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**SRGN aggravates cerebral ischemia/reperfusion injury by inducing SLC7A11
depletion and excessive mitophagy-dependent neuronal death**

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Abstract

Background: Cerebral ischemia/reperfusion injury (CI/RI) is a critical secondary injury that occurs after recanalization therapy for acute ischemic stroke, in which aberrant mitophagy triggers neuronal loss and neurological dysfunction. Serglycin (SRGN) is a secretory proteoglycan that regulates inflammation and protein stability, and it is implicated in multiple inflammatory diseases and tumors. However, its role in CI/RI-induced neuronal injury remains unknown. This study aimed to investigate the function and underlying mechanism of SRGN in CI/RI, and to explore a targeted therapeutic strategy based on SRGN silencing.

Methods: SRGN expression was measured in serum samples from patients with acute cerebral infarction, in brain tissues of middle cerebral artery occlusion/reperfusion (MCAO/R) mice, and in oxygen glucose deprivation/reoxygenation (OGD/R)-exposed neurons. Loss- and gain-of-function experiments were performed in vitro using SRGN knockdown and overexpression, with neuronal injury assessed by LDH release, ROS accumulation, glutathione content, and pro-inflammatory cytokine levels. The interaction between SRGN and SLC7A11 was evaluated by molecular docking, and the involvement of ubiquitin–proteasomal degradation and mitophagy was examined. HG106-mediated SLC7A11 inhibition was used to validate the mechanistic axis. In vivo, red blood cell-derived extracellular vesicles loaded with siRNA targeting SRGN (RBC-EVs@siSRGN) were evaluated in MCAO/R mice for blood-brain barrier penetration, gene silencing efficiency, and therapeutic efficacy by measuring infarct volume, neuroinflammation, autophagy balance, and neurological deficits.

Results: SRGN was elevated in the serum of acute cerebral infarction patients, as well as in brain tissues of MCAO/R mice and OGD/R-exposed neurons. In vitro, SRGN knockdown alleviated OGD/R-induced neuronal damage, evidenced by decreased LDH release, ROS accumulation, and pro-inflammatory cytokines, together with increased glutathione content, whereas SRGN overexpression aggravated neuronal injury. Mechanistically, SRGN directly interacted with SLC7A11 and promoted its ubiquitin–proteasomal degradation, leading to mitochondrial dysfunction and excessive mitophagy; HG106-mediated SLC7A11 inhibition abolished the neuroprotective effects of SRGN silencing, confirming the SRGN/SLC7A11/mitophagy axis in CI/RI. In MCAO/R mice, RBC-EVs@siSRGN efficiently crossed the blood–brain barrier, and effectively silenced cerebral SRGN in vivo, resulting in reduced infarct volume, relieved neuroinflammation, restored mitophagy balance, and improved neurological deficits.

Conclusions: SRGN exacerbates CI/RI by promoting SLC7A11 degradation and pathological mitophagy. Circulating SRGN may serve as a promising non-invasive biomarker for acute cerebral infarction, and RBC-EVs@siSRGN represents an effective targeted therapy for ischemic stroke.

Keywords: cerebral ischemia/reperfusion injury; SRGN; SLC7A11; mitophagy-dependent cell death; oxidative stress; red blood cell-derived extracellular vesicles

Bullet points

- SRGN is upregulated in the serum of patients with acute cerebral infarction and in the

66 brains of MCAO/R mice and OGD/R-injured neurons.

67 • SRGN promotes excessive mitophagy-dependent neuronal death by suppressing

68 SLC7A11 protein stability through the ubiquitin-proteasome pathway.

69 • Pharmacological blockade of SLC7A11 reverses the neuroprotection of SRGN

70 knockdown, defining the SRGN/SLC7A11/excessive mitophagy axis.

71 • RBC-EVs@siSRGN crosses the blood-brain barrier, silences SRGN, and confers

72 neuroprotection against CI/RI in MCAO/R mice.

1. Introduction

Acute ischemic stroke (AIS) is a leading global cause of death and permanent disability [1]. Thrombolytic therapy and mechanical thrombectomy are the first-line reperfusion treatments for AIS patients [2, 3]. Despite satisfactory recanalization efficacy, the restoration of blood flow often triggers secondary cerebral ischemia/reperfusion injury (CI/RI) [4]. This complication undermines therapeutic benefits and limits functional recovery and long-term outcomes [5-13]. CI/RI pathogenesis involves multiple detrimental cascades. Overactivated mitophagy, redox disruption, and neuronal loss are core hallmarks that together worsen neurological impairment [3, 5, 14]. Identifying key pathogenic molecules that control neuronal survival and restore mitophagy balance is therefore critical for developing effective CI/RI interventions.

Mitophagy is a vital mitochondrial quality-control mechanism that selectively clears impaired mitochondria, exerting dual regulatory effects on neuronal survival during CI/RI pathogenesis [15-18]. Moderate activation of mitophagy in the early stage of ischemia can promptly eliminate damaged mitochondria, reduce ROS release, and block the activation of the apoptosis pathway, thereby exerting neuroprotective effects and mitigating primary ischemic injury [19, 20]. Conversely, if the ischemia-reperfusion stimulus persists or its intensity is excessive, mitophagy becomes uncontrolled and overactivated, indiscriminately degrading normally functional mitochondria, causing collapse of energy metabolism, accelerating neuronal death, and aggravating brain tissue damage [16, 21]. Therefore, precise regulation of the balance of mitophagy has become a core breakthrough point for CI/RI intervention. Moderately

promoting mitophagy can alleviate CI/RI, whereas either insufficient or excessive activation of mitophagy will worsen brain injury. Nevertheless, the critical upstream regulators responsible for triggering lethal mitophagy overactivation in CI/RI have not been fully characterized.

Serglycin (SRGN) is a highly conserved secretory sulfated proteoglycan that regulates the stability, distribution, and signal transduction activity of target proteins by binding to various effector molecules such as cytokines, chemokines, proteases, and membrane receptors, thereby broadly mediating key biological processes including cell adhesion, migration, invasion, and inflammatory responses [22, 23]. It plays an important regulatory role in various pathological processes such as the occurrence and development of tumors, immune-inflammatory disorders, and cardiovascular diseases [24]. Existing studies have confirmed that SRGN can mediate the malignant phenotype of tumors and chemotherapy resistance, making it a potential diagnostic and therapeutic target for tumors; at the same time, it regulates immune-inflammatory activation and participates in cardiovascular lesions such as atherosclerosis and myocardial injury, possessing value for inflammation monitoring [23, 25-32], suggesting that SRGN may become a potential molecular target for the diagnosis, prognostic assessment, and targeted intervention of various diseases. Although previous studies have confirmed that SRGN as a driver of microglia-mediated neuroinflammation followingr ischemic stroke [33], the direct effect of SRGN on neurons after CI/RI and its downstream molecular mechanism have not been elucidated. The biological functions of SRGN in the central nervous system, particularly its regulatory mechanism in CI/RI, have not yet

117 been systematically reported.

118 In this study, we first measured peripheral circulating SRGN levels in patients with
119 acute cerebral infarction to evaluate its diagnostic value for CI/RI. Subsequently, we
120 investigated the pathogenic role of SRGN in oxygen glucose deprivation/reoxygenation
121 (OGD/R) neuronal models and middle cerebral artery occlusion/reperfusion (MCAO/R)
122 mice models. Previous studies have found that SLC7A11 can regulate mitophagy
123 activity via the ROS/ α KG-AMPK signaling axis, thereby aggravating liver injury [34].
124 SLC7A11 can also regulate mitophagy in esophageal squamous cell carcinoma cells,
125 and its expression level is positively correlated with mitophagy activity [35]. This study
126 further investigated whether SRGN promotes SLC7A11 degradation, thereby inducing
127 excessive activation of mitophagy and ultimately aggravating CI/RI. In addition, we
128 constructed red blood cell-derived extracellular vesicles loaded with siRNA targeting
129 SRGN (RBC-EVs@siSRGN) and validated their neuroprotective effects both in vitro
130 and in vivo. This study elucidates a novel molecular mechanism underlying
131 reperfusion-induced CI/RI, while also demonstrating that SRGN may serve as a
132 noninvasive biomarker and a targeted therapeutic candidate for ischemic stroke.

133 **2. Materials and methods**

134 **2.1 Cell culture**

135 The human neuroblastoma cell line SH-SY5Y (Cat.No. BC-C-HU-023) and the mouse
136 hippocampal neuron cell line HT22 (Cat.No. BC-C-MI-004) were purchased from
137 Nanjing BioChannel Biotechnology Co., Ltd. (Nanjing, China). The cell identities of
138 SH-SY5Y and HT22 have been verified through STR profiling. SH-SY5Y cells were

cultured in DMEM/F12 medium (VivaCell, China, C3132-0500) supplemented with 15% fetal bovine serum (FBS, Excell Bio, China, FSS500) and 100 U penicillin/streptomycin (Beyotime, China, C0222). HT22 cells were cultured in High-DMEM medium (Cytiva, China, BC-M-005) supplemented with 10% FBS and 100 U penicillin/streptomycin. All cells were maintained in an incubator at 37°C with 5% CO₂ and passaged upon reaching 80%–90% confluence. Each cell line was authenticated by STR analysis and confirmed to be free of mycoplasma contamination using the Myco-LumiTM Luminescent Mycoplasma Detection Kit (Beyotime, China, C0297S).

2.2 Establishment of the OGD/R Cell Model

Preparation of sodium dithionite: Weigh 0.174 g of sodium dithionite powder and dissolve it in 1 mL of glucose-free cell culture medium to prepare a 1 M sodium dithionite stock solution. Serially dilute the stock solution with the corresponding glucose-free cell culture medium to obtain 10 mM and 5 mM sodium dithionite working solutions.

After confirming that SH-SY5Y cells and HT22 cells are in a stable and optimal growth state, treat both cell types with different concentrations of sodium dithionite working solution for 2 h to induce oxygen glucose deprivation. Subsequently, discard the treatment solution, replace it with normal cell culture medium, and perform reoxygenation and glucose restoration culture of the cells for 22 h, thereby successfully establishing moderate and severe OGD/R cell models. The normal cell culture medium group was set as the blank control group to exclude any interference from the culture medium itself on the experimental results.

2.3 Experimental animals and treatment intervention

C57BL/6 mice (male, 7–8 weeks, 19 ± 1 g) were purchased from GemPharmatech Co., Ltd. (Jiangsu, China). The animals were housed in a clean, ventilated environment maintained at 23 ± 2 °C and $55\% \pm 5\%$ relative humidity, a daily temperature fluctuation of no more than 3 °C, and a 10 h/14 h light/dark cycle, and were maintained strictly under a specific-pathogen-free (SPF) barrier system. All animal care and experimental procedures were approved by the Experimental Animal Management and Use Committee of Jiangsu University (Approval no. UJS-IACUC-AP-2026022811). All animal experiments complied with national animal welfare regulations and the guidelines endorsed by the International Council for Laboratory Animal Science (ICLAS).

After one week of acclimation, the mice were randomly assigned to four groups for model establishment. In order to establish the MCAO/R mouse model, once the mental status, dietary intake, and activity of the mice were stable, they were anesthetized. The neck skin was incised, and the common carotid artery, internal carotid artery, and external carotid artery were dissected layer by layer to fully expose the bifurcation. A microincision was made on the proximal side of the bifurcation of the left common carotid artery in the prone position, and a filament was slowly inserted through the incision into the internal carotid artery until it reached the bifurcation of the middle cerebral artery; the insertion length was approximately 9 ± 1 mm. The insertion was stopped immediately upon feeling slight resistance to establish the middle cerebral artery occlusion model. After 90 minutes of ischemia, the filament was slowly

withdrawn to restore cerebral blood supply, completing the reperfusion procedure. Subsequently, the neck skin was sutured, and the mice were placed on a thermostatic heating pad to maintain body temperature. After they regained consciousness spontaneously, they were transferred to housing cages for conventional rearing. Two hours after the MCAO/R procedure, the mice received 200 μ L of PBS solution (MCAO/R+PBS group, n=10), 200 μ L of RBC-EVs@siCTR complex (MCAO/R+RBC-EVs@siCTR group, n=8), or 200 μ L of RBC-EVs@siSRGN complex (MCAO/R+RBC-EVs@siSRGN group, n=8) via tail vein injection, respectively. Meanwhile, mice that underwent anesthesia, cervical skin incision, and cervical vessel dissection without filament insertion, while the remaining procedures were identical to those in the MCAO/R experimental group, received no additional intervention and were maintained under conventional housing post-surgery as the sham group (Sham group, n=6). At the end of the study, all animals were euthanized. Blood and brain tissue samples were collected from the mice for further investigation.

2.4 Human serum samples

Blood samples were collected from 20 patients with a clinical diagnosis of acute cerebral infarction at Wujin Hospital Affiliated with Jiangsu University, Changzhou, China, after obtaining informed consent. All included acute cerebral infarction cases were met the relevant diagnostic criteria for acute cerebral infarction. Twenty healthy volunteers matched in baseline characteristics such as age and sex were recruited as the control group from Wujin Hospital Affiliated with Jiangsu University. All subjects received EDTA anticoagulant peripheral venous blood on fasting condition, and the

serum was separated by high-speed centrifugation and stored at -80 °C. All subjects in this study signed a written informed consent form. The human serum samples (human material) involved in this study have been reviewed and approved by the Medical Ethics Committee of Jiangsu University. The ethical approval document explicitly states that this research complies with the principles of the Declaration of Helsinki and relevant national regulations of China. All experiments using human serum samples in this work were performed strictly in accordance with the approved ethical protocol.

2.5 Measurement of serum SRGN concentration

The serum SRGN concentration was measured in both patients and controls using commercial ELISA kits (Shanghai Jianglai Biotechnology Co., Ltd, China, JL53890). Following the manufacturer's instructions, 100 µL of serum samples to be tested and serially diluted standards were added to a 96-well plate pre-coated with a specific capture antibody. Biotin-labeled secondary antibody and enzyme-conjugated antibody were then added sequentially, and the plate was incubated to form a stable immune sandwich complex. After washing away unbound substances, TMB chromogenic solution was added for color development in the dark, and the reaction was terminated with stop solution. The absorbance readings were recorded at a specific wavelength of 450 nm using the SpectraMax M2 multifunctional microplate reader from Molecular Devices. And the final concentration of SRGN in each sample was calculated based on the standard curve.

2.6 Transcriptome data retrieval

Relevant transcriptome data were downloaded from the Gene Expression Omnibus

(GEO) database, with dataset accession numbers GSE22255, GSE270958, and GSE232244. According to Article 32 of Chapter 3 of the "Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects" issued by the National Science and Technology Ethics Committee of China, the use of legally obtained, publicly available personal information data or biological samples for life science and medical research is exempt from ethical review.

2.7 Cell transfection of plasmid and siRNA

The Homo-SRGN overexpression plasmid (pLent-SV40-Puro-CAG-MCS-pSRGN) and control plasmid (pLent-SV40-Puro-CAG-MCS-pCTR) were purchased from Vigene Biosciences Co., Ltd., Shandong, China. And the Mus-SRGN siRNA and control siRNA were purchased from GeneCreate Biological Engineering Co., Ltd., Wuhan, China. Specific sequences are detailed in Table 1.

The well-grown cells were seeded into a 6-well plate at a density of 1.0×10^5 /well. Transfection was performed when the cell confluence reached 60%–70%. One hour before transfection, the original culture medium was discarded, and 1.5 mL of Opti-MEM medium (Gibco, USA, 31985070) was added to each well, after which the plate was returned to the cell culture incubator for incubation until use. Plasmids and siRNA were transfected into the corresponding cells using Lipofectamine 2000 (Invitrogen, USA, 11668019) transfection reagent in strict accordance with the manufacturer's instructions. Cells were collected 72 hours after transfection, and transfection efficiency was verified at the mRNA and protein levels by qPCR and Western blot respectively.

