COP9 Signalosome Subunit 3 Restricts Neuroinflammatory Responses During Cerebral Ischemia/Reperfusion Injury Through Stabilizing Suppressor of Cytokine Signaling 3 Protein

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Background: The suppressor of cytokine signaling 3 (SOCS3) is a specific negative regulator of signal transducer and activator of transcription 3 (STAT3) signaling, which is predominantly activated to induce neuroinflammatory response in microglia and functions essential roles during cerebral ischemia-reperfusion (I/R) injury. Constitutive photomorphogenesis 9 (COP9) signalosome (CSN) is a signaling platform controlling protein stability by remodeling of cullin-RING ubiquitin ligases, which is recently reported to specifically recognize proteins with SOCS-box domains. However, whether SOCS3 is related to COP9 signalosome in neuroinflammation during cerebral I/R injury is completely unclear.

Methods: Mice subjected to transient middle cerebral artery occlusion (MCAO) and reperfusion, and BV2 microglia cells treated with oxygen-glucose deprivation and reoxygenation (OGD/R) were used to mimic cerebral I/R injury. Western blot, qRTPCR, immunofluorescence, and co-Immunoprecipitation assays were performed to explore the regulatory mechanism of SOCS3 on neuroinflammation and the relationship of SOCS3 and COP9 signalosome during cerebral I/R injury.

Results: SOCS3 expression is significantly upregulated in microglia during OGD/R treatment, and overexpression of SOCS3 suppresses OGD/R-induced STAT3 activation and inflammatory factor expression. Furthermore, we find that COP9 signalosome subunit 3 (CSN3) interacts with SOCS3 protein to enhance its stability, thereby resulting in restricting OGD/R-induced STAT3 activation and inflammatory response. Moreover, we find that knockdown of CSN3 evidently accelerates STAT3 activation, and aggravates cerebral I/R injury in vivo.

Conclusion: CSN3 restricts neuroinflammatory responses during cerebral I/R injury through stabilizing SOCS3 protein and indicates that CSN3 a potential therapeutic target for cerebral I/R injury.

Keywords: cerebral ischemia-reperfusion injury, neuroinflammation, suppressor of cytokine signaling 3, constitutive photomorphogenesis 9 signalosome, signal transducer and activator of transcription 3

Introduction
Ischemic stroke is a major cause of disability and death all over the world.1,2 Nowadays, thrombolysis treatment is a common therapeutic strategy for patients with acute cerebral ischemia in clinical, whereas recanalization of occluded arteries usually leads to a secondary injury, namely cerebral ischemia-reperfusion (I/R)
injury. Many studies have reported that local excessive neuroinflammatory response in brain tissue is an essential pathogenesis of cerebral I/R. Thus, understanding the underlying mechanisms of neuroinflammation during cerebral I/R injury is important for developing new drugs for cerebral I/R therapy.

Microglia, as a resident macrophage in the brain, is activated during cerebral I/R process and produces many pro-inflammatory factors, such as tumor necrosis factor (TNFα) and interleukin-6 (IL-6), to aggravate brain injury. The signal transducer and activator of transcription 3 (STAT3), an important transcriptional regulator of inflammatory gene, is phosphorylated and activated in macroglia by various stimuli, and induces pro-inflammatory immune reaction, such as IL-6, in response to various central nervous system insults, including cerebral I/R injury. Excreted IL-6 binds to the soluble IL-6 receptor on the cell membrane, subsequently resulting in STAT3 phosphorylation, and induction of IL-6 expression, which forms a positive feedback to intensify neuroinflammation, suggesting that STAT3 as a new therapeutic target for cerebral I/R injury. The suppressor of cytokine signaling (SOCS) 3 is found as a specific negative regulator of STAT3 signaling by inhibiting STAT3 phosphorylation. In addition, activation of SOCS3-STAT3 signaling by G4-humanin has been reported to have neuroprotective effects on cerebral I/R injury in rats. However, how SOCS3 is regulated in neuroinflammation during cerebral I/R injury is still not clear.

