Blocking SUR1-TRPM4 reduces neuronal loss in peri-infarct area via stimulating TGFα released by microglia

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Research Article

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Abstract

Background

As a sequel of ischemic stroke, selective neuronal loss (SNL) mediated by activated microglia and consequential neuroinflammation affects the salvageable peri-infarct area (PIA) and hampers the functional recovery following reperfusion therapy. Recent evidence indicates that inhibition of sulfonylurea receptor 1-transient receptor potential M4 (SUR1-TRPM4) exerts a robust protective effect against inflammation. Hence, we hypothesized that blocking SUR1-TRPM4 channels reduced SNL after brain ischemia in the PIA.

Methods

Mice subjected to temporary middle cerebral artery occlusion (tMCAO) for 1 h and reperfusion for 24 h were adopted to mimic the pathophysiological changes of ischemic stroke. Gene expression, neuronal apoptosis, and protein content were tested by RNA-sequencing, TUNEL staining, Western blot, respectively.

Results

After tMCAO, abundant neuronal apoptosis appeared in the PIA, with remarkable up-regulation and co-localization of SUR1 and TRPM4. Blocking the SUR1-TRPM4 channel by glibenclamide (GLB, a SUR inhibitor) and *Trpm4* gene deletion (*Trpm4*$^{-/-}$) distinctly alleviated apoptotic neurons in the PIA. To explore the potential mechanism of blocking SUR1-TRPM4, we compared gene expression in brain tissues of *Trpm4*$^{-/-}$ and wild-type mice after tMCAO modeling using RNA-sequencing and identified 217 differentially expressed genes. Among them, the expression of *Tgfα* was significantly higher in *Trpm4*$^{-/-}$ mice compared with that in wild-type mice after tMCAO. GLB treatment significantly increased the expression of TGFα in microglia, as validated by Western Blot and immunofluorescence staining. Moreover, intracerebroventricular injection of recombinant TGFα significantly alleviated the neuronal loss in the PIA and improved neurological outcome after tMCAO. *In vitro*, we subjected BV-2 microglia cells to oxygen-glucose deprivation or lipopolysaccharide stimulation and found significant up-regulation and co-localization of SUR1 and TRPM4, while blocking SUR1-TRPM4 with GLB and 9-phenanthrol (9-Phe, a TRPM4 inhibitor) increased the expression and release of TGFα by activating the CaMKII/CREB pathway. BV-2 microglia derived conditioned medium after oxygen-glucose deprivation or lipopolysaccharide stimulation induced apoptosis of SH-SY5Y cells, which could be inhibited by applying GLB and 9-Phe on BV-2. Furthermore, direct application of recombinant TGFα alleviated neuronal apoptosis mediated by BV-2 microglia conditioned medium.

Conclusions

Collectively, our findings indicate that blocking SUR1-TRPM4 in microglia alleviates SNL, probably by up-regulating the expression and release of TGFα.

Background

The undisputable significance of reperfusion therapy in acute ischemic stroke (AIS) is underpinned by increasing experimental and clinical evidence[1, 2]. Infarct core and peri-infarct area (PIA) are two key regions in the pathophysiological process of AIS[3, 4]: 1) infarct core, characterized by irreversible neuronal necrosis in a short period due to the sudden interruption of cerebral blood flow; 2) PIA, featured by incomplete occlusion of blood flow in which vulnerable neuronal cells are salvaged with timely vascular recanalization. Nevertheless, recent evidence renders that the salvable PIA may be affected by selective neuronal loss, which may both hinder the early clinical benefit of reperfusion therapy and dampen long-term peri-infarct plasticity [5, 6]. Previous studies also indicated the occurrence of selective neuronal loss was strongly linked to the action of microglia nearby[6, 7], but the underlying mechanism remains unclear.