Table 1. Sequences of siRNAs

Name	Sense sequence 5'–3'	Antisense sequence 5'–3'
Mus-siSRGN-1	CGAACUGCAUCGAGGAGAATT	UUCUCCUCGAUGCAGUUCGTT
Mus-siSRGN-2	CUCCCAUGAAUAAUCCUGUUUTT	AAACAGGAUUAUUCAUGGGAGTT
Mus-siSRGN-3	CCAACAGAUGAAAGCAAUAUUTT	AAUAUUGCUUUCAUCUGUUGGTT
Mus-siCTR	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

250 **2.8 Lentivirus-mediated construction of SRGN knockdown cell lines**

251 The Homo-SRGN lentivirus and control lentivirus were purchased from Vigene
252 Biosciences Co., Ltd., Shandong, China. The sequences are detailed in Table 2.
253 The well-grown cells were seeded into a 6-well plate at a density of 1.0×10^5 /well.
254 Transfection was performed when the cell confluence reached 30%–50%. Discard the
255 original culture medium, add 1 mL of basal medium containing the corresponding
256 lentivirus, and incubate the cells in a CO₂ incubator for 24 h, after which supplement
257 with 1 mL of complete medium to continue the culture. When the cells reach full
258 confluency, observe their condition under a microscope and add complete medium
259 containing 0.02% puromycin for selection to establish a cell line with stable knockdown
260 of SRGN.

261 **Table 2. Sequences of shRNAs**

Name	Sequence 5'–3'
Homo-shSRGN-1	CCAGGACTTGAATCGTATCTT
Homo-shSRGN-2	GCTGCAATCCAGACAGTAATT
Homo-shSRGN-3	CCTCAGTTCAAGGTTATCCTA

2.9 HG106-treated cells

Dissolve 1 mg of HG106 (SLC7A11 inhibitor, MCE, USA, HY-W451275) powder in 631.433 μ L of dimethyl sulfoxide (DMSO) to prepare a 5 mM stock solution. When cells cultured in 6-well or 24-well plates reach 50–60% confluence, subject them to OGD/R treatment, followed by treatment with a HG106 working solution at a final concentration of 5 μ M for 24 h.

2.10 Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from cultured cells and mouse brain tissue using Trizol reagent (Ambion, USA, 15596026), following the kit instructions strictly. After determining the RNA concentration and purity, the RNA was reverse-transcribed into cDNA using the HiScript III 1st Strand cDNA Kit (Vazyme, China, R312-02). For real-time quantitative PCR, the reaction mixture was prepared using ChamQ SYBR Color qPCR Master Mix (Vazyme, China, Q312-02), and amplification and detection were performed on an ABI Prism 2720 quantitative PCR instrument (Applied Biosystems, USA). β -actin was used as the internal control, and the expression levels of the target genes were normalized using relative quantification. The primer sequences used in this study are detailed in Tables 3 and 4.

Table 3. Primer sequences used in RT-qPCR for Human

Gene	Forward 5'-3'	Reverse 5'-3'
<i>SRGN</i>	GGACTGACCTTTTCCAAAGAC	GTCAAGAGACCTAAGGTTGTCA
<i>IL1β</i>	GGAGAATGACCTGAGCACCT	GGAGGTGGAGAGCTTTCAGT

<i>IL6</i>	CCTGATCCAGTTCCTGCAGA	GAACTCCTTAAAGCTGCGCA
<i>IL18</i>	TGCACCCCGGACCATATTTA	TCCTGGGACACTTCTCTGAA
<i>TGFβ</i>	TACAGCAACAATTCCTGGCG	GTGAACCCGTTGATGTCCAC
<i>β-actin</i>	TCCTTCCTGGGCATGGAGT	CAGGAGGAGCAATGATCTTGAT

Table 4. Primer sequences used in RT-qPCR for Mice

Gene	Forward 5'-3'	Reverse 5'-3'
<i>SRGN</i>	CTCGCCTTCGTCCTGGTTT	CCTCGATGCAGTTCGCAAAAA
<i>IL1β</i>	ACTCATTGTGGCTGTGGAGA	TTGTTTCATCTCGGAGCCTGT
<i>IL6</i>	TACCACTCCCAACAGACCTG	ACTCCAGAAGACCAGAGGAA
<i>IL18</i>	ACAACCTTGGCCGACTTCAC	GGTCACAGCCAGTCCTCTTA
<i>TGFβ</i>	TCGCTTTGTACAACAGCACC	ACTGCTTCCCGAATGTCTGA
<i>β-actin</i>	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC

2.11 Western blot

Proteins were extracted from SH-SY5Y, HT22, mouse brain tissue, and RBC-EVs using RIPA lysis buffer (Beyotime, China, P0013B) containing phenylmethylsulfonyl fluoride (PMSF, Biosharp, China, BL507A). Protein sample concentrations were determined using the BCA protein assay kit (Vazyme, China, E112-01). Equal volumes of protein samples were separated by 10% or 15% SDS-PAGE gel electrophoresis and then transferred to a 0.2 μm PVDF membrane (Merck, China, ISEQ00010). The membrane was blocked with 5% skim milk at room temperature for 2 hours, followed by incubation with the corresponding primary antibodies overnight at 4°C. After washing the membrane, HRP-conjugated secondary antibodies were added and

incubated at room temperature for 2 hours. Immune complexes were visualized using the Chemi-Doc system (Tanon, Shanghai, China) along with enhanced chemiluminescence detection kit (Tanon, China, 180-5001), and grayscale values were quantitatively analyzed using ImageJ software. The relative expression levels of the target protein were normalized using GAPDH as an internal reference protein. Detailed information on the antibodies used in the experiment is provided in Table 5.

Table 5. Antibodies used in Western blot

Antibody	Dilution	Supplier	Reference
Anti-SRGN	1:800	Abclonal	A25426
Anti-P62	1:10000	Proteintech	18420-1-AP
Anti-TOM20	1:10000	Proteintech	11802-1-AP
Anti-LC3B	1:2000	Abclonal	A19665
Anti-SLC7A11	1:1000	Abclonal	A13685
Anti-ALIX	1:10000	Proteintech	12422-1-AP
Anti-TSG101	1:5000	Proteintech	28283-1-AP
Anti-CD63	1:1000	Abclonal	A19023
Anti-HBA1	1:1000	Abclonal	A9293
Anti-calnexin	1:1000	Abclonal	A15631
Anti-GAPDH	1:2000	Abclonal	AC001
HRP Goat Anti-rabbit IgG (H+L)	1:2000	Abclonal	AS014
HRP Goat Anti-mouse	1:2000	Abclonal	AS003

2.12 Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with Triton X-100 (Beyotime, China, P0096) for 2 minutes, then blocked with 5% BSA for 1 hours. After removing the blocking solution, add the primary antibodies diluted with 5% BSA and incubate overnight at 4 °C. The next day, incubate with the fluorescent secondary antibodies diluted with PBS for 1 hour at 37 °C in the dark. After washing with PBS, stain the nuclei with Hoechst 33342 dye (Beyotime, China, C1026) for 5 minutes at room temperature. Invert the cell smear onto a microscope slide onto which 5 µL of anti-fluorescence quenching mountant (LEAGENE, China, IH0252) had been added, acquire images using a fluorescence microscope (OLYMPUS microscopy CX41, Tokyo, Japan). Details of the antibodies used are shown in Table 6.

Table 6. Antibodies used in Immunofluorescence staining

Antibody	Dilution	Supplier	Reference
Anti-SRGN	1:200	Santa	sc-374657
Anti-LC3B	1:200	Abclonal	A19665
Anti-SLC7A11	1:200	Abclonal	A13685
FITC Goat Anti-Rabbit IgG (H+L)	1:200	Abclonal	AS011
Cy3 Goat Anti-Mouse IgG (H+L)	1:200	Abclonal	AS008

2.13 Immunohistochemistry staining

First, paraffin sections of striatum brain tissue were placed in xylene for gradient dewaxing, followed by dehydration in a gradient of ethanol. The sections were then immersed in citrate buffer (pH 6.0) and heated in a constant-temperature water bath at 100°C for 30 minutes to perform antigen retrieval. After allowing the sections to cool naturally to room temperature, add a 3% H₂O₂ solution and incubate at room temperature in the dark for 30 minutes to inactivate endogenous tissue peroxidase. Next, add 5% BSA blocking solution dropwise and block at room temperature for 1 hour. Discard the blocking solution, then add the anti-SRGN primary antibody (1:200 dilution) diluted in 5% BSA, and incubate overnight in a humid chamber at 4°C. The next day, the sections were incubated with the corresponding secondary antibody (Biotin-coupled goat anti-mouse/rabbit IgG) and incubated at room temperature for 30 minutes. The sections were then add Streptavidin-Biotin Complex (SABC incubation buffer, BOSTER, China, SA1064) and incubated at 37°C for 30 minutes. Subsequently, the tissue sections were visualized with DAB Horseradish Peroxidase Color Development Kit (Boster, Wuhan, AR1025).at room temperature in the dark. The degree of staining was observed under a microscope; once the positive signal was clear and there was no significant background staining, the reaction was terminated by rinsing with distilled water. Then, the nucleus were stained with a hematoxylin solution and rinsed with tap water to restore the blue color. The sections were then dehydrated using a gradient of ethanol and cleared with xylene before being mounted with neutral resin. Finally, images were captured using a histology scanner (3DHISTECH, Hungary).

2.14 Lactate dehydrogenase (LDH) Activity Assay

An LDH activity assay kit (Beyotime, China, P0393S) was used to measure cell-released LDH activity according to the manufacturer's protocol to assess cell damage. Cell culture supernatants from each group and tissue lysates were collected, centrifuged to remove impurities, and the supernatants were then tested. Add the supernatant and the LDH assay working solution to a 96-well plate in sequence, incubate at room temperature away from light until complete color development, measure the absorbance (OD value) of each well at a wavelength of 450 nm using a microplate reader, and calculate the LDH activity levels in the samples based on the kit's standard curve.

2.15 Reactive oxygen species (ROS) assay

Following the manufacturer's instructions, DCFH-DA was diluted in serum-free culture medium at a ratio of 1:1000 to prepare a working solution with a final concentration of 10 μ M. The cells were then incubated with the DCFH-DA working solution at 37°C for 20 minutes, gently washed with PBS to remove unbound probe, stained with Hoechst 33342 for 5 minutes, and the staining results were observed under a fluorescence microscope.

2.16 Glutathione (GSH) Level Assay

A commercial micro-GSH assay kit (Nanjing Jiancheng Bioengineering Institute, China, A006-2-1) was used to measure the levels of reduced GSH in samples via the DTNB colorimetric method at 420 nm. Cells and serum were centrifuged and deproteinized according to the instructions. The samples were incubated in a 96-well plate at room temperature for 5 minutes to develop color, after which absorbance was measured. GSH concentrations were calculated using the provided standard curve and formula to evaluate the antioxidant levels of the samples.

2.17 mRNA-seq analysis

mRNA-seq analysis was performed on OGD/R-damaged and control HT22 cell lines (n=3). The RNA libraries were generated utilizing the Illumina NovaSeqTM 6000 platform, and subsequent bioinformatics analysis was performed using the OmicStudio platform (available at <https://www.omicstudio.cn/tool>).

2.18 Mitochondrial membrane potential (MMP) assay

MMP in cells was analyzed using JC-1 staining solution (Beyotime, China, C2003S). Following the manufacturer's instructions, 5 μ L of JC-1 staining solution (200 $\times$) was added to 1 mL of JC-1 staining buffer, mixed thoroughly, and set aside. Next, cells were incubated with the diluted JC-1 probes in the dark at 37°C for 20 minutes, then stained with Hoechst 33342 for 5 minutes. Use a fluorescence microscope to detect JC-1 monomers at an excitation wavelength of 490 nm and an emission wavelength of 530 nm, JC-1 polymers at an excitation wavelength of 525 nm and an emission wavelength of 590 nm, and Hoechst 33342 at an excitation wavelength of 346 nm and an emission wavelength of 460 nm.

2.19 Mitochondria and lysosome probe staining

Cells were stained using Mito-Tracker red fluorescent probe (Beyotime, China, C1035) and Lyso-Tracker green fluorescent probe (Beyotime, China, C1047S) to detect the occurrence of mitophagy. The corresponding working solution was prepared by diluting the probe dye with complete medium according to the preparation ratio of the manual, and preincubated at 37°C. The original cell culture medium was discarded, and the pre-incubated staining working solution was added. The cells were incubated for 30 min at 37°C. The staining solution was discarded and Hoechst 33342 was added to stain nuclei for 5 min at room temperature. The fluorescence signal was observed by fluorescence microscope.

2.20 Protein-protein docking

The X-ray crystal structure of SRGN was obtained from the Protein Data Bank. The predicted structure of SLC7A11 was generated using AlphaFold. To ensure the accuracy of the docking results, the proteins were prepared using AutoDockTools-1.5.7. Protein-protein docking was performed using the Docking Web Server (GRAMM). Finally, protein-protein interactions were predicted, and the interaction diagrams were generated using PyMOL (<https://pymol.org>).

2.21 Co-immunoprecipitation (Co-IP) assay

SH-SY5Y cells transfected with the designated plasmid were lysed and sonicated to extract total protein. In accordance with the manufacturer's protocol (Beyotime, China, P2179S), specific primary antibodies and negative control IgG were bound to Protein A/G magnetic beads and incubated on a rotary mixer at room temperature for 1 hour. Subsequently, the samples were incubated with the antibody- or normal IgG-bound Protein A+G magnetic beads on a rotary mixer at 4°C overnight. Immunoprecipitated proteins were recovered by capturing the beads using a magnetic separator. After washing the beads 3 times with lysis buffer, the beads were resuspended in 30 µL of lysis buffer, heated with 1× SDS-PAGE protein loading buffer (Biosharp, China, BL502B) at 95 for 5 minutes, and the protein samples were analyzed by Western blot analysis.

2.22 Proteasome and lysosome inhibition assay

Cells were seeded into 6-well plates and cultured until approximately 70-80% confluence. Cells were transfected with an SRGN overexpression plasmid for 48 hours. Subsequently, the cells were treated with MG132 (MCE, USA, HY-13259, 10 µM), chloroquine (CQ, MCE, USA, HY-17589A, 50 µM), or an equivalent volume of DMSO as a vehicle control for 4 hours prior to harvesting. Whole-cell lysates were prepared using RIPA buffer supplemented with protease inhibitors, and Western blot analysis was

then performed to determine protein expression levels.

2.23 Cycloheximide (CHX) chase assay

To evaluate protein stability, cells were transfected with an SRGN overexpression plasmid for 48 hours. The cells were then treated with CHX (MCE, USA, HY-12320, 20 μ M) to inhibit de novo protein synthesis. Cells were harvested at the indicated time points (0, 3, 6, and 9 hours) after CHX treatment, and total protein was extracted for Western blot analysis. Relative protein abundance was quantified using ImageJ and normalized to GAPDH.

2.24 Extraction of RBC-EVs

Peripheral venous blood was collected from healthy type O donors and centrifuged at $2,000 \times g$ for 10 minutes to isolate red blood cells (RBCs) and remove plasma and white blood cell components. The purified RBCs were resuspended in PBS. The RBCs suspension was sequentially extruded through 5 μ m and 1 μ m polycarbonate membrane filters 4 times each using a mini-extruder (Avanti Polar Lipids, USA). The extruded sample was ultracentrifuged at $100,000 \times g$ for 70 minutes at 4 $^{\circ}$ C to enrich RBC-EVs. The RBC-EVs were resuspended in PBS and stored at -80 $^{\circ}$ C for future use. The exosomal protein concentration was determined using a detergent-free BCA protein assay kit.

2.25 Nanoparticle tracking analysis (NTA)

The quantity and particle size distribution of RBC-EVs were determined by NTA, using a NanoSight NS500 detection system (Malvern, UK). Prior to analysis, the instrument was calibrated using 100 nm polystyrene microspheres (Thermo Fisher Scientific, Canada) to ensure measurement accuracy. RBC-EVs samples were automatically injected into the detection chamber at a constant flow rate via the Malvern NanoSight syringe pump system. Three consecutive 60 seconds fields of view were captured for

each sample, with the detection threshold set to 5. Data were analyzed using NTA 3.3 Suite Software.