The constitutive morphophosphosome 9 (COP9) signalosome (CSN) is an evolutionarily conserved multi-protein complex, consisting of eight subunits termed CSN1-CSN8, and is a signaling platform controlling the cellular ubiquitylation by remodeling cullin-RING ubiquitin ligases and interacting with deubiquitylating enzymes to rescue ubiquitylated proteins from degradation. COP9 signalosome is reported to be involved in regulating cell cycle DNA repair, and inflammatory gene expression through controlling protein degradation via the ubiquitin-proteasome system. For example, COP9 signalosome subunit 3 (CSN3), binds transcriptional factor interferon regulatory factor 5 to maintain its stability. CSN3 interacts with ATP-binding cassette transporters A1 (ABCA1) to mediate ABCA1 stability to promote cholesterol efflux. Recently, proteins with SOCS-box domains could be specifically recognized by cullin-RING ubiquitin ligases, which is regulated by COP9 signalosome. Thus, we suspected whether SOCS3 is associated with COP9 signalosome in neuroinflammation during cerebral I/R injury.

In this study, the regulatory mechanism of SOCS3 and the relationship between SOCS3 and COP9 signalosome in neuroinflammation during cerebral I/R injury were investigated using BV2 microglia cells treated with oxygen-glucose deprivation and reoxygenation (OGD/R) in vitro and mice subjected to focal cerebral I/R in vivo. This study firstly indicates CSN3 restricts neuroinflammatory responses during cerebral I/R injury through stabilizing SOCS3 protein.

Materials and Methods

Cell Culture and Oxygen–Glucose Deprivation/Reoxygenation (OGD/R) Treatment

Mouse microglial BV-2 cells were obtained from ATCC (Rockville, USA) and cultured in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO2 in a humidified incubator. For OGD/R treatment, after 80% confluence, the cells were switched from a normal feeding medium containing serum and glucose to an oxygen-glucose-deprived medium, and incubated in a hypoxic chamber with CO2/N2 (5%/95%) for 3 h. The cells were then returned to the normal feeding medium and incubated under normal conditions for different hours as reperfusion.

Small Interfering RNA (siRNA) and Plasmid Transfection

siRNA against SOCS3 (si-SOCS3, 5'-CAAGACCUUCA GCUCCAGTT-3'), and siRNA negative control (si-NC, 5'-UUCUCCGAACGUGUCAGCTT-3') were synthesized by Ribobio (Guangzhou, China). pCMV3-N-FLAG-SOCS3, pCMV3-N-Mye-CSN3 plasmids were purchased from SiboBiological (Beijing, China). siRNAs or plasmids were transfected into BV2 cells with Lipofectamine 3000 (Invitrogen), as the manufacturers’ instructions described.

Immunofluorescence

After OGD/R-0h/24h treatment, BV2 cells were fixed with 4% paraformaldehyde and then permeabilized with Triton X-100. Subsequently, cells were incubated with anti-SOCS3 antibody (ab14939, Abcam) at 4°C overnight, and then incubated with secondary antibodies [goat anti-mouse Alexa Fluor 594 (A11032, Invitrogen)] at room temperature for 1 h. Nuclei were stained with DAPI.
(Roche, USA) for 10 min at room temperature. The cells were examined with a fluorescence microscope (Nikon TE300, Japan).

**Quantitative Real-Time PCR (qPCR)**

After treatment, total RNA of the BV-2 cells was isolated with TRIzol, and then was reverse-transcribed into cDNA using a reverse transcriptase kit (Promega, USA). qPCR was performed using SYBR green PCR Master Mix (Applied Biosystems, USA). The relative mRNA expressions were calculated by 2^ΔΔCT method. The qPCR primers were as follows: IL-6, Forward5′-GTACTCCAGA AGACCAGGG-3′; Reverse5′-TGCTGGGT ACAACCA CCGCC-3′; SOCS3, Forward5′-ATGGTCACCACACGC AAGTTT-3′; Reverse5′-TCCAGTAGAACCGGTCTGCC T-3′; Cyclin D1, Forward5′-ATGGAAGGACCCCTTGGAG C-3′; Reverse5′-CTTCACCAGGCTTCTGTCCT-3′; myeloid cell leukemia sequence 1 (Mcl1), Forward5′-AAAG GCAGGTCACTACTGTC-3′; Reverse5′-TGGCGGTATAG GTCGCCTCTC-3′; GAPDH, Forward 5′-GCCGAAGGT TGAGCAAGGT-3′; Reverse5′-TCTCCAGGCGGCACGT CAGA-3′;