Transient Receptor Potential M4 (TRPM4) is a calcium-activated non-selective cation channel activated by intracellular Ca^{2+} and ATP depletion, mainly mediating Na^{+} influx[8, 9]. In previous studies, Sulfonylurea Receptor 1 (SUR1) and TRPM4 were proved to be increasingly expressed and integrated into a newly-formed SURl-TRPM4 channel in the components of neurovascular unit in the infarct core after ischemia, inducing cell swelling and death (oncosis) and blood-brain barrier disruption[10–12]. Recently, we and other researchers both proposed that SUR1-TRPM4 channels were also extensively expressed in microglia after ischemia with unclear function[13, 14]. In addition, application of glibenclamide (GLB), a specific SUR1 inhibitor, and gene knockout of *Trpm4* (*Trpm4*$^{-/-}$) not only prevented brain edema, but also alleviated neuroinflammation in AIS and other brain injury models[15–18]. Interestingly, in an animal model of experimental autoimmune encephalomyelitis (EAE) which appeared minimal brain edema, inhibition of SUR1-TRPM4 also exerted a protective effect and improved the functional recovery[19]. Therefore, we speculated that SUR1-TRPM4 may not only participate in the formation of brain edema but directly engage in adjusting the function of microglia, blocking SUR1-TRPM4 might provide benefit to the PIA by alleviating neuroinflammation mediated by the maladjusted microglia.

In this study, we investigated the neuroprotective effect and underlying mechanism of blocking SUR1-TRPM4 channels on PIA after AIS by using a mouse model of cerebral ischemia/reperfusion and two kinds of BV-2 microglia cell models in vitro.We provided molecular evidence that blocking SUR1-TRPM4 channels with GLB or gene deletion of *Trpm4* significantly alleviated selective neuronal death in PIA after AIS, probably via inducing the up-regulation and release of TGFα in PIA microglia (Fig.1).

Methods

Reagents

Lipopolysaccharide (LPS) was purchased from Solarbio (Peking, China). Glibenclamide (GLB, an inhibitor of SUR1) and 9-phenanthrol (9-Phe, an inhibitor of TRMP4) were purchased from Sigma-Aldrich (St.Louis, MO, USA). TGFα recombinant proteins were purchased from Abcam (Cambridge, UK). CaMKII-IM-1 (an inhibitor of CaMKII) and KG-501 (an inhibitor of CREB) were purchased from MedChemExpress (NJ, USA). NCGC00067819 (an agonist of CREB) was purchased from Probechem (IL, USA). STAT3 inhibitor VI was purchased from Santa Cruz (CA, USA).

Animal

All animal experiments in this study were approved by the Animal Care and Use Committee of the Nanfang Hospital, Southern Medical University (Guangzhou, China), and were adhered to the National Institute of Health Guide for the care and use of laboratory animals. In this research, male *Trpm4*$^{-/-}$ mice on C57BL/6J background (6-8 weeks, 22-24g, Shanghai Model Organisms, China) and wild-type (WT) littermates propagated by homozygous mating were adopted. In RNA-sequencing, 10 mice (5 *Trpm4*$^{-/-}$ mice, 5 WT mice) were selected. In other experiments, 108 mice (36 *Trpm4*$^{-/-}$ mice, 36 WT mice, 36 sham mice) were adopted. A total of 118 mice were used in all experiments.

Temporary Middle Artery Occlusion (tMCAO)

The intraluminal filament model of focal ischemia was adopted[20]. Briefly, right common carotid artery, external and internal carotid arteries were isolated from surrounding nerves and fascia through a midline incision under anesthesia of isoflurane (induced with 3%, maintained with 1%). A monofilament nylon suture with a silicone-coated tip (silicone diameter: 0.22 ± 0.02 mm) was then inserted through an arteriotomy of the external carotid artery and gently advanced into the internal carotid artery to the opening of the middle cerebral artery. After 1 h of occlusion, the suture was removed to allow reperfusion for 24 h. Rectal temperature was maintained at 37 ± 0.2 °C during surgery using a heating pad (RWD Life Science, Shenzhen, China). Successful modeling of tMCAO was defined as a reduction of focal cerebral blood flow by more than 75% of baseline and complete recovery of blood flow after reperfusion, which were monitored by a laser Doppler flowmetry (Moor Instruments, Wilmington, USA). After 24 h of reperfusion, the brain tissues of the PIA were isolated and reserved for subsequent experiments. Sham surgery included the exposure of the common, external, and internal carotid arteries with all ligations and transections; no occlusion occurred in the sham group.

Treatment and Drug Administration

In the first part, GLB and *Trpm4*$^{-/-}$ are the main interventions inhibiting SUR1-TRPM4 channels. In the tMCAO model, mice were randomly allocated to receive vehicle or GLB at 1 h after ischemia. Based on previous studies, GLB (Sigma-Aldrich, St Louis, United States) was administrated with an initial dose of 10 μg/kg and additional doses of 0.2 μg every 8 hours[14]. *Trpm4*$^{-/-}$ mice undergone tMCAO received vehicle injection at the same time point with GLB injection. To elucidate the influence of infarction volume, delayed GLB injection at 10 h after ischemia and reperfusion were set as a control group. GLB

was prepared for intraperitoneal injection by dissolving in dimethyl sulfoxide (DMSO) and clarifying the solution as needed using a minimum amount of NaOH to a pH approximately 8 to 8.5 and 7.8. Mice in different groups randomly received equivalent volumes of vehicle (normal saline containing 0.05% DMSO) and GLB (vehicle and 2.5 μg/ml GLB).