2.26 Preparation of RBC-EVs@siSRGN and RBC-EVs@siCTR by

Electroporation

In vitro synthesized Mus-siSRGN (the sense strand: 5'-CCAACAGAUGAAAGCAAUAUUTT-3', the antisense strand: 5'-AAUAUUGCUUUCAUCUGUUGGTT-3'), FAM-labeled siSRGN, and negative control siCTR were used. 45 μ L aliquot of RBC-EVs at a concentration of 10^{11} particles/mL was mixed with 5 μ g of siSRGN, FAM-siSRGN, or siCTR, respectively. The mixture was transferred to a 4 mm electroporation cuvette and pulsed twice using an electroporator at 0.7 kV and 0.35 seconds. Subsequently, the samples were incubated at 37 °C for 2 hours to restore the intact membrane structure of the RBC-EVs. To remove unencapsulated free siRNA, the mixture was resuspended and washed with pre-cooled PBS, followed by ultracentrifugation at $100,000 \times g$ at 4 °C for 70 minutes. The resulting pellet was resuspended in PBS for future use.

2.27 Transmission electron microscopy

The ultrastructure of mitochondria and autolysosomes in cell samples and the morphology of RBC-EVs were observed using TEM according to routine procedures. SH-SY5Y cells were prefixed in pre-cooled 2.5% glutaraldehyde solution. After thorough rinsing with PBS, post-fixation was performed with 1% osmium tetroxide solution. Subsequently, the samples were dehydrated through a graded ethanol-acetone series, embedded in epoxy resin, cut into 70-nm ultrathin sections using an ultramicrotome, and double-stained with uranyl acetate and lead citrate. Purified RBC-EVs were dropped onto 200-mesh carbon-coated copper grids and allowed to adsorb at room temperature for 2 min. Excess suspension was gently blotted

with filter paper, followed by negative staining with uranyl acetate staining solution at room temperature for 5 min. After staining, the samples were washed twice with PBS and allowed to air-dry. Finally, mitochondrial morphology, mitophagosomes, autolysosomes, and the characteristic bilayer membrane structure of RBC-EVs were observed, and electron micrographs were captured using a Tecnai T10 transmission electron microscope (TEM, FEI, Hillsboro, USA) at an accelerating voltage of 100 kV.

2.28 RBC-EVs internalization assay

According to the manufacturer's instructions, RBC-EVs were labeled with Dir dye (UElandy, China, D4006). The labeled RBC-EVs were loaded with FAM-labeled siSRGN via electroporation and then co-incubated with HT22 cells for 24 hours. The cells were fixed with 4% paraformaldehyde for 30 minutes, and the nuclei were stained with Hoechst 33342 for 5 minutes. The internalization efficiency of RBC-EVs was evaluated using a fluorescence microscope.

2.29 Intravital imaging of small animals

To observe the distribution and targeting localization of RBC-EVs in mice, RBC-EVs were labeled with Dir fluorescent dye according to the manufacturer's instructions. A total of 2.1×10^9 particles/mL of labeled RBC-EVs were injected into mice via the tail vein. Fluorescence signals were captured using a small animal in vivo imaging system (BLT FluoView400, China) to record and analyze the distribution characteristics of RBC-EVs in mice.

2.30 Laser speckle blood flow imaging

Approximately 20-30 minutes after the insertion of the suture into the common carotid artery of the mouse, the scalp was carefully peeled back to fully expose the skull, and the head of the mouse was secured in a stereotaxic frame. A laser speckle blood flow imaging system (SIM BFI ZOOM, China) was used to monitor and record the cerebral

blood perfusion in the middle cerebral artery territory in real time, in order to evaluate the success rate of model establishment and changes in regional cerebral blood flow.

2.31 TTC staining

After 24 hours of cerebral ischemia-reperfusion in mice, the animals were anesthetized and their brains were harvested to evaluate brain tissue infarction using TTC staining. Briefly, the mouse brains were rapidly removed and quick-frozen at -20°C for 10 minutes. The prepared brain slices were placed in 0.2% TTC staining solution and incubated at 37°C in the dark for 10-15 minutes. Subsequently, the slices were fixed in 4% paraformaldehyde. Automated analysis of the staining results was performed using ImageJ software, which included key technical steps such as image grayscale conversion, threshold segmentation, and particle analysis.

2.32 Neurological deficit assessment

After 24 hours of cerebral ischemia-reperfusion in mice, neurobehavioral scores were evaluated using the Longa scale. Score 0: no neurological deficits, normal; Score 1: failure to fully extend the left forelimb, mild neurological deficit; Score 2: circling to the left while walking, moderate neurological deficit; Score 3: falling to the left side while walking, severe neurological deficit; Score 4: inability to walk spontaneously or loss of consciousness, impaired consciousness.

2.33 Open field test

The open field test was conducted in a quiet, evenly illuminated environment. Mice were gently placed in the center of the bottom of the open field box, and the video recording system was activated to start timing. The mice were allowed to explore freely for 15 minutes, during which behavioral parameters such as movement trajectory and distance traveled were recorded. After each mouse was tested, the inner wall and bottom surface of the open field box were thoroughly cleaned with 75% ethanol. After the

ethanol was completely evaporated, the experiment of the next mouse was carried out, in order to avoid the interference of residual odor or feces on the subsequent test results.

2.34 Serum biochemical assays

According to the manufacturer's instructions, the serum levels of lipoprotein (a) (LP(a)), alanine transaminase (ALT) and aspartate transaminase (AST) in mice were detected with a Beckman AU5800 automated biochemistry analyzer (Beckman Coulter, China). Specifically, we used blood collection tubes containing procoagulant and separator gel to take whole blood from experimental mice, which was left to stand for 1 hour at room temperature and then centrifuged at 3,000 rpm for 10 minutes to obtain serum. Subsequently, the triglyceride test kits (LP(a) kit-immunoturbidimetric assay, ALT kit-Lactate dehydrogenase, and AST kit-enzyme activity) were put into the analyzer. Next, approximately 200 μ L of test sample was transferred to the test tank into the analyzer for automated testing. All of these procedures are similar to biochemical testing in a hospital laboratory. Reference ranges for normal approximately 20-week-old male C57BL/6 mice: LP(a) 0–50 mg/L; ALT 10-50 U/L; AST 35.7-135.7 U/L.

2.35 Hematoxylin and Eosin (H&E) staining

Fresh mouse brain tissues were fixed in 4% paraformaldehyde, routinely embedded in paraffin, sliced into 4 μ m-thick paraffin sections, and stained in accordance with the manufacturer's instructions. First, the sections were treated with xylene to remove the paraffin, followed by rehydration using a series of ethanol solutions of decreasing concentration (100%, 95%, 85% and 75%). The sections were stained in Hematoxylin Staining Solution (Beyotime, China, C0105S) at room temperature for 5 minutes to stain the nuclei and rinsed in tap water to counteract the blue color. Subsequently, sections were stained in Eosin Solution staining (Beyotime, China, C0105S) for 1 minute. Finally, the sections were dehydrated sequentially using a gradient of ethanol

solutions (75%, 85%, 95%, 100%), cleared with xylene, mounted with neutral resin, and left in a fume cupboard overnight. Images of the sections were captured using a histology scanner (Sigma, USA).

2.36 Nissl staining

Paraffin sections of mouse brain tissue were deparaffinised and dehydrated. Methyl violet staining solution was applied dropwise to cover the tissue sections, and staining was carried out at room temperature for 20 minutes. Rinse gently with distilled water to remove excess stain, then rapidly decolourise using Nissl decolourising solution for 5 seconds, until the background is essentially colourless and the outlines of the neurons are clearly defined. Following decolourisation, dehydrate directly with anhydrous ethanol, clear with xylene, and finally mount with neutral resin. Images were acquired using a histology scanner to observe the morphology of neurons in the brain tissue and to count the number of surviving neurons.

2.37 Statistical analysis

All experiments (including cell and mouse-based experiments) included biological replicates and were repeated at least three times. Statistical analyses were performed using GraphPad Prism 9.0. All data are presented as mean $\pm$ standard deviation (SD). For comparisons between two groups, an independent samples t-test was used; for comparisons involving multiple groups, one-way analysis of variance (ANOVA) was employed. All statistical tests were two-tailed; $P < 0.05$ was defined as significant, and 95% confidence intervals were reported.

3. Results

3.1 SRGN is markedly upregulated following cerebral ischemia/reperfusion injury

To clarify the expression pattern and clinical implication of SRGN in CI/RI, we initially analyzed three independent public transcriptome datasets (GSE22255, GSE270958, GSE232244) retrieved from the GEO database. Bioinformatics analysis consistently revealed elevated SRGN transcription in peripheral blood samples from patients with AIS, as well as in brain tissues collected from MCAO/R mice (Figure 1A). Subsequent validation in our clinical cohort further verified significantly higher serum SRGN levels in patients diagnosed with acute cerebral infarction relative to healthy subjects (Figure 1B). Furthermore, receiver operating characteristic (ROC) curve analysis yielded an area under the curve (AUC) value of 0.78, suggesting that SRGN may have potential biological value for evaluating acute cerebral infarction. In addition, we validated SRGN expression changes at both cellular and animal levels (Figure 1C-K). Consistent results from qRT-PCR, Western blot, immunofluorescence and immunohistochemistry demonstrated that SRGN mRNA and protein abundances were distinctly increased in OGD/R-stimulated HT22 (Figure 1C-E) and SH-SY5Y (Figure 1F-H) neuronal cells, as well as in ischemic brain tissues of MCAO/R mice (Figure 1I-K). Notably, the upregulation of SRGN was closely correlated with the severity of neuronal damage, supporting its potential involvement in CI/RI pathogenesis.

3.2 SRGN knockdown protects neurons against OGD/R-induced damage

To elucidate the biological function of SRGN during CI/RI progression, we established SRGN knockdown models in neuronal cells. Based on interference efficiency screening, siSRGN-3 was selected for transient SRGN silencing in HT22 cells (Figure 2A-B), while shSRGN-1 was used to construct stably transfected SRGN-knockdown SH-

SY5Y cell lines (Figure 2G-H). Functional phenotypic assays showed that OGD/R stimulation induced severe neuronal injury, which was characterized by increased LDH release, excessive ROS accumulation, intracellular GSH depletion, and elevated expression levels of pro-inflammatory cytokines including IL-1 β , IL-6, IL-18 and TGF- β (Figure 2C-F, I-L). Intriguingly, genetic ablation of SRGN effectively reversed OGD/R-triggered cellular damage, redox imbalance and neuroinflammatory activation (Figure 2C-F, I-L). The consistent protective phenotypes observed in two distinct neuronal cell lines strongly suggest that SRGN acts as a vital pathogenic regulator that exacerbates neuronal injury under ischemia/reperfusion stress.

3.3 SRGN aggravates CI/RI by inducing excessive pathological mitophagy

To further explore the molecular mechanism by which SRGN modulates CI/RI-associated neuronal dysfunction, we performed transcriptome sequencing and functional enrichment analysis in OGD/R-injured neurons (Figure 3A). GO and KEGG enrichment results indicated that SRGN-regulated differentially expressed genes were primarily enriched in biological processes related to autophagy activation, mitochondrial structural and functional maintenance, as well as oxidative phosphorylation, PI3K-Akt and MAPK signaling cascades, implying a potential regulatory effect of SRGN on mitophagy. TEM ultrastructural observation showed that OGD/R stimulation triggered evident mitochondrial swelling, structural disruption and massive mitophagosome formation (Figure 3B). SRGN overexpression further aggravated mitochondrial morphological damage and mitophagic overactivation, whereas SRGN knockdown effectively mitigated mitophagosome accumulation and

preserved mitochondrial structural integrity (Figure 3B). Collectively, these TEM findings visually confirmed, at the ultrastructural morphological level, that SRGN accelerates excessive mitophagy under OGD/R conditions.

Subsequent functional validation corroborated these morphological changes. SRGN overexpression led to a significant decrease in red-green ratio and MMP, accompanied by aggravated GSH consumption and enhanced oxidative stress (Figure 3C-D).

Mitochondrial co-localization analysis with LC3B and LysoTracker demonstrated that SRGN overexpression facilitated abnormal recruitment of mitochondria to autophagosomes and lysosomes, ultimately resulting in mitochondrial loss and dysfunction (Figure 3E). Western blot results revealed that both OGD/R injury and

SRGN overexpression reduced P62 and TOM20 protein expression while increasing the LC3B-II/LC3B-I ratio, which are representative molecular features of excessive pathological mitophagy (Figure 3F). Conversely, SRGN deficiency restored MMP

levels, ameliorated OGD/R-induced damage (Figure 4A-C). This was accompanied by reduced colocalization of mitochondria with autophagosomes and lysosomes, increased mitochondrial fluorescence intensity, and normalized aberrant expression of

mitophagy-related proteins (Figure 4B-C). In summary, OGD/R injury can induce

excessive mitophagy in neurons, and SRGN overexpression similarly promotes

excessive activation of mitophagy, whereas SRGN knockdown alleviates OGD/R-

induced cell injury by inhibiting excessive mitophagy. The above positive and negative

experiments fully confirmed that SRGN acts as a key regulator of CI/R I by mediating

excessive mitophagy.

3.4 SRGN impairs neuronal antioxidant defense via post-transcriptional downregulation of SLC7A11

SLC7A11 serves as a core antioxidant protein that sustains cellular redox homeostasis and mitochondrial function [34, 36, 37]. To identify the downstream effector mediating SRGN-induced mitophagy and neuronal injury, we focused on SLC7A11 for mechanistic verification. Protein interaction prediction, Co-IP assays and immunofluorescence co-localization analysis confirmed a direct physical interaction and intracellular co-localization between SRGN and SLC7A11 in neurons (Figure 5A-C). Transcriptional detection showed that OGD/R stress significantly upregulated SRGN mRNA expression while suppressing SLC7A11 transcription (Figure 5D). Further protein expression analysis revealed that SRGN overexpression reduced SLC7A11 protein abundance in a dose-dependent manner, without altering its transcriptional level (Figure 5D-F). In contrast, SRGN knockdown efficiently restored SLC7A11 protein expression suppressed by OGD/R injury, while its mRNA underwent only a slight change (Figure 5D-F). These observations indicate that SRGN negatively modulates SLC7A11 expression mainly at the post-transcriptional level.

To clarify the precise post-transcriptional regulatory mechanism, we conducted pharmacological intervention and protein stability assays. Treatment with the proteasome inhibitor MG132 substantially rescued SRGN-induced SLC7A11 protein degradation, whereas the lysosomal autophagy inhibitor CQ exhibited no obvious rescue effect (Figure 5G, Figure S1A). Furthermore, CHX chase assays verified that SRGN overexpression significantly shortened the half-life of SLC7A11 protein and

accelerated its intracellular degradation (Figure 5H, Figure S1B). Taken together, these results confirm that SRGN facilitates SLC7A11 protein turnover primarily through the ubiquitin–proteasome pathway, thereby disabling SLC7A11-mediated antioxidant defense and exacerbating neuronal oxidative damage under CI/RI conditions.

3.5 SLC7A11 inhibition abolishes the neuroprotective effects of SRGN knockdown

To validate whether SLC7A11 is the essential downstream mediator responsible for SRGN-regulated mitophagy and neuronal injury, we performed rescue experiments using HG106, a specific pharmacological inhibitor of SLC7A11. HG106 treatment effectively downregulated SLC7A11 protein expression without affecting SRGN transcription or protein abundance, ruling out potential off-target interference with upstream SRGN expression (Figure 6A-B). Functional phenotypic detection showed that HG106 administration completely abrogated the neuroprotective effects induced by SRGN knockdown. Specifically, SRGN-deficient neurons exhibited re-elevated LDH release, excessive ROS accumulation, reduced GSH content, and restored pro-inflammatory cytokine expression after HG106 intervention (Figure 6C-F).

Further detection of mitochondrial function and mitophagy biomarkers demonstrated that HG106 reversed the MMP restoration mediated by SRGN knockdown, and restored the abnormal co-localization of mitochondria with autophagosomes and lysosomes (Figure 6G-H). Consistent with immunofluorescence results, Western blot analysis confirmed that HG106 treatment counteracted the upregulation of P62 and TOM20, as well as the reduced LC3B-II/LC3B-I ratio induced by SRGN silencing, thereby reactivating pathological excessive mitophagy (Figure 6I). Collectively,

pharmacological blockade of SLC7A11 completely abolished the neuroprotective effect produced by SRGN knockdown, confirming that SRGN induces excessive mitophagy by suppressing SLC7A11 signaling, thereby exacerbating neuronal injury during the CI/RI process.