**Protein Extraction and Western Blot**

After treatments, BV2 cells were collected and lysed using NP40 lysis buffer (Beyotime Biotechnology, China) on ice. Lysates were quantified by the BCA kit (Beyotime Biotechnology, China), separated by 10% SDS-PAGE gel and then transferred onto polyvinylidene difluoride membranes. Membranes were blocked, incubated sequentially with primary and secondary antibodies. The immunoblots were detected using chemiluminescence (ECL Plus detection system). Bands were quantified using Image J software. The primary antibodies used were as follows: SOCS3 (1:1000, ab14939, Abcam), STAT3 (1:1000, ab68153, Abcam), phosphorylated STAT3 (pSTAT3, 1:1000, ab76315, Abcam), flag (1:1000, 80010-1-RR, ProteinTech), GAPDH (1:1000, 60004-1-Ig, ProteinTech), Myc (1:1000, 16286-1-AP, ProteinTech), CSN2 (1:1000, 10969-2-AP, ProteinTech), CSN3 (1:1000, ab229807, Abcam), CSN4 (1:1000, 10464-1-AP, ProteinTech), CSN5 (1:1000, 27511-1-AP, ProteinTech), CSN6 (1:1000, ab77299, Abcam), CSN7 (1:1000, ab133548, Abcam), CSN8 (1:1000, 10089-2-AP, ProteinTech). The secondary antibodies used in our work were as follows: goat anti-rabbit IgG-HRP (1:3000, SA00001-2, ProteinTech), goat anti-mouse IgG-HRP (1:3000, SA00001-1, ProteinTech).

**Protein Stability Assay**

After OGD/R-24h treatment, BV2 cells were treated with 50 μg/mL CHX (cycloheximide, Beyotime Biotechnology, China), 10 μM MG132 (Beyotime Biotechnology, Chin) or DMSO for 24 h, and then collected for the following experiments.

**Co-Immunoprecipitation (Co-IP)**

BV-2 cells were transfected with 4 μg pCMV3-N-FLAG-SOCS3 and pCMV3-N-Myc-CSN3 plasmids for 48 h. Cell lysates were incubated with an anti-Flag antibody overnight at 4°C, and then incubated with protein A/G-agarose beads for 4 h at 4°C. The immunoprecipitated proteins were separated by 10% SDS-PAGE and detected by Western blot with anti-Myc or anti-Flag antibodies. For endogenous immunoprecipitations, non-transfected cell lysates were incubated with anti-SOCS3 antibodies or IgG (control). Western blot was performed using anti-CSN2/3/4/5/6/7 antibodies.

**Middle Cerebral Artery Occlusion (MCAO) Surgery**

Experiments were performed under a project license (No.20200810001) granted by institutional ethics board of Guangzhou University of Chinese Medicine. MCAO surgery was performed as previous description. In short, mice were anesthetized with 4% isoflurane, and then the focal cerebral ischemia was produced by intraluminal occlusion of the right middle cerebral artery using a silicone coated nylon (6.0) monofilament. After 60 min, the occluding filament was withdrawn to allow blood reperfusion for 24 h. Then, the mice were used for further study.

**Lentiviral Gene Transfer into Mouse Brains**

Briefly, mice were kept immobile on a stereotactic apparatus under 4% isoflurane. Then, 3 μL of lentivirus solution (Lenti-shCSN3, or Lenti-NC 4.0×10^5 IU, obtained from Genechem) was directly injected (0.2 μL/min) into the right striatum at 2 mm lateral and 0.8 mm anterior to the bregma at a depth of 3.0 mm with a Stoelting injection system, as previously described. At 10 days after viral vector injection, the cerebral I/R operation was prepared using these mice.