In the second part, intracerebroventricular injection of recombinant TGFα was performed using stereotaxic surgery. Mice after ischemia for 1 h received 50 ng recombinant TGFα (n = 6) in the lateral ventricle according to Paxinos atlas. Phosphate buffered saline (PBS) treated vehicle group (n = 6) underwent stereotaxic surgery and received 2 μl PBS in the right lateral ventricle set as a control group.

Agarose gel electrophoresis of tail DNA

Genotyping of each mouse was identified by using the Mighty Amp Genotyping kit (TAKARA), according to the manufacturer's protocol. Firstly, DNA was extracted from tail tissue and amplified. Then, by mixing DNA with primers and 5x loading dye for agarose gel electrophoresis, genotypes were identified.

Different primer sequences were listed as follows:

Trpm4$^{-/-}$: Forward primer 5'-3': GGAGCAACACCTGAGCTTATGAC;

Reverse primer 5'-3': AGGCTGAGCTGGAACTCAGAG.

Trpm4$^{+/+}$: Forward primer 5'-3': CTATCTGGAGCTGCATCCCTG;

Reverse primer 5'-3': GTTCCACCTATCCTTAGGACCTG

RNA isolation and cDNA synthesis

Total RNA from the cerebral PIA was isolated using the Animal Total RNA Isolation Kit (Foregene, China), according to the manufacturer's protocol. cDNA synthesis was conducted in 20 μl of reaction mixture containing 2 mg of RNA by using PrimeScript RT reagent Kit with gDNA Eraser (TAKARA).

Differentially expressed genes analysis

After ischemia for 1 h and reperfusion for 4 h, the total RNA was extracted in the PIA. In this analysis, two groups (WT versus *Trpm4*$^{-/-}$) were compared for differential gene expression. Transcriptomic analysis was performed using the HISAT2-Stringtie-DESeq pipeline. Genes that exhibited changes in expression > 2-fold and had a *P*-value adjusted using the Benjamini–Hochberg procedure lower than 0.05 (Padj < 0.05) were identified as differentially expressed genes (DEGs). Enrichment Analysis based on Gene Ontology Database annotates gene functions of DEGs to obtain the related pathways that the genes participate in, and then calculates the significance level (P-value) and misjudgment rate (FDR) of each pathway by using hypergeometric test (or Fisher's exact test) and multiple comparison test.

Immunofluorescence

Mice were euthanized after reperfusion for 24 h and transcardially perfused with saline. Following fixation with 4% PFA overnight, brains were immersed in 15% and 30% sucrose at 4°C for cryoprotection. Sequential 6 μm-thick coronal sections of the brain were prepared by cryoutramicrotomy (CM1950, Leica, Germany). The slices were simultaneously incubated at 4°C overnight with two types of primary antibody from different species for the co-localization staining: primary antibody from mouse: anti-SUR1 (1:100, Sigma-Aldrich), anti-Iba1 (1:100, Sigma-Aldrich), anti-GFAP (1:100, Abcam), anti-NeuN (1:100, Abcam) with rabbit anti-TRPM4 (1:100, Sigma-Aldrich), respectively; mouse anti-Iba1 (1:100) with rabbit anti-TGFα (1:100, Santa Cruz, CA, USA). Afterwards, the slices were washed and detected with secondary antibodies: Goat anti-mouse Cy3 and Goat anti-rabbit FITC (Beyotime, China).

Immunofluorescence staining for neuronal apoptosis

Double staining of Terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) and neuronal nuclei (NeuN) was conducted to determine the co-localization of apoptotic cells and neurons. In brief, frozen sections were immunostained with mouse anti-NeuN antibody (1:100, Abcam) at 4 °C overnight and subsequently subjected to TUNEL staining using a One Step TUNEL Apoptosis Assay Kit (Beyotime, China) according to the manufacturer's protocol. Afterwards, the slices were washed and detected with Goat anti-mouse Cy3 at 37 °C for 2 h. Finally, the sections were covered with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Positive cells around the injured cortex were calculated per square millimeter from six random microscopic fields of each section (three sections per animal) under a fluorescence microscope (Olympus).