3.6 RBC-EVs@siSRGN exerts neuroprotective effects against OGD/R injury in vitro

Given the pathogenic role of SRGN in CI/RI, we further developed a targeted nanotherapeutic strategy by constructing RBC-derived extracellular vesicle-loaded siSRGN (RBC-EVs@siSRGN), and evaluated its protective efficacy and molecular mechanism in vitro. NTA showed that high-purity RBC-EVs with uniform particle size were successfully isolated from type O-negative RBCs, with the resulting vesicle particle size concentrated around 160 nm and a particle concentration of 2.9×10^{13} particles/mL (Figure 7A). These vesicles positively expressed canonical EV markers (ALIX, TSG101, CD63) and erythrocyte-specific marker HBA1, while barely expressing the endoplasmic reticulum contamination marker Calnexin, confirming high purity and qualified vesicle properties (Figure 7B). The typical cup-shaped or spherical bilayer membrane structure of RBC-EVs was observed by TEM (Figure 7C). The above results demonstrate that RBC-EVs of high purity were successfully isolated, providing a foundation for their application as siRNA carriers. Subsequently, siSRGN was loaded into RBC-EVs by electroporation to construct RBC-EVs@siSRGN, with RBC-EVs@siCTR simultaneously established as the negative control (Figure 7D). The FAM fluorescence-labeled siRNA uptake assay confirmed that the prepared RBC-

EVs@siSRGN exhibited stable structural integrity and high internalization efficiency in neuronal cells (Figure 7E). NTA quantification and TEM structural observation showed that the particle concentrations of RBC-EVs@siCTR and RBC-EVs@siSRGN were 2.1×10^{11} particles/mL and 1.8×10^{11} particles/mL, respectively, indicating that the electroporation procedure did not significantly reduce vesicle yield (Figure S2A-B). Collectively, these results suggest that the RBC-EVs@siSRGN we constructed has potential as an engineered exosome-based drug carrier.

In vitro functional experiments confirmed that RBC-EVs@siSRGN exerted a dose-dependent inhibitory effect on SRGN mRNA and protein expression in OGD/R-injured neurons (Figure 7F-H). Consistent with genetic knockdown results, RBC-EVs@siSRGN-mediated SRGN silencing effectively restored SLC7A11 protein levels and restrained pathological excessive mitophagy (Figure 7G). Further phenotypic assays demonstrated that RBC-EVs@siSRGN treatment markedly reduced LDH release and ROS accumulation, rescued intracellular GSH depletion, and suppressed the expression of pro-inflammatory cytokines (Figure 7I-L). In summary, the engineered RBC-EVs@siSRGN nanocarriers efficiently alleviate OGD/R-induced oxidative stress, neuroinflammation and aberrant mitophagy, thereby exerting prominent neuroprotective effects in cultured neurons.

3.7 RBC-EVs@siSRGN ameliorates cerebral ischemia/reperfusion injury in vivo

We further established the mouse MCAO/R model to verify the in vivo neuroprotective efficacy of RBC-EVs@siSRGN. Insertion of the MCAO filament effectively induced focal cerebral ischemia in the mouse cerebral hemisphere; withdrawal of the filament

achieved reperfusion, but blood flow in the ischemic region could not be fully restored within a short period. Compared with the sham operation group, these findings visually demonstrate the hemodynamic characteristics of the ischemia-reperfusion injury model (Figure 8A). Intravital fluorescence imaging results showed that after tail vein injection of DiI fluorescence-labeled RBC-EVs, the vesicles were specifically enriched in the cerebral ischemic injury region of mice, indicating excellent lesion-targeting capability (Figure 8B). Therapeutic outcome assessment showed that RBC-EVs@siSRGN treatment significantly reduced cerebral infarct volume, rescued impaired spontaneous locomotor activity, and improved neurological deficit scores in MCAO/R mice (Figure 8C-E, Figure S3A). Detection of the serum injury markers LP, ALT, and AST indicated that RBC-EVs@siSRGN could alleviate tissue damage (Figure 8F). Histopathological examination further revealed that RBC-EVs@siSRGN administration reduced neuronal necrosis and cytoplasmic vacuolization, and prevented Nissl body loss in ischemic brain regions (Figure 8G, Figure S3B).

Consistent with in vitro findings, in vivo mechanistic detection demonstrated that RBC-EVs@siSRGN effectively silenced SRGN expression in ischemic brain tissues, thereby restoring SLC7A11 protein abundance (Figure 8G-H, L). Functional index assays revealed that RBC-EVs@SISRGN reduced serum LDH, increased brain tissue GSH content, and alleviated oxidative stress injury (Figure 8I-J). Transcriptional detection of inflammatory factors in brain tissue demonstrated that RBC-EVs@siSRGN downregulated the expression of pro-inflammatory factors (Figure 8K). Moreover, RBC-EVs@siSRGN treatment corrected the abnormal expression of mitophagy-related

proteins and suppressed pathological excessive mitophagy in ischemic brain tissues (Figure 8L). In conclusion, RBC-EVs@siSRGN exerts robust neuroprotective effects against CI/RI in mice. The underlying mechanism is dependent on targeted SRGN silencing, which activates SLC7A11-mediated antioxidant signaling and inhibits oxidative stress, neuroinflammation and aberrant mitophagy, ultimately promoting neurological functional recovery after ischemic stroke.

4. Discussion

Growing evidence has revealed that CI/RI pathogenesis is multifactorial, driven by the crosstalk among oxidative stress, dysregulated mitophagy, neuroinflammation and ferroptosis, rather than relying on any single pathological pathway [5]. Among these detrimental events, disrupted mitochondrial homeostasis and impaired endogenous antioxidant defenses act as key upstream triggers that initiate and continuously amplify neuronal reperfusion damage [4, 38-41]. Our clinical data showed significantly elevated serum SRGN levels in patients diagnosed with acute cerebral infarction, implying that circulating SRGN has potential as a non-invasive peripheral biomarker for evaluating CI/RI severity. Previous studies have shown that SRGN, as a secreted glycoprotein, can participate in the pathological development of various diseases by regulating processes such as inflammatory response and apoptosis [22, 23]. However, its expression features and biological roles in CI/RI remain largely uncharacterized. Through integrated analysis of clinical specimens, cellular phenotypes and animal phenotypic validation, our study confirmed aberrant SRGN upregulation in the CI/RI pathological context and verified its close correlation with ischemic brain injury, providing solid clinical ground

for subsequent functional validation and mechanistic exploration.

Mitophagy serves as a core mitochondrial quality-control mechanism that sustains neuronal energy metabolism and cellular homeostasis, exerting dual protective and detrimental roles during cerebral ischemic injury. Under physiological conditions, moderate mitophagy selectively eliminates damaged mitochondria and maintains neuronal survival [42-44]. In the reperfusion stage, nevertheless, burst oxidative stress excessively activates mitophagic flux, leading to non-specific degradation of functional mitochondria, energy metabolic collapse and subsequent neuronal apoptosis, which further exacerbates brain tissue damage [4, 41]. Consistent with recent reports that excessive mitophagy aggravates ischemic injury via crosstalk with oxidative stress and ferroptosis [45], our ultrastructural observation, MMP detection, immunofluorescence co-localization and immunoblotting data demonstrated that SRGN overexpression compromised mitochondrial integrity, reduced membrane potential, and promoted abnormal association between mitochondria, autophagosomes and lysosomes. At the molecular level, SRGN overexpression increased the LC3B-II/LC3B-I ratio while decreasing P62 and TOM20 protein levels, which are typical molecular signatures of pathological mitophagy. In contrast, SRGN knockdown efficiently restrained overactivated mitophagy and restored mitochondrial homeostasis. These results suggest that SRGN may aggravate the injury of CI/RI neurons by regulating the excessive occurrence of mitophagy and disrupting mitophagy homeostasis, which may be one of the important ways for SRGN to aggravate the injury of CI/RI neurons, this also further enriches the molecular network of mitophagy regulation in CI/RI and provides a new

perspective for elucidating the pathophysiological mechanisms of CI/RI.

As a cystine/glutamate transporter, SLC7A11 can maintain cellular oxidative stress homeostasis by regulating intracellular GSH synthesis and inhibiting excessive ROS accumulation, and can also participate in the process of mitophagy by regulating mitochondrial function [34, 36]. Here, molecular docking, Co-IP and immunofluorescence co-localization assays consistently verified direct physical interaction and intracellular co-localization between SRGN and SLC7A11 in neurons. Mechanistically, SRGN did not regulate SLC7A11 transcription, but facilitated SLC7A11 protein ubiquitination and degradation through the ubiquitin–proteasome pathway, thereby reducing SLC7A11 protein stability at the post-transcriptional level. To verify whether SRGN affects the CI/RI process by regulating SLC7A11, this study introduced HG106, a specific inhibitor of SLC7A11, into the OGD/R-induced neuronal injury model. The results showed that HG106 intervention significantly reversed the protective effect of SRGN knockdown on neurons, increasing ROS accumulation, reducing GSH levels, promoting pathological excessive mitophagy, and aggravating neuronal injury. These results further confirmed that SRGN, through negative regulation of SLC7A11 expression, inhibits intracellular GSH synthesis and promotes ROS accumulation, thereby inducing excessive mitophagy and ultimately aggravating CI/RI-induced neuronal injury. These findings demonstrate that the SRGN–SLC7A11 signaling axis dominates oxidative stress imbalance and pathological mitophagy during CI/RI progression.

Although the injury-promoting role and regulatory mechanism of SRGN in CI/RI have

been clarified, how to achieve precise targeted inhibition of SRGN while avoiding the toxic side effects caused by systemic intervention remains a key challenge for its clinical translation. RBC-EVs, as a novel drug delivery vehicle, possess notable advantages such as good biocompatibility, low immunogenicity, and efficient BBB penetration [46-49]. As a vesicular system, they have been widely applied in research on targeted therapy for central nervous system diseases [50, 51]. In this study, RBC-EVs were isolated and extracted from type O-negative blood, and siSRGN was loaded into RBC-EVs via electroporation, successfully constructing the RBC-EVs@siSRGN delivery system [52]. In vitro neuronal experiments confirmed that RBC-EVs@siSRGN could be efficiently internalized by neurons to specifically silence pathological SRGN overexpression. By restoring SLC7A11 protein function, this targeted treatment relieved neuronal oxidative damage, suppressed neuroinflammatory responses and corrected aberrant mitophagy, ultimately reducing ischemic lesion volume and improving neurological function in model animals. Compared with conventional delivery vectors, this RBC-EV-based nanoplatform effectively avoids off-target effects and systemic side effects, achieving precise CI/RI intervention with promising clinical translational prospects.

Several limitations still exist in the present study. First, the clinical cohort enrolled in this work is relatively small. Future large-scale and multicenter clinical investigations are required to further validate the diagnostic value of SRGN for CI/RI, and to clarify the correlation between SRGN expression levels and patient long-term prognosis. Second, our current study mainly focuses on the regulatory effects of SRGN on

mitophagy and oxidative stress. Whether the SRGN–SLC7A11 axis modulates neuronal ferroptosis remains undefined. In addition, the precise binding domains and interactive sites between SRGN and SLC7A11, as well as other unexploited downstream signaling pathways, require further in-depth exploration. Third, the long-term in vivo metabolic characteristics, biological stability and safety of repeated RBC-EVs@siSRGN administration have not been fully evaluated, which warrants systematic preclinical safety assessment in future studies.

In summary, pathological SRGN upregulation during CI/RI accelerates SLC7A11 protein degradation via the ubiquitin–proteasome pathway, depletes intracellular antioxidant reserves, triggers excessive ROS accumulation and induces uncontrolled pathological mitophagy, thereby ultimately promoting neuronal apoptosis and cerebral infarction. In contrast, targeted SRGN silencing stabilizes endogenous SLC7A11 expression, remodels mitochondrial homeostasis, and inhibits oxidative stress as well as neuroinflammation, which efficiently alleviates CI/RI-induced brain injury. The present study firstly identifies the crucial regulatory role of the SRGN–SLC7A11 axis in ischemic brain injury. These findings provide a novel non-invasive biomarker and targeted therapeutic candidate for CI/RI, enrich the pathological regulatory network of ischemic stroke, and offer valuable experimental evidence to support the development of precise therapeutic strategies for stroke treatment.

5. Conclusion

In this study, we first confirmed, based on clinical samples, that serum SRGN expression levels were significantly increased in patients with acute cerebral infarction,

suggesting that serum SRGN may serve as a potential diagnostic biomarker for CI/RI. The results of in vitro and in vivo experiments showed that SRGN was abnormally upregulated in an OGD/R-induced neuronal injury model and in an MCAO/R mouse model of CI/RI, which could exacerbate neuronal oxidative stress dysregulation and cellular damage. Mechanistic investigations further revealed that SRGN directly interacts with SLC7A11, promoting its degradation via the proteasomal pathway. The loss of SLC7A11 induced substantial ROS accumulation and pathological excessive mitophagy, ultimately aggravating neuronal injury and driving the progression of CI/RI. In addition, we constructed a delivery system based on RBC-EVs, loading siSRGN onto RBC-EVs for targeted intervention. The results confirmed that RBC-EVs could effectively deliver siSRGN to ischemic brain tissue, inhibit SRGN expression, reduce the inflammatory response, restore mitophagy homeostasis, and alleviate CI/RI. Overall, serum SRGN may hold promise for application in the diagnosis and prognostic evaluation of CI/RI, whereas the RBC-EVs@siSRGN-mediated strategy for targeted SRGN inhibition could effectively attenuate ischemia-reperfusion-induced neuronal injury, providing a new experimental basis and technical direction for the targeted treatment of ischemic stroke.

6. List of abbreviations

CI/RI: Cerebral ischemia/reperfusion injury

OGD/R: oxygen glucose deprivation/reoxygenation

MCAO/R: middle cerebral artery occlusion/reperfusion

AIS: Acute ischemic stroke

863 **SRGN:** Serglycin

864 **SLC7A11:** Solute carrier family 7 member 11

865 **LDH:** Lactate dehydrogenase

866 **ROS:** Reactive oxygen species

867 **GSH:** Glutathione

868 **MMP:** Mitochondrial membrane potential

869 **Co-IP:** Co-immunoprecipitation

870 **CHX:** Cycloheximide

871 **RBCs:** Red blood cells

872 **RBC-EVs:** Red blood cell-derived extracellular vesicles

873 **NTA:** Nanoparticle tracking analysis

874 **TEM:** Transmission electron microscope

875 **H&E:** Hematoxylin and Eosin

876 **7. Declarations**

877 **Ethics approval and consent to participate**

878 All animal care and experimental procedures were approved by the Experimental

879 Animal Management and Use Committee of Jiangsu University (Approval no. UJS-

880 IACUC-AP-2026022811). All animal experiments complied with national animal

881 welfare regulations and the guidelines endorsed by the International Council for

882 Laboratory Animal Science (ICLAS). The human serum samples (human material)

883 involved in this study have been reviewed and approved by the Medical Ethics

884 Committee of Jiangsu University. The ethical approval document explicitly states that

this research complies with the principles of the Declaration of Helsinki and relevant national regulations of China. All experiments using human serum samples in this work were performed strictly in accordance with the approved ethical protocol.

Clinical trial number

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

D. H. and J. Z. for the Investigation, Methodology, and Writing - Original Draft. B. W., F. Y. and X. J. for the Software, Methodology, and Data curation. Y. Y. and J. Z. for the Writing - Review & Editing, Conceptualization, Funding acquisition, Project

administration, and Supervision.