**Neurobehavioral Assessment**

Neurological function was assessed by the modified neurological severity scores (mNSS), according to previous description. mNSS is graded on a scale of 0 to 18.
(normal score, 0; maximal deficit score, 18). The higher score, the more severe is the injury.

Measurement of Infarct Size
Two percent of TTC (Sigma, USA) staining was conducted to evaluate the infarct volume, as previously described, and infarct volume was quantified using Image J.

Immunohistochemistry
Mice were anesthetized and intracardially perfused with 0.9% sodium chloride followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% PFA overnight at 4°C. Coronal brain sections (10 μm) were cut on a freezing microtome. The sections were stained overnight at 4°C using anti-CSN3 antibody (1:50, ab229807, Abcam) or anti-SOCS3 (1:50, ab14939, Abcam). Then, the sections were incubated using an appropriate secondary antibody for 1 h at room temperature, and the sections were imaged by microscope.

Statistical Analysis
Differences between two groups or multiple groups were analyzed by Student’s t-test or one-way analysis of variance (ANOVA), respectively. Statistical analyses were performed using R software (v 3.4.2) and GraphPad Prism software (v 8.00). P value <0.05 was considered statistically significant.

Results
SOCS3 Suppresses OGD/R-Induced STAT3 Activation and Inflammatory Factor Expression in BV-2 Microglia Cells
To explore the roles of SOCS3 in neuroinflammatory responses during cerebral I/R, we firstly detected SOCS3 protein expressions in BV-2 microglia cells under OGD/R treatment. As shown in Figure 1A, OGD/R treatment significantly increased SOCS3 protein expression in microglia cells. Besides, immunofluorescence analysis found that OGD/R treatment induced the nuclear distribution of SOCS3 in microglia cells (Figure 1B). Furthermore, down-regulation of SOCS3 by transfecting siRNA-SOCS3 (si-SOCS3) significantly enhanced OGD/R-induced inflammatory factor, IL-6 expression, whereas, overexpression of SOCS3 evidently suppressed (Figure 1C and D). Given that SOCS3 is an important negative regulator of STAT3 signaling, which mediates the activation of inflammatory responses,12 Next, we examined the effect of SOCS3 on STAT3 signaling in microglia cells under OGD/R condition and found that overexpression of SOCS3 significantly suppressed OGD/R-induced STAT3 activation, and transcription of STAT3 downstream gene, Cyclin D1 and myeloid cell leukemia sequence 1 (Mcl1) (Figure 1E and F). Overall, these results indicate that SOCS3 suppresses OGD/R-induced STAT3 activation and inflammatory factor expression in BV-2 microglia cells.

COP9 Signalosome Complex Interacts with SOCS3 Protein and is Involved in Regulating SOCS3 Protein in Microglia During OGD/R Injury
To explore the underlying mechanism of OGD/R-induced upregulation of SOCS3 in microglia, we examined the SOCS3 mRNA expressions in microglia under OGD/R condition. As shown in Figure 2A, OGD/R treatment significantly increased SOCS3 mRNA expression in microglia. Furthermore, we used the protein synthesis inhibitor CHX and the proteasome inhibitor MG132 to further explore the underlying mechanism of SOCS3 upregulated expression under OGD/R condition. Compared to DMSO (as control) or CHX treatment, MG132 treatment significantly suppressed OGD/R-induced upregulated expression of SOCS3 protein in microglia (Figure 2B), indicating that degradation of SOCS3 protein may be inhibited during OGD/R injury. Given that COP9 signalosome has been reported to regulate deubiquitination and protect proteins from proteasome-mediated degradation,24 we further examined whether COP9 signalosome interacts with SOCS3 protein in microglia. Co-IP analysis showed that COP9 signalosome subunits CSN2/3/4/5/6/7/8, especially CSN3, could be obviously immunoprecipitated by anti-SOCS3 antibody (Figure 2C). Moreover, the interaction between exogenous SOCS3 and exogenous CSN3 was also confirmed in 293T cells by Co-IP analysis (Figure 2D), indicating that SOCS3 indeed interacts with COP9 signalosome. In addition, OGD/R treatment obviously enhanced SOCS3 interacting with CSN3 (Figure 2E). Overall, these results indicate that COP9 signalosome complex interacts with SOCS3 protein and is involved in regulating SOCS3 protein in microglia during OGD/R injury.