Measure of Infarct Volume and the selection of the PIA

At 24 hours after ischemia, the whole mice brains were extracted after euthanasia and sectioned into 7 contiguous coronal slices from the frontal pole with placement in a brain matrix. Then, all slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich) for 30 minutes at 37 °C. After correction for edema, corrected infarct volume (%) was calculated by a researcher blinded to group allocation with Image J (NIH, Bethesda, United States) as follow: corrected infarct volume (%) = [contralateral hemisphere volume − (ipsilateral hemisphere volume − infarct volume)] / contralateral hemisphere volume × 100%. The peri-infarct territory, which is found in the 2 mm around the infarct core, was dissected for protein analysis.

Morris water maze

Spatial learning and memory were evaluated in animals 8 days post-tMCAO using the Morris water maze as described previously [40, 41]. Firstly, mice were trained to reach for the platform for 5 consecutive days with 4 trials per day. Movements of the mice were tracked by TSE VideoMot2 tracking system (Bad Homburg, Germany) to record the path and time taken to escape from 4 randomly assigned locations. The latency time required to locate the hidden platform was assessed among groups. After acquisition trial, the probe trial was performed on the following day, when mice were allowed 60 seconds to explore

the platform which had been removed. The percentage of total time that mice spent in the target quadrant and the number of platform location crossing were recorded and analyzed.

Neurological Test

The global neurological and motor function were evaluated with longa test and string test by a researcher blinded to the group allocation. The longa test is a 5-point scale with a minimum score of 0 for no neurological deficit and a maximum score of 5 for no spontaneous walking and depressed. The string test was performed by placing the mouse on a 50 cm string at a point between the supports and rated by the following system[14]:0, falls off; 1, hangs onto string by two forepaws; 2, as for 1, but attempt to climb onto string; 3, hangs onto string by two forepaws plus one tail wrapped around string; 4, hangs onto string by all four paws plus one tail wrapped around string 5, escape.

Nissl staining

After successful tMCAO modeling, mice were perfused with the use of 4% paraformaldehyde in PBS via the heart, followed by the extraction of the brains. After being fixed with 4% paraformaldehyde and dehydrated using 15% and 30% sucrose solution, the brain tissues were sliced into 10-μm sections, followed by 1-h staining with 0.04% cresyl violet dissolved in acetate buffer. Next, the sections were observed under a light microscope. Cell counting was performed in the peri-infarct area of tMCAO mice using five sections of each brain tissue.

Cell culture

The BV-2 mouse microglial cells (obtained from Bnbio, Peking, China) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) containing 10% fetal bovine serum (FBS; Gibco), 100 IU/mL penicillin, and 100 mg/mL streptomycin. SH-SY5Y cells (obtained from Bnbio, Peking, China) were cultured in DMEM/F12 containing 15% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin, under a humidified atmosphere of 5% CO2 at 37 °C.

Oxygen and glucose deprivation/reoxygenation (OGD) of BV-2

The cultured BV-2 cells were washed two times with PBS and incubated with DMEM without glucose in a chamber (Thermofisher, USA) that was filled with 95% N_2 and 5% CO_2 at 37°C. Control group cells were incubated under normal culture conditions for the same time period. After 3 h of OGD, the medium of both OGD and control group was replaced with the normal DMEM medium and cultured under normal conditions for 24 h.

Cell proliferation assay

SH-SY5Y were seeded in a 96-well culture plate at 1×10^4 cells/well in 100 μl for 24h to adhere. The medium was then replaced with a 100 μl conditioned medium from BV-2 cells. After 24 h incubation, 10

μl CCK-8 were added per well and then absorbance value at 450 nm was tested to examine the survival of SH-SY5Y.

ELISA assay

The concentration of TGFα in the cell-free supernatants was detected by means of a mouse TGFα Elisa detection kit (Boshen, Jiangsu, China) according to its manufacturer's protocol.

Measurement of Gene Expression

The mRNA levels of SUR1, TRPM4, TGFα, and GADPH were routinely measured by quantitative real-time polymerase chain reaction (q-PCR). Briefly, total RNA was isolated using a Total RNA/DNA isolation kit (Tiangen, Peking, China) and reverse transcribed to cDNA with PrimeScriptTM RT Master Mix Kit (TAKARA) according to the manufacturer's instructions. q-PCR was performed using the SYBR Green master mixes (TAKARA) and Roche LightCycler480 System. Relative changes of mRNA expression were normalized to the level of GADPH.