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References

- [1] N.A. Hilkens, B. Casolla, T.W. Leung, F.E. de Leeuw, *Stroke*, *Lancet* 403(10446) (2024) 2820-2836.
- [2] Z. Ruan, G. Cao, Y. Qian, L. Fu, J. Hu, T. Xu, Y. Wu, Y. Lv, Single-cell RNA sequencing unveils *Lrg1*'s role in cerebral ischemia–reperfusion injury by modulating various cells, *J Neuroinflammation* 20(1) (2023) 285.
- [3] J.P. Ho, W.J. Powers, *Contemporary Management of Acute Ischemic Stroke*, *Annu Rev Med* 76(1) (2025) 417-429.
- [4] X. Lan, Q. Wang, Y. Liu, Q. You, W. Wei, C. Zhu, D. Hai, Z. Cai, J. Yu, J. Zhang, N. Liu, Isoliquiritigenin alleviates cerebral ischemia-reperfusion injury by reducing oxidative stress and ameliorating mitochondrial dysfunction via activating the Nrf2 pathway, *Redox Biol* 77 (2024) 103406.
- [5] X. Zeng, Y.D. Zhang, R.Y. Ma, Y.J. Chen, X.M. Xiang, D.Y. Hou, X.H. Li, H. Huang, T. Li, C.Y. Duan, Activated Drp1 regulates p62-mediated autophagic flux and aggravates inflammation in cerebral ischemia-reperfusion via the ROS-RIP1/RIP3-exosome axis, *Mil Med Res* 9(1) (2022) 25.
- [6] S. Bernardo-Castro, J.A. Sousa, E. Martins, H. Donato, C. Nunes, O.C. d'Almeida, M. Castelo-Branco, A. Abrunhosa, L. Ferreira, J. Sargento-Freitas, The evolution of

928 blood-brain barrier permeability changes after stroke and its implications on clinical
 929 outcome: A systematic review and meta-analysis, *Int J Stroke* 18(7) (2023) 783-794.

930 [7] P. Xiao, H. Huang, H. Zhao, R. Liu, Z. Sun, Y. Liu, N. Chen, Z. Zhang, Edaravone
 931 dexborneol protects against cerebral ischemia/reperfusion-induced blood-brain barrier
 932 damage by inhibiting ferroptosis via activation of nrf-2/HO-1/GPX4 signaling, *Free*
 933 *Radic Biol Med* 217 (2024) 116-125.

934 [8] J.J. Lochhead, P.T. Ronaldson, T.P. Davis, The role of oxidative stress in blood-brain
 935 barrier disruption during ischemic stroke: Antioxidants in clinical trials, *Biochem*
 936 *Pharmacol* 228 (2024) 116186.

937 [9] C.H. Chen, C.L. Hsieh, Effect of Acupuncture on Oxidative Stress Induced by
 938 Cerebral Ischemia-Reperfusion Injury, *Antioxidants (Basel)* 9(3) (2020).

939 [10] D.N. Granger, P.R. Kvietys, Reperfusion injury and reactive oxygen species: The
 940 evolution of a concept, *Redox Biol* 6 (2015) 524-551.

941 [11] Q. Zhang, M. Jia, Y. Wang, Q. Wang, J. Wu, Cell Death Mechanisms in Cerebral
 942 Ischemia-Reperfusion Injury, *Neurochem Res* 47(12) (2022) 3525-3542.

943 [12] R. Mao, N. Zong, Y. Hu, Y. Chen, Y. Xu, Neuronal Death Mechanisms and
 944 Therapeutic Strategy in Ischemic Stroke, *Neurosci Bull* 38(10) (2022) 1229-1247.

945 [13] A. Jurcau, A. Simion, Neuroinflammation in Cerebral Ischemia and
 946 Ischemia/Reperfusion Injuries: From Pathophysiology to Therapeutic Strategies, *Int J*
 947 *Mol Sci* 23(1) (2021).

948 [14] Y. Zhao, X. Zhang, X. Chen, Y. Wei, Neuronal injuries in cerebral infarction and
 949 ischemic stroke: From mechanisms to treatment (Review), *Int J Mol Med* 49(2) (2022).

950 [15] Y. Lu, Z. Li, S. Zhang, T. Zhang, Y. Liu, L. Zhang, Cellular mitophagy: Mechanism,
 951 roles in diseases and small molecule pharmacological regulation, *Theranostics* 13(2)
 952 (2023) 736-766.

953 [16] Y. Li, Y. Gao, G. Yu, Y. Ye, H. Zhu, J. Wang, Y. Li, L. Chen, L. Gu, G6PD protects
 954 against cerebral ischemia-reperfusion injury by inhibiting excessive mitophagy, *Life*
 955 *Sci* 362 (2025) 123367.

956 [17] L. Ai, H. Ren, Y. Wang, M. Liu, Y. Qiu, J. Feng, R. Dai, W. Fu, Y. Wang, Z. Xi, H.
 957 Xu, F. Wang, The Neuroprotective Role of Ginsenoside Rg1 Against Cerebral
 958 Ischemia-Reperfusion Damage Through Inhibition of Mitophagy via Blocking
 959 Mitophagosome-Lysosome Fusion, *Cell Prolif* (2025) e70071.

960 [18] Q. Zhang, J. Zhou, M. Shen, H. Xu, S. Yu, Q. Cheng, F. Ding, Pyrroloquinoline
 961 Quinone Inhibits Rotenone-Induced Microglia Inflammation by Enhancing Autophagy,
 962 *Molecules* 25(19) (2020).

963 [19] Y. Cai, E. Yang, X. Yao, X. Zhang, Q. Wang, Y. Wang, J. Liu, W. Fan, K. Yi, C.
 964 Kang, J. Wu, FUNDC1-dependent mitophagy induced by tPA protects neurons against
 965 cerebral ischemia-reperfusion injury, *Redox Biol* 38 (2021) 101792.

966 [20] T. Zhang, P. Wu, E. Budbazar, Q. Zhu, C. Sun, J. Mo, J. Peng, V. Gospodarev, J.
 967 Tang, H. Shi, J.H. Zhang, Mitophagy Reduces Oxidative Stress Via Keap1 (Kelch-Like
 968 Epichlorohydrin-Associated Protein 1)/Nrf2 (Nuclear Factor-E2-Related Factor
 969 2)/PHB2 (Prohibitin 2) Pathway After Subarachnoid Hemorrhage in Rats, *Stroke* 50(4)
 970 (2019) 978-988.

971 [21] Z.L. Yuan, Y.Z. Mo, D.L. Li, L. Xie, M.H. Chen, Inhibition of ERK downregulates
 972 autophagy via mitigating mitochondrial fragmentation to protect SH-SY5Y cells from
 973 OGD/R injury, *Cell Commun Signal* 21(1) (2023) 204.

974 [22] H. Chanzu, J. Lykins, S. Wigna-Kumar, S. Joshi, I. Pokrovskaya, B. Storrie, G.
 975 Pejler, J.P. Wood, S.W. Whiteheart, Platelet alpha-granule cargo packaging and release
 976 are affected by the luminal proteoglycan, serglycin, *J Thromb Haemost* 19(4) (2021)
 977 1082-1095.

978 [23] A. Korpetinou, S.S. Skandalis, V.T. Labropoulou, G. Smirlaki, A. Noulas, N.K.
 979 Karamanos, A.D. Theocharis, Serglycin: at the crossroad of inflammation and
 980 malignancy, *Front Oncol* 3 (2014) 327.

981 [24] Y. Zhu, A.K.Y. Lam, D.K.Y. Shum, D. Cui, J. Zhang, D.D. Yan, B. Li, W.W. Xu,
 982 N.P.Y. Lee, K.T. Chan, S. Law, S.W. Tsao, A.L.M. Cheung, Significance of serglycin
 983 and its binding partners in autocrine promotion of metastasis in esophageal cancer,
 984 *Theranostics* 11(6) (2021) 2722-2741.

985 [25] J.Y. Guo, H.S. Hsu, S.W. Tyan, F.Y. Li, J.Y. Shew, W.H. Lee, J.Y. Chen, Serglycin
 986 in tumor microenvironment promotes non-small cell lung cancer aggressiveness in a
 987 CD44-dependent manner, *Oncogene* 36(17) (2017) 2457-2471.

988 [26] Y. He, D. Cheng, C. Lian, Y. Liu, W. Luo, Y. Wang, C. Ma, Q. Wu, P. Tian, D. He,
 989 Z. Jia, X. Lv, X. Zhang, Z. Pan, J. Lu, Y. Xiao, P. Zhang, Y. Liang, Q. Yang, G. Hu,
 990 Serglycin induces osteoclastogenesis and promotes tumor growth in giant cell tumor of
 991 bone, *Cell Death Dis* 12(10) (2021) 868.

992 [27] Z. Zhang, N. Qiu, J. Yin, J. Zhang, H. Liu, W. Guo, M. Liu, T. Liu, D. Chen, K.
993 Luo, H. Li, Z. He, J. Liu, G. Zheng, SRGN crosstalks with YAP to maintain
994 chemoresistance and stemness in breast cancer cells by modulating HDAC2 expression,
995 *Theranostics* 10(10) (2020) 4290-4307.

996 [28] S. Zhang, H. Hu, X. Li, Q. Chen, Y. Zheng, H. Peng, Z. Mo, W. Hu, Y. Li, S. Hong,
997 B. Huang, W. Kuang, S. Zhang, Y. Cao, SRGN-mediated reactivation of the
998 YAP/CRISPLD2 axis promotes aggressiveness of hepatocellular carcinoma, *Int J Biol*
999 *Sci* 21(7) (2025) 3262-3285.

1000 [29] A.I. Doncheva, F.A. Norheim, M. Hjorth, M. Grujic, A. Paivandy, S.N. Dankel,
1001 J.K. Hertel, T.G. Valderhaug, Y. Bottcher, J. Ferno, G. Mellgren, K.T. Dalen, G. Pejler,
1002 S.O. Kolset, Serglycin Is Involved in Adipose Tissue Inflammation in Obesity, *J*
1003 *Immunol* 208(1) (2022) 121-132.

1004 [30] F.G. La Rosa, L.W. Moreland, L. Nibali, M. Curtis, K.D. Deane, C. Strickland, J.
1005 Seifert, C. Keeter, D. Simberg, R.I. Scheinman, R. Lau, C. Pitzalis, M.J. Lewis, V.M.
1006 Holers, N.K. Banda, R.A.S.L.E.N. Accelerating Medicines Partnership, Pathotype-
1007 Specific Expression of Granzyme-Perforin Pathway Genes and Their Association With
1008 Clinical Disease Activity in Early Rheumatoid Arthritis and in a Randomized Clinical
1009 Trial, *Immune Netw* 25(4) (2025) e25.

1010 [31] Q. Ma, W. Gu, T. Li, K. Zhang, Y. Cui, K. Qu, N. Wang, R. Humphry, C. Durkan,
1011 J. Qiu, G. Wang, SRGN, a new identified shear-stress-responsive gene in endothelial
1012 cells, *Mol Cell Biochem* 474(1-2) (2020) 15-26.

1013 [32] Q. Liang, J. Wang, Q. Nong, S. Tao, D. Fang, Integration of multi-omics and
1014 machine learning strategies identifies immune related candidate biomarkers in
1015 inflammation-associated hypertrophic cardiomyopathy, *Front Immunol* 16 (2025)
1016 1645382.

1017 [33] Y. Qian, L. Yang, J. Chen, C. Zhou, N. Zong, Y. Geng, S. Xia, H. Yang, X. Bao, Y.
1018 Chen, Y. Xu, SRGN amplifies microglia-mediated neuroinflammation and exacerbates
1019 ischemic brain injury, *J Neuroinflammation* 21(1) (2024) 35.

1020 [34] T. Lv, X. Fan, C. He, S. Zhu, X. Xiong, W. Yan, M. Liu, H. Xu, R. Shi, Q. He,
1021 SLC7A11-ROS/alphaKG-AMPK axis regulates liver inflammation through mitophagy
1022 and impairs liver fibrosis and NASH progression, *Redox Biol* 72 (2024) 103159.

1023 [35] S.M. Zhen, H.R. Zhang, J.X. Si, J.Q. Wang, Y. Zhao, Y.L. Jia, L.H. Liu, [MiR-1-
1024 3p inhibits mitophagy in esophageal squamous cell carcinoma by targeting SLC7A11],
1025 *Zhonghua Zhong Liu Za Zhi* 47(7) (2025) 645-656.

1026 [36] H. Liu, T.A. Zhang, W.Y. Zhang, S.R. Huang, Y. Hu, J. Sun, Rhein attenuates
1027 cerebral ischemia-reperfusion injury via inhibition of ferroptosis through
1028 NRF2/SLC7A11/GPX4 pathway, *Exp Neurol* 369 (2023) 114541.

1029 [37] A. Grosjean, A. Jalon, C. Leveau, M. Diedisheim, D.A. Bejarano, J. Cuenco, K.
1030 Mulder, Z. Liu, A. Le Guernic, M.L. Island, J. Walkiers, G. Ates, C. Materne, A.
1031 Goncalves, I. Nemazanyy, L.G. Baudrin, S. Baulande, M. Ropert, J.F. Gautier, A.
1032 Hamai, A. Schlitzer, F. Ginhoux, A. Massie, N. Venteclef, E. Dalmás, An islet-resident
1033 macrophage antioxidant program preserves beta cell physiology, *Sci Immunol* 10(113)
1034 (2025) eadz5181.

1035 [38] J. Zhang, S. Wang, H. Zhang, X. Yang, X. Ren, L. Wang, Y. Yang, Y. Yang, Y. Wen,
1036 Drp1 acetylation mediated by CDK5-AMPK-GCN5L1 axis promotes cerebral ischemic
1037 injury via facilitating mitochondrial fission, *Mol Med* 30(1) (2024) 173.

1038 [39] L. Yang, Y. Tao, L. Luo, Y. Zhang, X. Wang, X. Meng, Dengzhan Xixin injection
1039 derived from a traditional Chinese herb *Erigeron breviscapus* ameliorates cerebral
1040 ischemia/reperfusion injury in rats via modulation of mitophagy and mitochondrial
1041 apoptosis, *J Ethnopharmacol* 288 (2022) 114988.

1042 [40] L. Wang, X. Zhang, X. Xiong, H. Zhu, R. Chen, S. Zhang, G. Chen, Z. Jian, Nrf2
1043 Regulates Oxidative Stress and Its Role in Cerebral Ischemic Stroke, *Antioxidants*
1044 (Basel) 11(12) (2022).

1045 [41] L. Yang, X. Wei, Z. Liao, B. Chen, G. Zeng, Z. Mei, Role of mitochondria
1046 transmission in ischemic stroke: Friend or foe?, *Redox Biol* 87 (2025) 103868.

1047 [42] M. Dai, T. Lu, J. Li, H. Yu, Neural stem cell-derived exosomal PA2G4 induces
1048 ANXA2 degradation to promote mitophagy and alleviate neuronal oxidative stress in
1049 cerebral ischemia/reperfusion, *Apoptosis* 31(3) (2026).

1050 [43] X. Li, L. Guan, Z. Liu, Z. Du, Q. Yuan, F. Zhou, X. Yang, M. Lv, L. Lv,
1051 Ubiquitination of ATAD3A by TRIM25 exacerbates cerebral ischemia-reperfusion
1052 injury via regulating PINK1/Parkin signaling pathway-mediated mitophagy, *Free Radic*
1053 *Biol Med* 224 (2024) 757-769.

1054 [44] Q. Yao, G. Hu, C. Yin, A. Wu, G. Hu, D. Qin, X. Zhou, B.Y. Law, X. Du, L. Chen,
1055 J. Li, H. Lin, X. Long, J. Wu, L. Yu, Thonningianin A derived from *Penthorum chinense*
1056 Pursh alleviates cerebral ischemia/reperfusion-mediated apoptosis and pyroptosis

through the activation of PINK1/Parkin-dependent mitophagy, *Chin Med* 21(1) (2026)
40.

[45] Y. Wu, Z. Li, T. Ding, Y. Yang, C. Wei, S. Zhang, X. Fan, Bidirectional regulation
of neuronal autophagy in ischemic stroke: Mechanisms and therapeutic potential,
Ageing Res Rev 111 (2025) 102842.

[46] L. Yang, S. Huang, Z. Zhang, Z. Liu, L. Zhang, Roles and Applications of Red
Blood Cell-Derived Extracellular Vesicles in Health and Diseases, *Int J Mol Sci* 23(11)
(2022).

