophagy.
FIGURE 7  Expression of mitophagy-related proteins in 293T cells, which interfered with Pink1 or overexpressed Git1. A and C, Representative Western blot of the expression of total proteins (GIT1, PINK1, Beclin-1, p-Beclin-1, and cleaved caspase-9) (A) and mitochondrial proteins (Parkin, p-Parkin, and p-Ub) (C) in WT/OE-Git1 HE293T cells infected with Pink1 shRNA or scrambled shRNA 4 hours after OGD/R. GAPDH and VDAC were used as internal references for total protein and mitochondrial protein, respectively. B and D, Statistical analysis of the expression ratio of p-Beclin-1/Beclin-1 (B) and p-Parkin/Parkin (D) (values are the mean ± SD, *P < .05, two-tailed Student's t tests). E, Representative flow cytometry to detect the proportion of apoptotic cells. F, Statistical analysis of the apoptosis rate (values are the mean ± SD, *P < .05, two-tailed Student's t tests).
FIGURE 8  Interference with GIT1 reduced autophagic flux and the expression of LC3 in HEK293T cells. A, Transfection of lentivirus into 293T cells to label autolysosomes (in red) and autophagosomes (in yellow). Scale bars = 50 μm. B, Statistical analysis of the amount of autolysosome and autophagosomes (values are the mean ± SD, *P < .05, two-tailed Student’s t tests)
Overexpression of Git1 in vivo promoted mitophagy and protected neurons

The adeno-associated virus (AAV)2/9-Syn-MCS-Git1-3xFlag was constructed to explore the effects of Git1's overexpression in vivo. As a neuron-specific promoter, Syn can constrain GIT1 expression only in neuronal cells. Immunofluorescence identified the specific expression of AAV in neurons and the expression of GIT1 (Figure 9A-C). Phosphorylated Parkin increased after the overexpression of GIT1, indicating an increase in mitophagy (Figure 9D and E). TUNEL staining suggested less apoptotic cells in the Git1 overexpression group (Figure 9F,G). Git1-overexpressing mice had better gait 3 days after the injury (Figure 9H). The BMS scores showed that the recovery of Git1-overexpressing mice produced differences 3 days after the injury (Figure 9I). The overexpression of Git1 in vivo was consistent with the effect of overexpression of Git1 in vitro, both of which could increase mitophagy and reduce apoptosis of neurons.

Discussion

Mitophagy can remove damaged mitochondria and maintain cell function in time. Recent studies have revealed an important role of mitophagy in spinal cord ischemia-reperfusion injury. In the early stage of spinal cord ischemia-reperfusion, enhanced mitophagy can inhibit neuronal apoptosis and alleviate the symptoms of spinal cord injury. This study found that GIT1 played a regulatory role in mitophagy. GIT1 is vital in the development of mitochondria in the heart, but its role in neurons is unknown. This study found that the mitochondrial density, volume, and morphological structure in neuron cells from Git1 knockout mice did not change significantly compared with WT mice. However, after ischemia-reperfusion injury, the mitophagy of spinal cord neurons in Git1 knockout mice was inhibited, while the mitophagy was enhanced when Git1 was overexpressed. Mitophagy can reduce cell damage and inhibit apoptosis in the early stage of ischemia-reperfusion, which is consistent with the results of the present study. This study demonstrated that GIT1 enhanced the interaction between BECN1 and Parkin by phosphorylating BECN1 at Thr119 residues and promoted Parkin translocation to the mitochondrial outer membrane to enhance mitophagy.

Parkin, an E3 ubiquitin ligase, is closely related to Parkinson's disease and plays a role in mitophagy. When mitochondria are damaged, the membrane potential is reduced. Also, ROS are produced, and the translocation of PINK1 to the mitochondrial outer membrane is promoted. PINK1 activates the phosphorylation of Parkin and translocation to the mitochondrial outer membrane. Therefore, the ROS/PINK1/Parkin pathway is the main regulatory pathway of mitophagy, and Parkin's translocation to mitochondria is the most critical event. This study found that the phosphorylation of Beclin-1 caused by GIT1 would enhance the interaction with Parkin and promote Parkin's translocation to the mitochondrial membrane. This could explain the mechanism of GIT1 promoting mitophagy.