Tested genes and primer sequences were listed as follows:

Abcc8: Forward primer 5'-3': CATCCGGGTGAGGAGATACG;

Reverse primer 5'-3': CAGGTTAACGAAGGGCTGCA.

Trpm4: Forward primer 5'-3': TGATGAGCACACCACGGAGA;

Reverse primer 5'-3': ATCCGTGCGATCAGACAGC.

Tgfα: Forward primer 5'-3': CACTCTGGGTACGTGGGTG;

Reverse primer 5'-3': CACAGGTGATAATGAGGACAGC.

Gapdh: Forward primer 5'-3': AGGTCGGTGTGAACGGATTTG;

Reverse primer 5'-3': TGTAGACCATGTAGTTGAGGTCA.

Western Blot

Ischemic tissues in PIA were resolved in RIPA solution (Beyotime Biotechnology, Shanghai, China) and restored at -80 °C until used. Denatured protein was separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, United States). The membrane was incubated overnight at 4 °C with antibodies involving anti-SUR1 (1:1000; Bioss, Beijing, China; Rabbit), anti-TRPM4 (1:1000; Sigma-Aldrich; Rabbit), anti-TGFα (1:1000; Santa Cruz; Rabbit), and anti-GAPDH (1:10000; Proteintech; Mouse). Primary antibodies were detected with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies. All signals were detected by enhanced

chemiluminescence detection method followed by quantification with Image J and normalizing to GAPDH levels.

Preparation of microglia conditioned medium (MCM)

Cultured BV-2 microglia were plated in DMEM serum-free media at a density of $1x10^6$ cells/well, in six-well tissue culture-treated plates (Corning. Lowell, MA) and maintained in a 37 °C incubator under conditions of 5 % CO_2 and 95 % humidity for 24 h. After 24 h, media was aspirated out and 1 mL of fresh DMEM serum-free media was added. After modeling for 24 h, the medium was collected after centrifugation at 1000 g for 10 minutes with sterile filtration (0.22 μM filter) of the supernatant. The microglia conditioned medium after OGD or LPS were named OGD-MCM or LPS-MCM, respectively.

siRNA Transfection

siRNA duplex sequences were designed by RIB BIO (Guangzhou, China) and transfected using ribo FECT^{TM} CP (Guangzhou, China) according to its protocol.

Statistical analysis

The number of positive cells in immunofluorescence staining was compared using two independent sample t-test. Statistical differences in multiple groups were compared using one-way analysis of variance followed by the Tukey's multiple comparisons test, while two groups were analyzed by unpaired t-tests. All statistics were completed by SPSS 25.0, and GraphPad Prism 8.0 was used for image drawing and processing. $P < 0.05$ was statistically significant.

Results

tMCAO induces neuronal apoptosis and SUR1-TRPM4 up-regulation in PIA

The tMCAO model is widely used to mimic the pathophysiological process of cerebral artery occlusion and reperfusion. It has been confirmed that ischemic stroke can induce peri-infarct astrogliosis with a clear boundary surrounding the infarct core. By fluorescent staining with GFAP and NeuN, the infarct core and PIA can be classified and localized distinctly (Figure 2A). To evaluate neuronal apoptosis in the infarct core and PIA after ischemia, WT mice randomly assigned to receive sham operation or tMCAO were simultaneously stained with TUNEL and NeuN. The results showed that compared with the sham group, prominent apoptotic cells (TUNEL+) appeared in the PIA of tMCAO mice, with the majority of which were NeuN+ labeled neurons (Figure 2B).

The SUR1-TRPM4 channel is known to assemble in neurons, astrocytes, and endothelial cells after brain injury[14]. We first examined the expressions of SUR1 and TRPM4 and found that both the mRNA and protein levels of SUR1 (encoding gene *Abcc8*) and TRPM4 in the PIA were significantly up-regulated after tMCAO (Figure 2C-D). Double immunofluorescence staining showed evident up-regulation and co-localization of SUR1 and TRPM4 in the PIA after tMCAO, suggesting the potential formation of the SUR1-

TRPM4 channel (Figure 2E-F). We also used double immunofluorescence staining of anti-TRPM4 with anti-NeuN, anti-Iba1, and anti-GFAP to detect the distribution of TRPM4 in different cells in the brain. Strikingly, we found that unlike 79% of TRPM4+ cells in the infarct core were co-localized with NeuN+ neurons, 71% of TRPM4+ cells in the PIA were co-localized with Iba1+ microglia/macrophage (for convenience, microglia were used in the following text), suggesting that microglial SUR1-TRPM4 channel may play a central role in the PIA (Figure 2G-J).