[47] S. Biagiotti, B. Canonico, M. Tiboni, F. Abbas, E. Perla, M. Montanari, M.
Battistelli, S. Papa, L. Casettari, L. Rossi, M. Guescini, M. Magnani, Efficient and
highly reproducible production of red blood cell-derived extracellular vesicle mimetics
for the loading and delivery of RNA molecules, *Sci Rep* 14(1) (2024) 14610.

[48] B. Peng, T.M. Nguyen, M.K. Jayasinghe, C. Gao, T.T. Pham, L.T. Vu, E.Y.M. Yeo,
G. Yap, L. Wang, B.C. Goh, W.L. Tam, D. Luo, M.T. Le, Robust delivery of RIG-I
agonists using extracellular vesicles for anti-cancer immunotherapy, *J Extracell
Vesicles* 11(4) (2022) e12187.

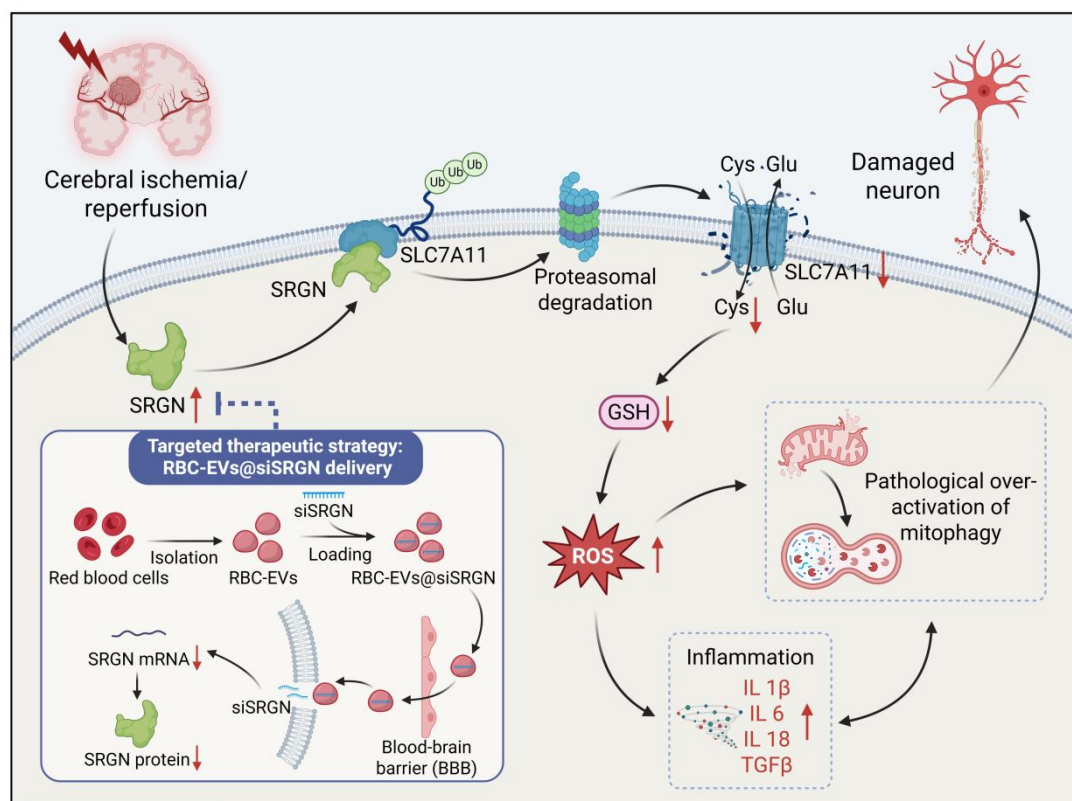
[49] M.K. Jayasinghe, C. Gao, G. Yap, B.Z.J. Yeo, L.T. Vu, D.J.W. Tay, W.X. Loh, Z.Q.
Aw, H. Chen, D.C. Phung, D.V. Hoang, R.C. Prajogo, L. Hooi, F.Q. Lim, M. Pirisinu,
C.K. Mok, K.W. Lim, S.J. Tang, K.S. Tan, E.K. Chow, L. Chen, A.T. Phan, J.J.H. Chu,
M.T. Le, Red Blood Cell-Derived Extracellular Vesicles Display Endogenous Antiviral
Effects and Enhance the Efficacy of Antiviral Oligonucleotide Therapy, *ACS Nano*
17(21) (2023) 21639-21661.

[50] B.W.S. Lam, M. Tan, C. Gao, T.T. Pham, L.T.N. Tran, L.N. Nguyen, H. Sidik, M.B.H. Lim, A.H. Le, T.T.T. Nguyen, N. Lim, L. Nguyen, T.H. Nguyen, Y.H. Loh, M.W. Usman, M.T.N. Le, Extracellular Vesicles Administered via Intrathecal Injection Mediate Safe Delivery of Nucleic Acids to the Central Nervous System for Gene Therapy, *J Extracell Vesicles* 14(7) (2025) e70116.

[51] Y. Su, B. Huang, D. Liu, L. Yu, Y. Chen, Cell-Membrane-Camouflaged Biomimetic Nanomedicines for Cerebral Ischemia-Reperfusion Injury Treatment, *Adv Healthc Mater* 15(4) (2026) e02936.

[52] Z. Zhang, S. Li, Y. Zhang, R. Xu, J. Zhou, Y. Wang, Y. Yan, NUP85 siRNA loaded red blood cell-derived extracellular vesicles alleviate hepatic steatosis in MASLD, *J Nanobiotechnology* 24(1) (2025) 63.

Figure legends



Graphical Abstract. SRGN-driven SLC7A11 depletion triggers excessive mitophagy-dependent neuronal death in cerebral ischemia/reperfusion injury and therapeutic intervention via RBC-EVs-delivered siSRGN

Cerebral ischemia-reperfusion-induced upregulation of SRGN expression accelerates the proteasomal degradation of SLC7A11, causing GSH exhaustion, ROS accumulation, pathological mitophagy and inflammatory activation, which contribute to neuronal damage. RBC-EVs-encapsulated siSRGN penetrates the blood-brain barrier to suppress SRGN expression and alleviate the aforementioned pathological cascade.

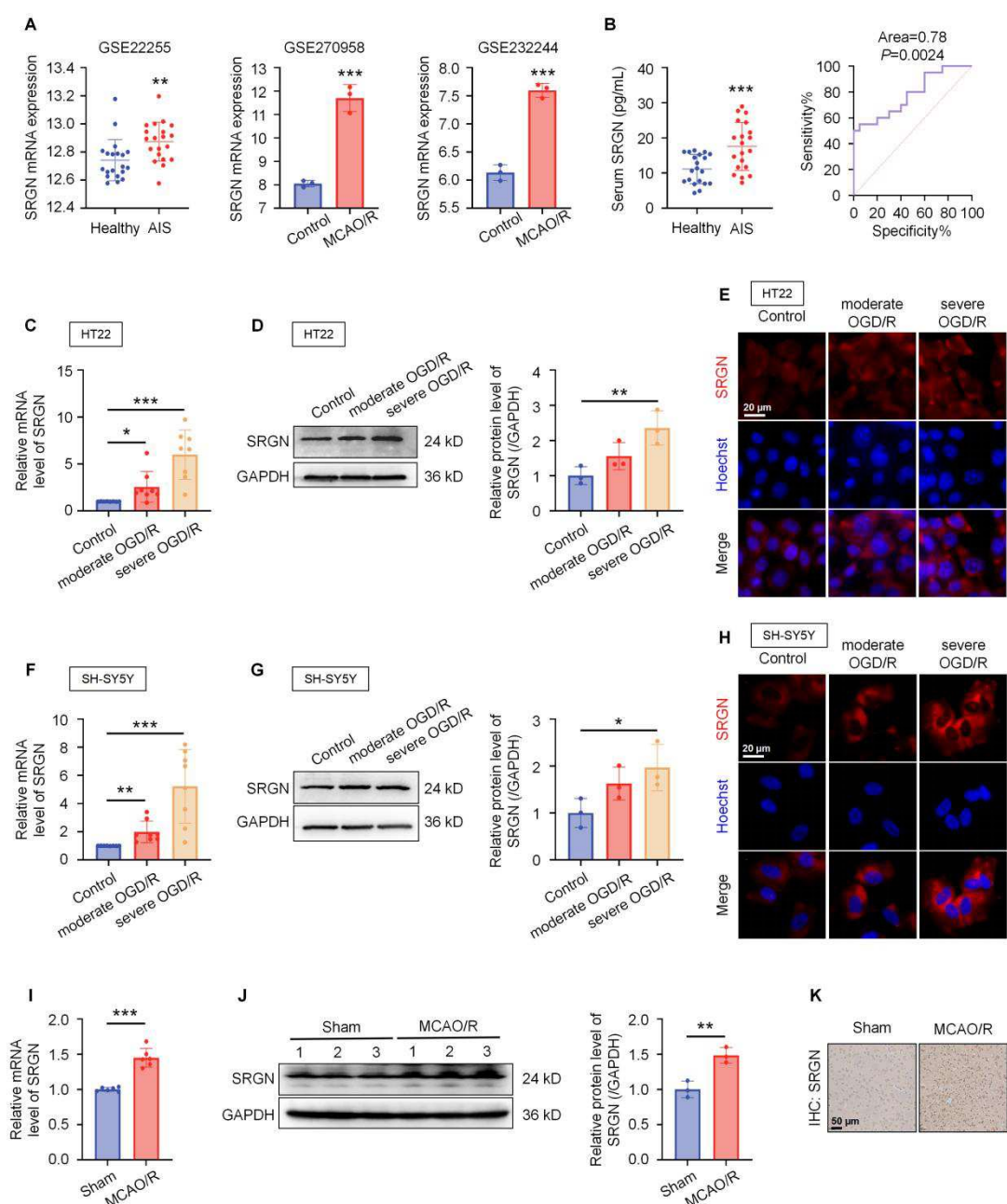


Figure 1. SRGN expression is upregulated in cerebral ischemia/reperfusion injury

A. Analysis of SRGN gene expression levels in 3 datasets (GSE22255, GSE270958, GSE232244) from the GEO database. B. Enzyme-linked immunosorbent assay (ELISA) results and receiver operating characteristic (ROC) curve analysis of serum SRGN protein in healthy controls and patients with CI/RI (n=20). C-E. qPCR (n=8), Western blot (n=3), and immunofluorescence (scale bar: 20 μ m) results of SRGN expression in normal, moderate, and severe OGD/R-injured HT22 cell models. F-H. qPCR (n=8), Western blot (n=3), and immunofluorescence (scale bar: 20 μ m) results of SRGN

expression in normal, moderate, and severe OGD/R-injured SH-SY5Y cell models. I-K. qPCR (n=6), Western blot (n=3), and immunohistochemical (scale bar: 50 μ m) results of SRGN expression in Sham and MCAO/R mice models. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

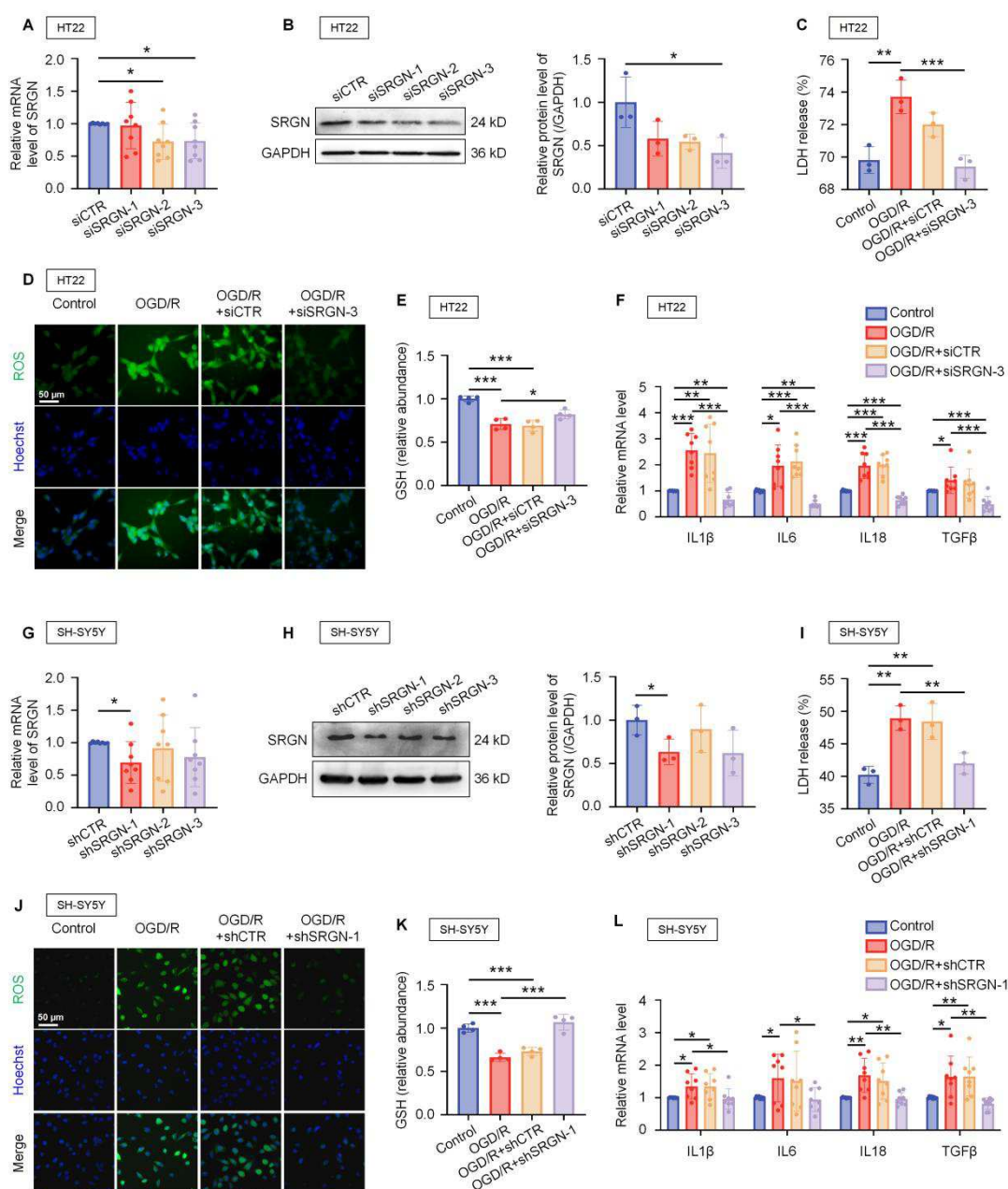


Figure 2. Knockdown of SRGN effectively alleviates OGD/R-induced neuronal

injuries

A-B. SRGN mRNA and protein expression levels detected by qPCR (n=8) and Western blot (n=3) after transfection with siCTR and different siSRGN sequences. C. HT22 cell injury degree in each group detected by LDH release assay (n=3). D. Intracellular ROS levels in HT22 cells across groups detected by ROS fluorescence staining (scale bar: 50 μ m). E. Oxidative stress status of HT22 cells in each group evaluated by GSH content detection (n=4). F. mRNA expression levels of inflammatory factors IL 1 β , IL 6, IL 18 and TGF β in HT22 cells detected by qPCR (n=8). G-H. SRGN mRNA and protein expression levels detected by qPCR (n=8) and Western blot (n=3) after transfection with shCTR and different shSRGN constructs. I. SH-SY5Y cell injury in each group detected by LDH release assay (n=3). J. Intracellular ROS levels in SH-SY5Y cells across groups detected by ROS fluorescence staining (scale bar: 50 μ m). K. Oxidative stress status of SH-SY5Y cells in each group evaluated by GSH content detection (n=4). L. mRNA expression levels of inflammatory factors IL 1 β , IL 6, IL 18 and TGF β in SH-SY5Y cells detected by qPCR (n=8). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