As an important protein in autophagy, Beclin-1 ubiquitination and phosphorylation at different residues can regulate different processes of autophagy. Choubey showed that Beclin-1 promoted Parkin's translocation, but had no significant effect on PINK1. While Gelmetti confirmed that PINK1 and Beclin1 acted early in mitophagy, promoting the binding of mitochondria and endoplasmic reticulum. The present study found that the phosphorylation of Beclin-1 at Thr119 changed the interaction with Parkin, but did not change the interaction with PINK1. The phosphorylation of Beclin-1 in serine-14 also promoted Parkin's translocation. Another residue, Thr119, was found, whose phosphorylation had the same effect of promoting mitophagy. The phosphorylation at Thr119 was activated by GIT1. Pink1 was knocked down, and the overexpression of Git1 did not rescue the inhibition of mitophagy. This suggested that the phosphorylation of Beclin-1 did not compensate for the knockdown of Pink1, which might be related to the phosphorylation of Parkin. Parkin phosphorylation was activated mainly by PINK1, while Beclin-1 promoted only Parkin translocation and did not promote Parkin phosphorylation. When Pink1 was knocked down, Parkin in the mitochondrial outer membrane could not be phosphorylated, and hence, mitophagy significantly reduced. However, when Pink1 expression was normal, the overexpression of Git1 could promote Beclin-1 phosphorylation and Parkin translocation, leading to an increase in mitophagy. Beclin-1 is a 450-amino acid protein and consists of three important domains: BH3 (amino acid position 114-123), CCD (amino acid position 144-269), and ECD (amino acid position 244-337). GIT1 was found to interact with Beclin-1 in the Thr119 residue, which is located in the BH3 domain. This indicated that Beclin-1 and GIT1 might be combined in the BH3 domain. Ubiquitin-specific processing enzyme 19 (USP19) interacts only with the CCD domain of Beclin-1, rather than BD (amino acid position 1-143) or Beclin-1 alone ECD domain (amino acid position 269-450). Parkin, as an E3 ubiquitin ligase, may also interact with Beclin-1 in the CCD domain. This may be the next step of research.

A previous study found that the overexpression of Git1 increased LC3 expression and enhanced autophagic flow. Ubiquitinated mitochondria bound to the autophagy receptor protein and were recognized by the receptor LC3 on the
FIGURE 9 Overexpression of Git1 increased mitophagy and protected neurons. A, Representative image of immunofluorescence to identify transfection and specificity of AAV that overexpressed Git1. 3xFlag used as a specific label. Scale bars = 100 μm. B, Representative image of immunofluorescence to detect the expression level of GIT1. Scale bars = 100 μm. D, Representative image of immunofluorescence to detect the expression level of p-Parkin, which represents the extent of mitophagy 4 hours after ischemia-reperfusion. Scale bars = 100 μm. F, Detection of apoptotic cells 4 hours after I/R using the TUNEL assay. Scale bars = 100 μm. C, E, and G, Data summary of (B, D, and F) (values are the mean ± SD, **P < .01, two-tailed Student’s t tests). H, Representative footprint tests 7 days after ischemia-reperfusion. I, The BMS scores of Git1-overexpressing (n = 10) and Git1 WT (n = 10) mice within 7 days (values are the mean ± SD, *P < .05, two-tailed Student’s t tests).
autophagic membrane to cause autophagy.\(^{13,17}\) Also, GIT1 could promote the production of autophagosomes after 4 hours of OGD/R, which also promoted the production of mitophagy.

In conclusion, this study demonstrated in vivo and in vitro that GIT1 promoted mitophagy in ischemic-reperfusion injury in neurons and protected neurons from apoptosis (Figure 10). GIT1 regulated the phosphorylation of Beclin-1 at Thr119, promoting the translocation of Parkin to the mitochondrial outer membrane. This process had nothing to do with PINK1, but it could not rescue the role in the absence of PINK1. The overexpression of Git1 enhanced mitophagy and protected against ischemia-reperfusion injury. GIT1 might serve as a new research site for the protection of ischemia-reperfusion injury.

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**CONFLICT OF INTEREST**

None of the authors have a conflict of interest to declare.

**AUTHOR CONTRIBUTIONS**

YH, CG, and QW performed the experiments; JC, WZ, WL, SZ, and FK collected data and prepared the manuscript. ZZ, LL, DQ, and XZ assisted in the experiments and analyzed the data. GY, JF, and QL designed the study, analyzed data, and edited the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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