Together, these results indicated that tMCAO induced neuronal apoptosis and up-regulation of SURl-TRPM4 in the PIA, in which the majority of SUR1-TRPM4 co-localized with microglia.

Blocking SUR1-TRPM4 by GLB treatment and *Trpm4*$^{-/-}$ alleviates neuronal apoptosis in PIA after tMCAO

We then examined whether blocking the SUR1-TRPM4 channel with GLB and *Trpm4*$^{-/-}$ alleviated neuronal apoptosis in PIA after tMCAO. Compared with WT mice with vehicle treatment, GLB treatment in WT mice and gene deletion of *Trpm4* both significantly decreased the neuronal loss in PIA after tMCAO, revealing as increased number of NeuN+ neurons (Figure 3A-C). Besides, the number of TUNEL+ cells in the PIA of the GLB group and *Trpm4*$^{-/-}$ group was significantly lower than that of the vehicle group, suggesting amelioration of neuronal apoptosis by the treatment (Figure 3D-E).

In our previous study, the application of GLB at 1 h after reperfusion significantly reduce the infarct core volume[14]. Here, to minimize the influence of GLB on PIA brought by protecting the infarct core and to explore the protective time window of GLB on PIA, we also observed the effect of GLB when administered 10 h after reperfusion, because the volume of infarct core had been basically fixed at this time

point (comparable to that observed at 24 h) [23]. We found that delayed application of GLB at 10 h after reperfusion did not lessen the infarct core volume or reduce the number of neuronal loss (NeuN+) in the infarct core at 24 h, compared with the vehicle group (Figure 3F-I). However, delayed application of GLB significantly decreased the number of TUNEL+ apoptotic cells in the PIA, suggesting that the protection of GLB on PIA might be independent of its effect on alleviating infarct core volume, and the protection of GLB on PIA may have a wider time window (Figure 3H-J).

Together, the above results suggested that cerebral ischemia and reperfusion induced abundant neuronal apoptosis in the PIA, which could be prevented by GLB treatment and gene deletion of *Trpm4*. The neuronal protection of GLB on PIA might be independent of its effect on salvaging the infarct core, and its therapeutic window should be at least 10 hours.

Blocking SUR1-TRPM4 by GLB treatment and *Trpm4* knockout induces up-regulation of TGFα after tMCAO

We then sought to find out why blockage of the SUR1-TRPM4 channel by applying GLB or gene deletion of *Trpm4* alleviated neuronal apoptosis in PIA after tMCAO. To minimize the confounding effect of brain edema, WT and *Trpm4*$^{-/-}$ mice underwent tMCAO and were euthanatized 4 h after reperfusion, when brain

edema appeared slightly. Total mRNA was extracted from the PIA of both groups and the expression of 31898 genes was detected using RNA-sequencing (Figure 4A). A total of 241 DEGs (147 up- and 74 down-regulated) were found between the *Trpm4*$^{-/-}$ and WT mice. The top five genes with the greatest fold change were presented in Figure 4B. In the GO analysis, these DEGs were enriched in TGF-regulated and inflammation-related signaling pathways, suggesting the protective mechanisms of *Trpm4*$^{-/-}$ might be associated with its regulation of inflammation and the activation of protective growth factors (Figure 4C). Q-PCR analysis of the expression of DEGs was used to verify the RNA-Sequencing results, and both methods of analysis identified that *Tgfa* had a maximum positive expression change in *Trpm4*$^{-/-}$ mice (Figure 4B). Transforming growth factor alpha (TGFα) encoded by *Tgfa* has been reported to promote neuronal survival and enhance neurogenesis and angiogenesis in models of stroke and neurodegenerative diseases [28-32]. Hence, we focused on the function of *Tgfa* in our subsequent research.

Consistently, in immunoblotting, the protein expression of TGFα was significantly increased in *Trpm4*$^{-/-}$ and GLB groups (Figure 5A-B). Additionally, increased TGFα was found to co-localize with Iba1+ cells in the PIA by immunostaining, and more TGFα+ cells in the *Trpm4*$^{-/-}$ group and GLB group were observed than that in the WT vehicle group (Figure 5C-D).