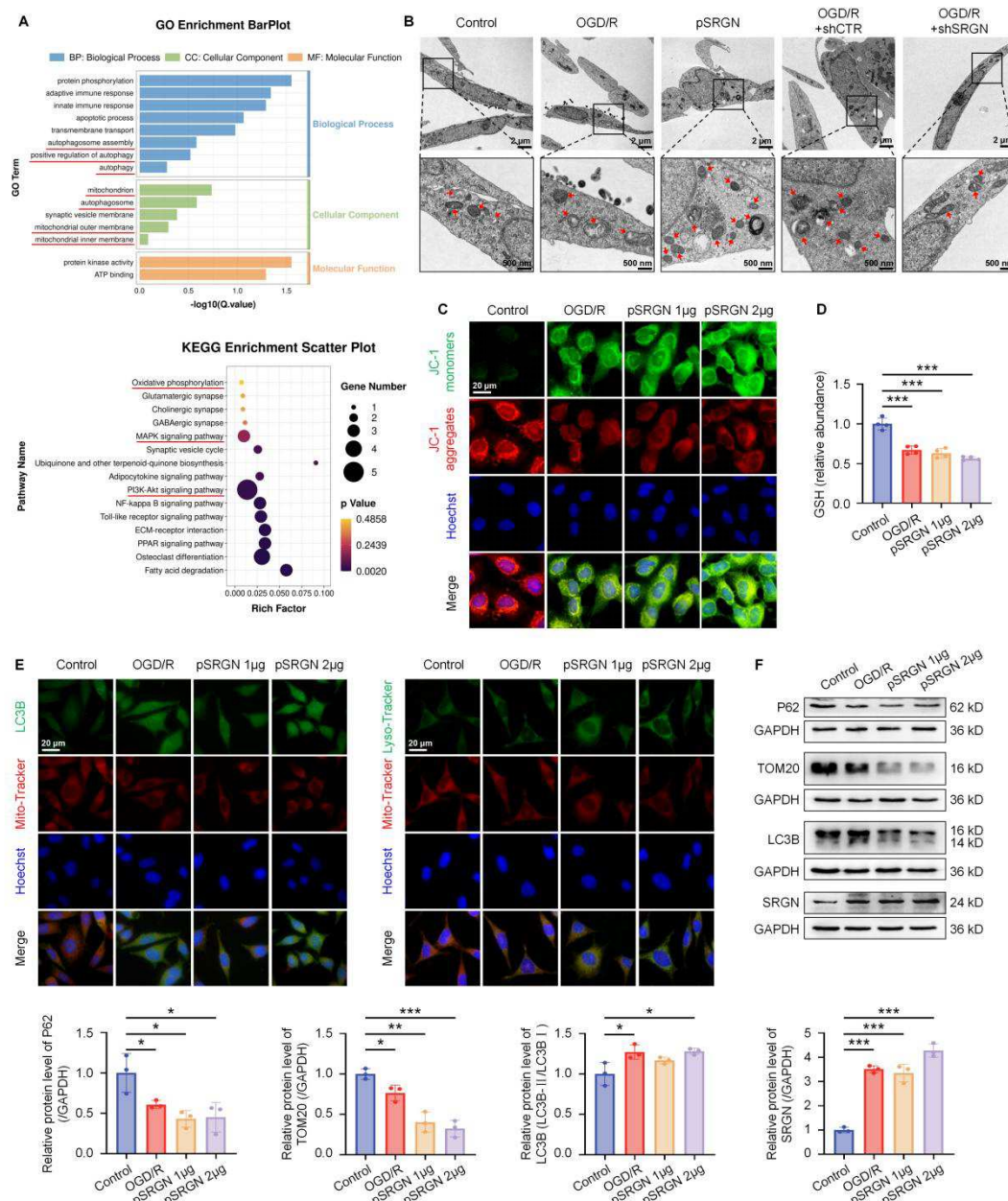


Figure 3. SRGN drives excessive mitophagy in neurons after OGD-R injury

A. GO enrichment bar chart showing enriched terms of differentially expressed genes (DEGs) in biological process (BP, blue), cellular component (CC, green), and molecular function (MF, orange), with the horizontal axis representing $-\log_{10}(Q.value)$; higher values indicate more significant enrichment. In the KEGG enrichment bubble chart, the horizontal axis represents the Rich Factor, the dot size represents the number of DEGs, and the color reflects enrichment significance. B. Transmission electron microscopy (TEM) observation of mitophagosomes in SH-SY5Y cells of each group (red arrows

indicate mitophagosomes; scale bars: 2 μm for the upper panel, 500 nm for the magnified panel). C. MMP of SH-SY5Y cells in each group was detected by JC-1 staining (scale bar: 20 μm). D. Statistical results of the relative GSH content in SH-SY5Y cells of each group ($n = 4$). E. Immunofluorescence colocalization of Mito-Tracker with LC3B and Lyso-Tracker was used to observe the binding of mitochondria with autophagosomes and lysosomes (scale bar: 20 μm). F. Western blot detection of the expression levels of P62, TOM20, LC3B, and SRGN proteins and their statistical quantitative analysis results ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

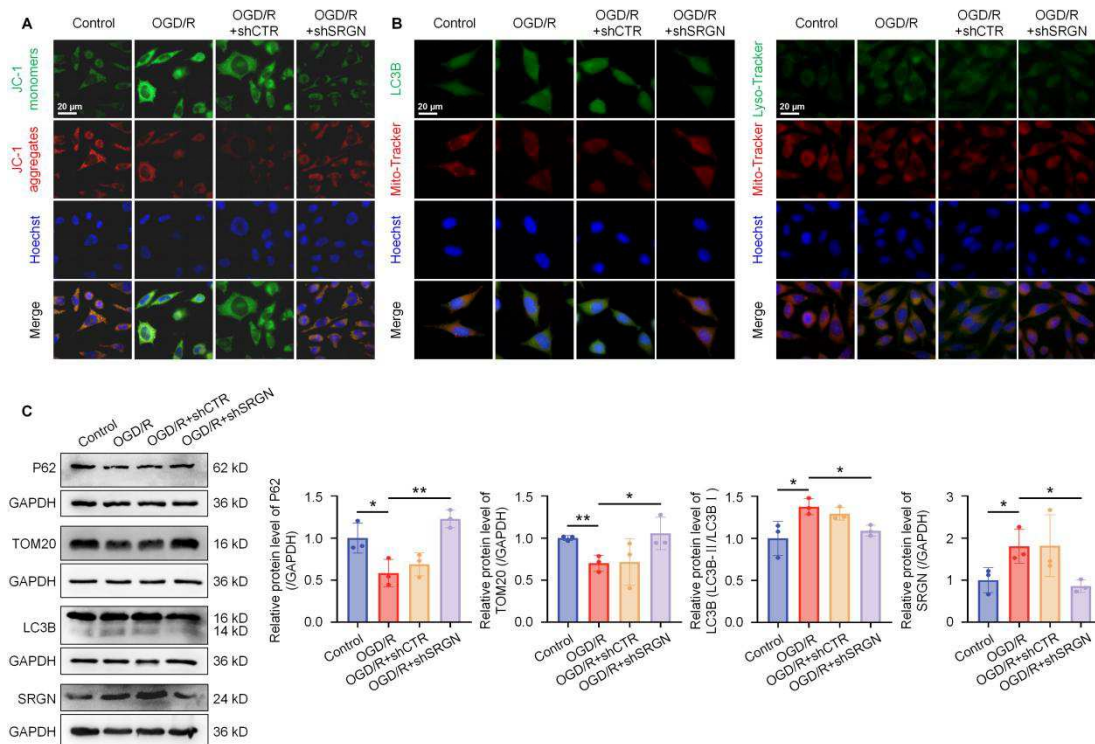


Figure 4. SRGN knockdown attenuates OGD/R-induced excessive mitophagy

A. In SH-SY5Y cells, following SRGN knockdown, MMP of SH-SY5Y cells in each group was detected by JC-1 staining (scale bar: 20 μm). B. Immunofluorescence colocalization of Mito-Tracker with LC3B and Lyso-Tracker was used to observe the binding of mitochondria with autophagosomes and lysosomes (scale bar: 20 μm). C. Western blot detection of the expression levels of P62, TOM20, LC3B, and SRGN

proteins and their statistical quantitative analysis results (n = 3). * $P < 0.05$; ** $P < 0.01$.

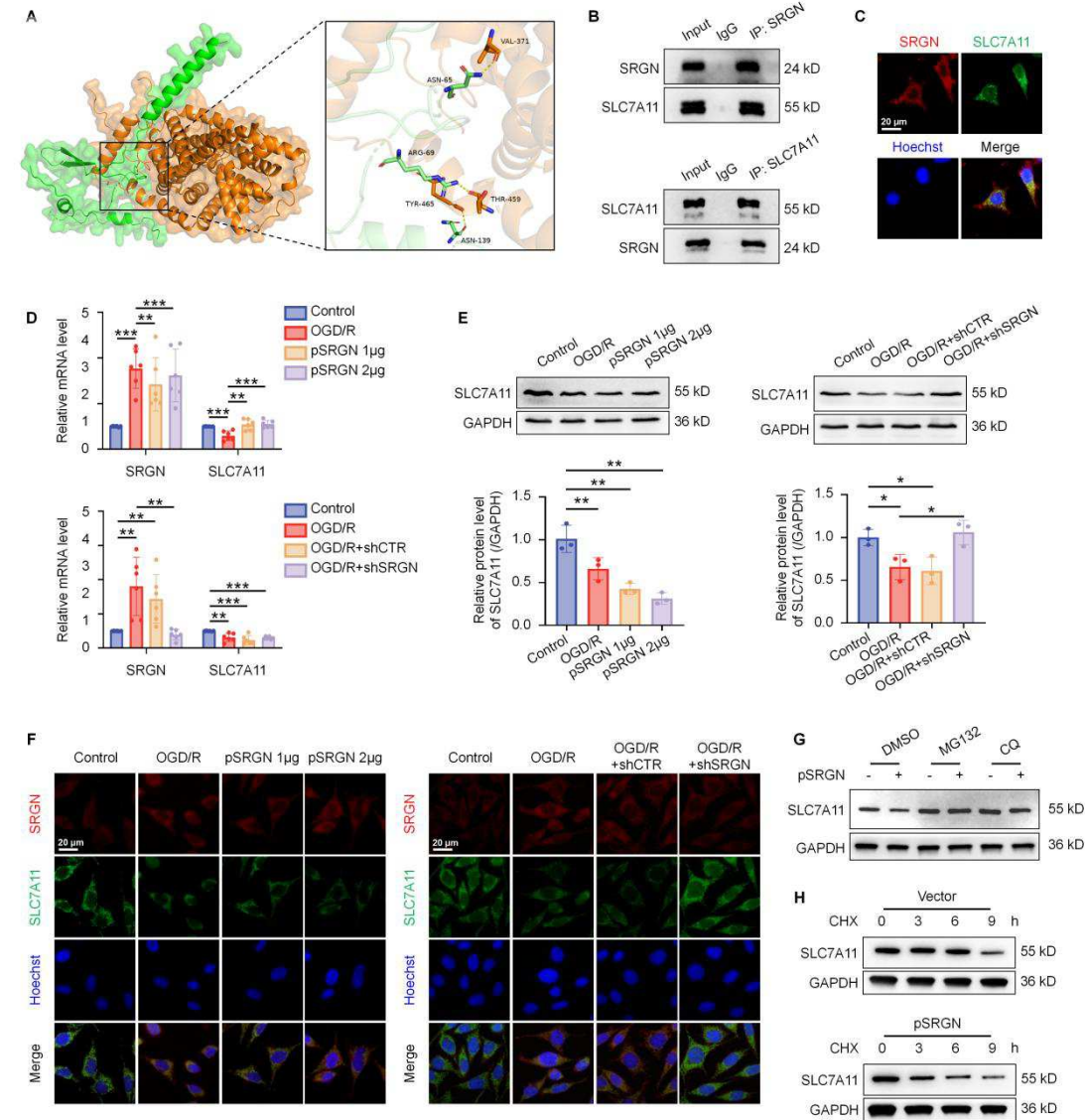


Figure 5. SRGN interacts with SLC7A11 at the protein level and negatively regulates SLC7A11 stability through the proteasome pathway

A. Protein-protein docking simulation predicts the binding interface and key amino acid residues between SRGN and SLC7A11. B. Co-IP assay verifies the endogenous protein interaction between SRGN and SLC7A11 in SH-SY5Y cells. C. Immunofluorescence staining shows the colocalization of SRGN (red) and SLC7A11

(green) in neurons (scale bar: 20 μ m). D. qRT-PCR detection of SRGN and SLC7A11 mRNA expression levels after SRGN overexpression or knockdown (n=6). E. Western blot detection and quantitative analysis of the effect of SRGN overexpression or knockdown on SLC7A11 protein expression (n=3). F. After SRGN overexpression or knockdown, the expression and localization of SRGN and SLC7A11 proteins within cells were observed by immunofluorescence staining (scale bar: 20 μ m). G. Western blot detection of SLC7A11 protein expression in SH-SY5Y cells with/without SRGN overexpression treated with the proteasome inhibitor MG132 (10 μ M), the lysosome inhibitor CQ (50 μ M), or DMSO, respectively (n=3). H. CHX (20 μ M) chase assay measures SLC7A11 protein degradation with/without SRGN overexpression (n=3). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

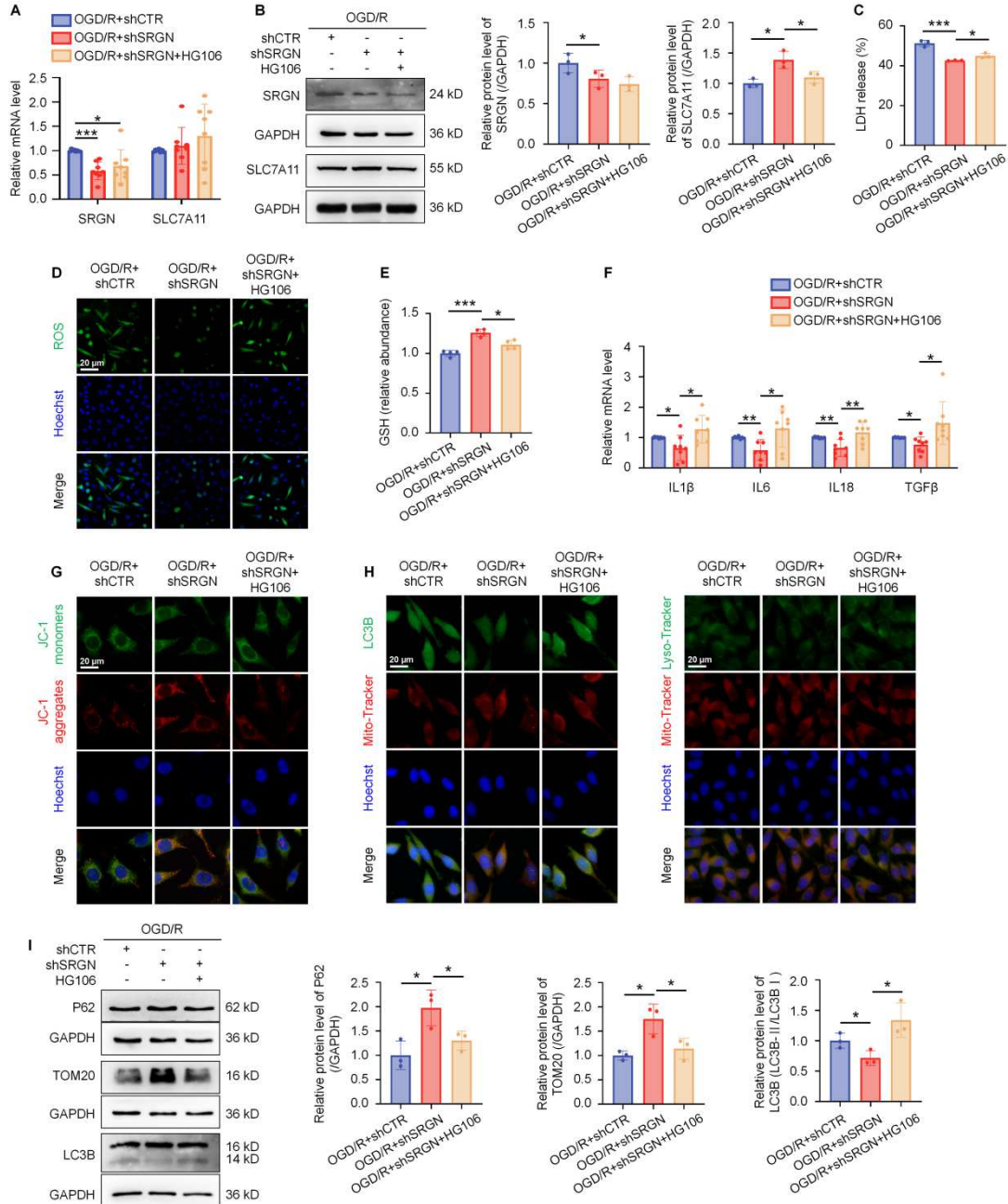


Figure 6. HG106 treatment reverses the neuroprotective effect produced by SRGN knockdown

A. qRT-PCR detection of the relative mRNA expression levels of SRGN and SLC7A11 in SH-SY5Y cells of each group (n = 8). B. Western blot detection and quantitative statistics of the protein expression levels of SRGN and SLC7A11 in cells of each group (n = 3). C. LDH release quantification to detect the degree of cell damage in each group (n = 3). D. Intracellular ROS levels detected by ROS fluorescence staining (scale bar:

20 μm). E. GSH content detection to assess the oxidative stress status of cells ($n = 4$).
F. qPCR detection of the expression levels of inflammatory factors IL 1 β , IL 6, IL 18,
and TGF β in cells of each group ($n = 8$). G. JC-1 staining to assess MMP (scale bar: 20
 μm). H. Immunofluorescence colocalization of Mito-Tracker with LC3B and Lyso-
Tracker to observe the binding of mitochondria with autophagosomes and lysosomes
(scale bar: 20 μm). I. Western blot detection and quantitative statistics of the protein
expression levels of P62, TOM20, and LC3B in cells of each group ($n = 3$). * $P < 0.05$;
** $P < 0.01$; *** $P < 0.001$.