CSN3 Promotes SOCS3 Protein Stabilization in Microglia During OGD/R Injury
To further illuminate the molecular mechanism of COP9 signalosome complex regulating SOCS3 protein, we
subsequently examined the effect of knockdown of COP9 signalosome subunit CSN3 on SOCS3 protein expression in microglia. As shown in Figure 3A and B, knockdown of CSN3 evidently decreased SOCS3 interacting with other subunits of COP9 signalosome, such as CSN4 and CSN5. Furthermore, we also found that knockdown of CSN3 not only obviously inhibited OGD/R-induced upregulation of SOCS3 protein in microglia but also decreased SOCS3 interacting with CSN4 and CSN5 (Figure 3C). To further confirm that CSN3 promotes SOCS3 protein stabilization through proteasome pathway, BV-2 microglia cells were transfected with si-CSN3, and then treated with CHX for different hours. Western blot analysis showed that under CHX treatment, SOCS3 protein level was significantly decreased in CSN3-knockdown BV-2 microglia cells, compared to control (Figure 3D). Overall, these results
indicate that CSN3 promotes SOCS3 protein stabilization in microglia during OGD/R injury.

CSN3 Restricting OGD/R-Induced STAT3 Activation and Inflammatory Responses Dependent on SOCS3

To further explore the role of CSN3 on inflammatory responses in microglia during OGD/R injury, we then investigated the effect of overexpression of CSN3 on inflammatory factor IL-6 expression. As shown in Figure 4A and B, overexpression of CSN3 did not evidently affect IL-6 expression in microglia under normal condition, whereas overexpression of CSN3 significantly inhibited OGD/R-induced IL-6 expression. Furthermore, we found that overexpression of CSN3 significantly suppressed OGD/R-induced STAT3 activation (Figure 4C). However, knockdown of SOCS3 evidently suppressed the effects of overexpression of CSN3 on STAT3 activation and transcription of STAT3 downstream genes, Cyclin D1 and Mcl1 in microglia under OGD/R condition (Figure 4D and E). Overall, these results indicate that CSN3 restricting OGD/R-induced STAT3 activation and inflammatory responses dependents on SOCS3.

Knockdown of CSN3 Aggravates Cerebral I/R Injury in vivo

We further examined the roles of CSN3 on cerebral I/R injury in vivo using a mice cerebral I/R model by occluding the middle cerebral artery (MCAO) for 1 h, and then reperfusing for 24 h. CSN3 expression in the brain was downregulated by injection of lentivirus-mediated shRNA-
CSN3 (Lenti-shCSN3) into the lateral ventricle of mice before MCAO operation (Figure 5A). As expected, pre-injection of Lenti-shRNA-CSN3 obviously aggravated cerebral I/R-induced neurological deficit (Figure 5B) and increased cerebral infarction size (Figure 5C and D). Furthermore, we examined the STAT3 expression level in the ischemic boundary zone of brain tissue by immunohistochemistry and found that STAT3 expression was significantly increased and more STAT3 protein translocated into nucleus in the ischemic boundary zone of mice pre-injected with Lenti-shCSN3, compared to those mice pre-injected with Lenti-shNC (Figure 5E). Overall, knockdown of CSN3 aggravated cerebral I/R injury.