Overall, these findings rendered that blocking the SUR1-TRPM4 complex by GLB and *Trpm4*$^{-/-}$ induced the up-regulation of TGFα in the PIA microglia after tMCAO.

Intracerebroventricular administration of recombinant TGFα alleviates neuronal apoptosis and improves neurologic function after tMCAO

We next sought to identify the neuroprotective effect of TGFα on PIA after brain ischemia by direct injection of recombinant TGFα into lateral ventricles after tMCAO. Results showed that, compared with vehicle control, intracerebroventricular injection of recombinant TGFα significantly decreased the number of apoptotic neurons (TUNEL+) in the PIA (Figure 6A-B) and improved the neurologic function of tMCAO mice (Figure 6C-D).

To further evaluate the long-term protection of recombinant TGFα, mice were subjected to the Morris water maze test at day 8 to 12 after tMCAO. Before the Morris water maze test, we made an initial evaluation on the swimming speed of the mice to roughly exclude the confounding of the basic athletic ability changes after tMCAO (Figure 6G). Compared with vehicle-treated tMCAO mice, recombinant TGFα injected mice showed superior learning and memorial ability, revealing as shortened latency in the training trial and increased crossing platform times in the probe trial. (Figure 6E-H). Besides, mice with recombinant TGFα treatment showed significantly more viable neurons, less dendritic damage, and less glial proliferation in the PIA at day 12 after tMCAO (Figure 6I-N)

Overall, our data suggested that intracerebroventricular administration of recombinant TGFα provided both short-term and long-term neuroprotection on PIA after tMCAO.

OGD and LPS stimulation induces assembly of SUR1-TRPM4 complex in BV-2 microglia

Two major mechanisms may be involved in the pathophysiological evolvement of PIA[3]: 1) direct stimulation caused by ischemia and reperfusion; 2) inflammatory response induced by danger-associated molecule patterns (DAMPs) released from the infarct core. Based on that, we used BV-2 microglia to receive OGD as an in vitro model of cerebral ischemia and reperfusion and LPS stimulation to mimic the inflammatory reaction in the brain. After exposure to OGD and LPS for 24 h, more SUR1+ and TRPM4+ BV-2 cells were observed compared with the control. In addition, SUR1 and TRPM4 were significantly co-localized after modeling, in accordance with the results of the in vivo experiments (Figure 7A-B). Results from Western blot also presented up-regulation of SUR1 and TRPM4 (Figure 7C-D). Moreover, the mRNA levels of *Abcc8* and *Trpm4* were significantly up-regulated in BV-2 cells (Figure 7E). These results indicated the up-regulation and assembly of the SUR1-TRPM4 channel after OGD and LPS stimulation in BV-2 microglia.

Blocking SUR1-TRPM4 by GLB and 9-Phe induces up-regulation and release of TGFα in BV-2 microglia

To determine whether blocking the SUR1-TRPM4 channel in microglia would elicit the up-regulation and release of TGFα in vitro, we first detected the mRNA levels of *Tgfa* via qPCR, finding that pre-treatment of GLB and 9-Phe significantly induced the up-regulation of *Tgfa,* in both OGD and LPS-stimulated models (Figure 7F). Then, the concentrations of TGFα in the culture medium of BV-2 cells were tested using an ELISA kit. Pre-treatment with GLB and 9-Phe before exposure to OGD or LPS both significantly increased the concentration of TGFα in culture medium by approximate 3-folds, as compared with these exposures to OGD or LPS alone (Figure 7G).

The above data suggested that GLB and 9-Phe directly induced the up-regulation and release of TGFα from microglia after OGD and LPS modeling.

Release of TGFα by blocking SUR1-TRPM4 prevents neuronal apoptosis after OGD or LPS stimulation

Conditioned medium culture provides an approach to explore glial-neuronal signaling interactions via the release of soluble mediators. By adding LPS-MCM or OGD-MCM to cultured SH-SY5Y cells, abundant cell apoptosis was detected via TUNEL staining. Interestingly, both GLB-LPS-MCM and GLB-OGD-MCM exerted protective effects against apoptosis (Figure 8A-C). However, siTGFα-MCM weaken the protective effect of GLB and 9-Phe by gene silencing of TGFα, suggesting that TGFα plays as a downstream effector of GLB and 9-Phe (Figure 8A-E). Via direct application of mouse TGF*a* recombinant protein into SH-SY5Y cells with OGD-MCM and LPS-MCM, the number of TUNEL+ SH-SY5Y cells was considerably lower than that without recombinant TGFα (Figure 8A-E).