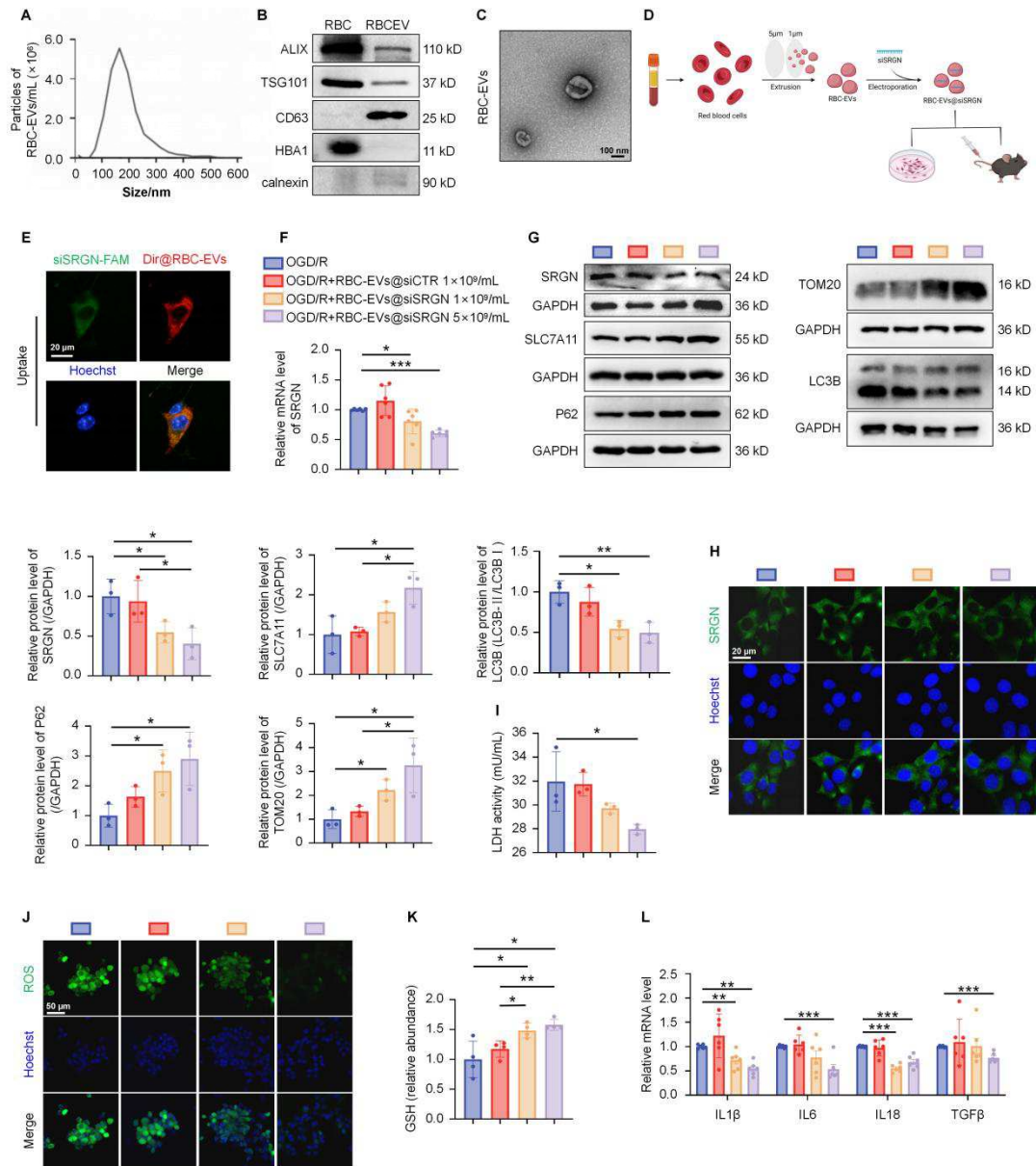


Figure 7. RBC-EVs@siSRGN alleviates OGD/R-induced HT22 neuronal injury in vitro

A. NTA detecting the particle size distribution and particle concentration of RBC-EVs.

B. Western blot detecting the extracellular vesicle markers ALIX, TSG101, and CD63, the RBC marker HBA1, and the negative endoplasmic reticulum marker Calnexin.

C. TEM images of RBC-EVs (scale bar: 100 nm).

D. Schematic diagram of the construction of RBC-EVs@siSRGN, cellular delivery, and subsequent in vitro and in vivo experimental procedures.

E. FAM fluorescent-labeled siRNA uptake assay verifying the internalization and uptake of RBC-EVs by HT22 cells (scale bar: 20 μ m).

F. qRT-PCR detecting SRGN mRNA levels after treatment with gradient concentrations of RBC-EVs@siSRGN (n=6). G. Western blot and quantitative analysis of the protein levels of SRGN, SLC7A11, P62, TOM20, and LC3B (n=3). H. Immunofluorescence staining verifying the SRGN silencing efficiency of RBC-EVs@siSRGN (scale bar: 20 μ m). I-K. LDH release (n=3), ROS (scale bar: 50 μ m), GSH (n=4), and mRNA expression of the pro-inflammatory and anti-inflammatory factors IL 1 β , IL 6, IL 18, and TGF β (n=6) in OGD/R-injured HT22 cells treated with RBC-EVs@siSRGN. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

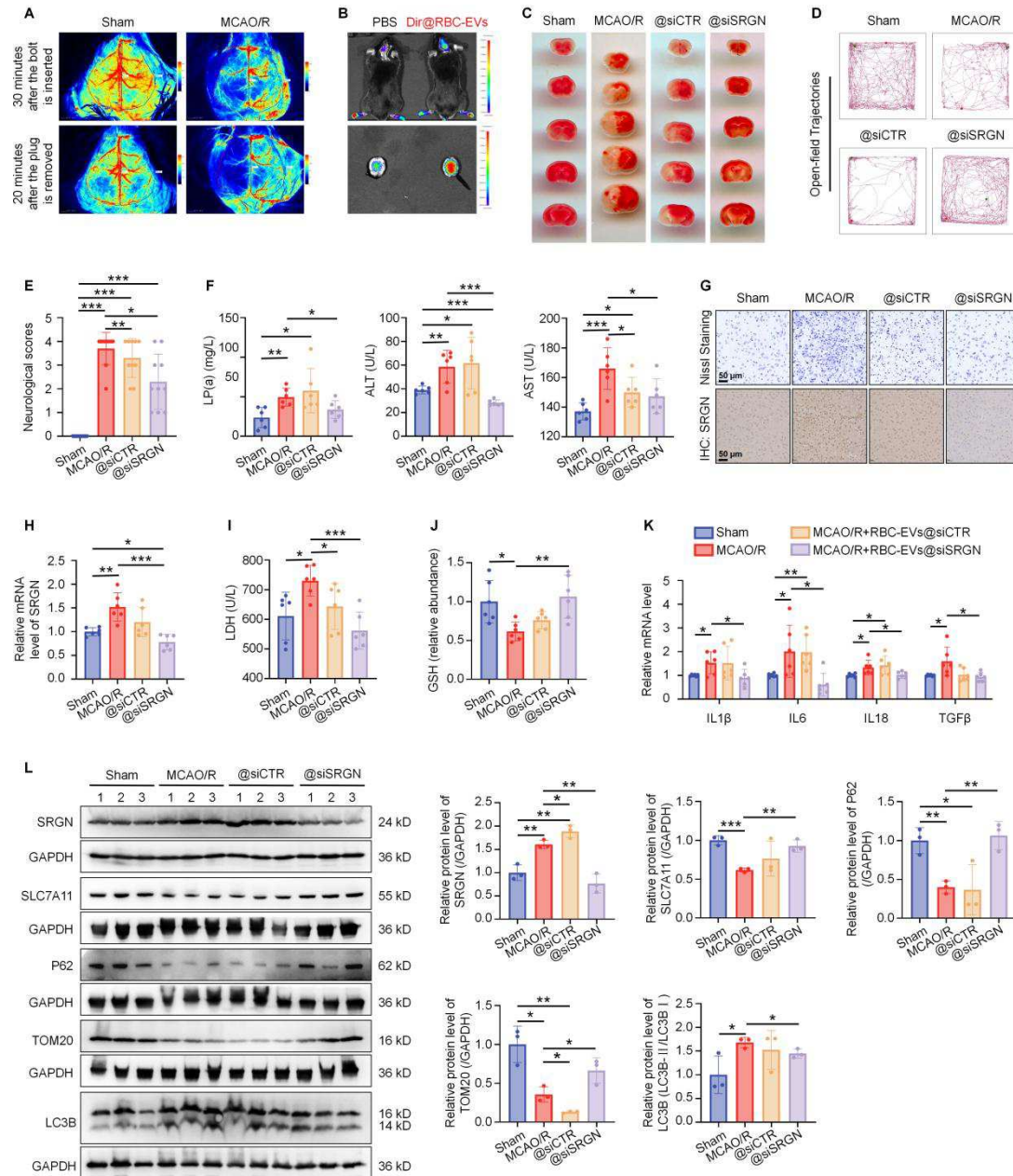


Figure 8. RBC-EVs@siSRGN ameliorates MCAO/R-induced CI/RI in mice in vivo

A. Laser speckle cerebral blood flow imaging to record the representative images of cerebral blood flow distribution in Sham and MCAO/R model mice at two time points: 30 minutes after filament insertion (ischemic phase) and 20 minutes after filament withdrawal and reperfusion (reperfusion phase). Colors represent blood flow levels, with red/yellow indicating high perfusion and blue indicating low perfusion. B. Intravital fluorescence imaging verifying the distribution and localization of Dir-

labeled RBC-EVs in mice. C. TTC staining detecting cerebral infarct volume in each group of mice at 24 hours after MCAO/R. D. Representative open-field trajectories of mice on day 7 after MCAO/R. E. Neurological deficit assessment of mice 24 hours after MCAO/R (n = 10). F. Quantitative detection of serum injury markers LP, ALT, and AST (n = 6). G. Nissl staining of the striatum region of mouse brain tissue for observation of neuronal injury (scale bar: 50 μ m) and immunohistochemistry for observation of SRGN expression (scale bar: 50 μ m). H. qRT-PCR (n = 6) verifying the silencing efficiency of SRGN in brain tissue. I. Quantitative detection of serum LDH activity to evaluate neuronal injury (n = 6). J. Detection of relative GSH content in brain tissue to reflect the level of oxidative stress (n = 6). K. qRT-PCR detecting mRNA expression of inflammatory factors IL 1 β , IL 6, IL 18, and TGF β in brain tissue (n = 6). L. Western blot and quantitative analysis of SRGN, SLC7A11, P62, TOM20, and LC3B expression in brain tissue (n = 3). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. @siCTR: MCAO/R+RBC-EVs@siCTR; @siSRGN: MCAO/R+RBC-EVs@siSRGN.

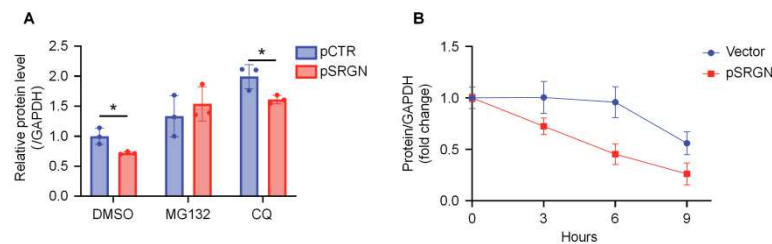


Figure S1. SRGN promotes SLC7A11 protein degradation through the proteasome pathway

A. Quantification of SLC7A11 protein expression in SH-SY5Y cells with/without SRGN overexpression treated with the proteasome inhibitor MG132 (10 μ M), the lysosome inhibitor CQ (50 μ M), or DMSO (n=3). B. Quantification of SLC7A11 protein degradation with/without SRGN overexpression tracked by CHX (20 μ M) chase assay (n=3). * $P < 0.05$.

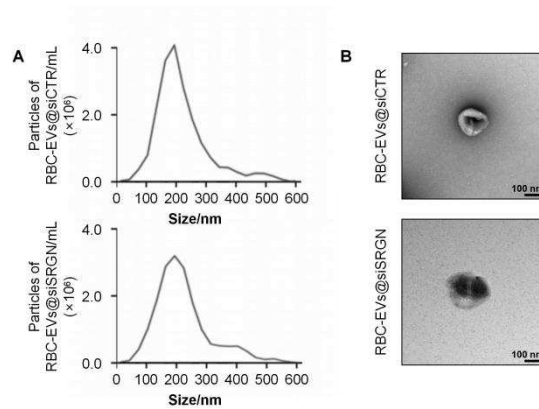


Figure S2. Validation of RBC-EVs@siCTR and RBC-EVs@siSRGN

A. NTA of the particle size distribution and particle concentration of RBC-EVs@siCTR and RBC-EVs@siSRGN. B. TEM observation of the vesicle morphology of RBC-EVs@siCTR and RBC-EVs@siSRGN (scale bar: 100 nm).

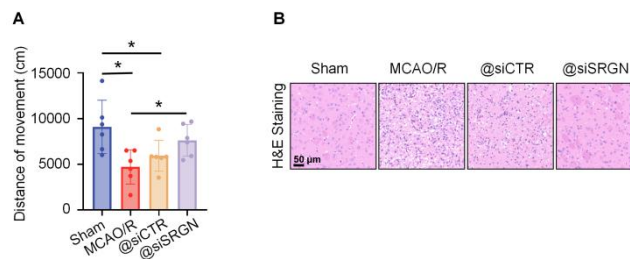


Figure S3. Inhibition of SRGN ameliorates neurobehavioral deficits and brain tissue pathology in MCAO/R mice

A. Statistical analysis of total distance of movement in the open field test in mice on day 7 after MCAO/R (n = 6). B. H&E staining of the striatal region of mouse brain tissue to observe brain tissue pathological injury (scale bar: 50 μm). * $P < 0.05$. @siCTR: MCAO/R+RBC-EVs@siCTR; @siSRGN: MCAO/R+RBC-EVs@siSRGN.

Supplementary Files

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