Discussion

SOCS3 is a well-known negative regulator of the Janus kinases-signal transducer and activator of transcription 3 (JAK/STAT3) signaling pathway, which has been reported to be activated to induce inflammatory responses and be involved in various diseases, such as cerebral I/R injury, myocardial cerebral I/R injury, and inflammatory bowel disease. Through binding tyrosine kinase receptor, primary receptor shared IL-6 receptor subunit gp130 and bindinJAK2, SOCS3 inhibits STAT3 phosphorylation to restrain inflammation. Here, we find that SOCS3 expression is gradually upregulated in microglia during I/R injury, and overexpression of SOCS3 evidently inhibits I/R-induced STAT3 phosphorylation and inflammatory factor expression, further suggesting SOCS3 functions protective effects in brain inflammation.

SOCS3 protein has two functional domains: a central SH2 domain, which binds the defined tyrosine-phosphorylated substrates on the cytokine receptor, and a SOCS-box domain at the C-terminus, which could be recognized as substrate by a Cullin-RING E3 ubiquitin-protein ligase complex, indicating that SOCS3 could also be regulated by deubiquitination. In this study, we explored the relationship between SOCS3 and COP9 signalosome which regulates the activity of Cullin-RING ubiquitin E3 ligase, during cerebral I/R injury. We find that SOCS3 interacts with COP9 subunit CSN3, which is essential for stabilize SOCS3 protein in microglia during cerebral I/R injury, thereby suppressing STAT3 activation and induction of pro-inflammatory factors. Besides, Nuclear factor kappa B (NF-kB) Signaling is also activated and essential for neuroinflammation during cerebral I/R injury. Generally, upon stimulation, the inhibitor of NF-kB, IκBα, is ubiquitinated and then degraded by the
26S proteasome, thereby leading to that NF-κB is released and translocated into the nucleus to induce expressions of pro-inflammatory factors.\(^{34,35}\) Recently, COP9 signalosome has been reported to function in the protection of Iκβα from degradation by regulating the deubiquitination of CRL1, resulting in reduction of NF-κB activation upon tumor necrosis factor (TNF) stimulation.\(^{36,37}\) Whether NF-κB signaling is also regulated by the same COP9 signalosome during cerebral I/R injury is still unknown and the relationship among SOCS3, NF-κB and COP9 signalosome complex needs to be investigated in the following studies. We also identify other subunits of COP9 signalosome interact with SOCS3 in microglia. Given that these CSNs cooperate with each other to form COP9 signalosome complex to function, and that the association between CSN4/5 and SOCS3 is decreased by CSN3 knockdown in microglia, it is probably that SOCS3 interacting with COP9 signalosome depends on CSN3, which needs further confirmation in the future studies. The detailed mechanism of COP9 signalosome stabilizing SOCS3 by deubiquitination during cerebral I/R injury is also worthy of further study. In addition, there are also some limitations in our present study. First, BV2 microglia cell line was used to explore the underlying mechanism of COP9 signalosome complex regulating SOCS3 protein during neuroinflammation in vitro. In the following studies, primary microglia should be used. Second, for the in vivo experiments, CSN3 knockout mice should be used.
in the following studies. Third, SOCS family has eight members: SOCS1-7 and the cytokine-inducible SH2-containing protein, which all negatively regulate the JAK-STAT signaling pathway.\(^{38}\) Except SOCS3, whether COP9 signalosome complex also regulating other SOCS members in neuroinflammation during cerebral I/R injury should be further investigated in the future studies.

**Conclusion**

This study firstly reports that CSN3 restricts neuroinflammatory responses during cerebral I/R injury through stabilizing STAT3 protein and identifies a protective role of COP9 signalosome in cerebral I/R. Our results contribute to deeply understanding the regulatory mechanism of SOCS3 in neuroinflammation and also providing potential therapeutic targets for cerebral I/R injury.

**Availability of Supporting Data**

The data will be available on request.

**Ethical Approval**

All animals were maintained according to the American Animal Protection Legislation. This study was approved by the Institutional Animal Care and Use Committee of the Guangzhou University of Chinese Medicine.
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Disclosure
The authors reported no conflicts of interest for this work.

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