To rule out the likely effect of the remaining GLB and 9-Phe in the MCM, we directly applied GLB and 9-Phe on OGD/LPS-MCM treated SH-SY5Y cells, but did not find that they had the ability to protect SH-SY5Y cells from apoptosis (Figure 8F).

In sum, these findings suggested that microglia mediated the neuronal apoptosis after OGD and LPS stimulation, whereas blocking SUR1-TRPM4 channels elicited the release of TGFα interacting between microglia and neurons as a protective growth factor capable of promoting neuronal survival.

CaMKII/CREB signaling pathway is involved in regulating the up-regulation of TGFα after blocking the SUR1-TRPM4 channel

Inhibition of the SUR1-TRPM4 using GLB has been shown to increase the phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[13], which is a multifunctional serine/threonine kinase that involves in synaptic plasticity, learning, and memory[21]. The activation of CaMKII is also known to participate in the phosphorylation of cyclic adenosine monophosphate response element-binding protein (CREB)[21], with the latter being a critical transcription factor regulating the function of microglia. Therefore, we tested whether the CaMKII/CREB signaling pathway is involved in regulating the up-regulation of TGFα after blocking the SUR1-TRPM4 channel.

We first tested the protein expression of CaMKII, CREB, p-CaMKII, and p-CREB by immunoblotting. The results showed that the phosphorylated levels of CaMKII and CREB were both significantly increased after GLB and 9-Phe treatment (Figure 9A-B), suggesting that blocking the SUR1-TRPM4 channel leads to activation of the CaMKII/CREB pathway.

To further investigate the involvement of the CaMKII/CREB pathway in the regulation of TGFα expression and release, selective inhibitors of CaMKII (CaMKII-IN-1, 100 nM) and antagonist of CREB (KG-501, 10 μM) were applied 1 h before OGD and LPS-stimulated modeling. The results showed that pre-treatment with CaMKII-IN-1 and KG-501 both inhibited the up-regulation of TGFα elicited by the application of GLB and 9-Phe (Figure 9E-F). In contrast, agonists of CREB (NCGC00067819, 100 nM) directly triggered the up-regulation of TGFα (Figure 9C-D).

These data indicated that blocking SUR1-TRPM4 induced the up-regulation and release of TGFα from microglia by activating the CaMKII/CREB pathway.

The transcription factor STAT3 mediates the protective effects of recombinant TGFα in SH-SY5Y cells

Previous studies indicate that STAT3 is an essential mediator of signal transduction downstream of TGFα-EGFR engagement after ischemia [28]. Blockade of STAT3 in cultured cancer cells was found to inhibit cell proliferation, induce apoptosis, and stimulate immune responses[35]. To verify the participation of STAT3 in the TGFα-afforded neuronal survival, we measured the effect of recombinant TGFα on STAT3 activity in SH-SY5Y cells after OGD-MCM treatment in vitro. In OGD-MCM cultured SH-SY5Y cells, there was a slight decline in p-STAT3 level, whereas recombinant TGFα treatment significantly increased the p-STAT3 level (Figure9G-H).

To further investigate the involvement of the STAT3 pathway in the protective effects of TGFα, a specific STAT3 inhibitor (STAT3 inhibitor VI) was used to interrupt STAT3 activity. The apoptotic activity of SH-

SY5Y cells was assessed by the protein levels of the typical apoptotic and anti-apoptotic protein, Caspase-3 and Bcl-2, respectively. The result of immunoblotting revealed that co-administration of the STAT3 inhibitor diminished the protective effects of recombinant TGFα, increasing the expression of cleaved Caspase-3 and decreasing the protein level of Bcl-2 (Figure 9G-H). Overall, these results suggested that the protective effect of recombinant TGFα in SH-SY5Y cells may be mediated, at least in part, by the activation of the STAT3 pathway.

Discussion

Salvable PIA determines clinical recovery after ischemic stroke, but it may be affected by selective neuronal loss. Here, we rendered that ischemia and reperfusion for 24 h induced neuronal apoptosis and up-regulation of SUR1-TRPM4 in the PIA. Blocking the SUR1-TRPM4 channel in microglia by pharmacological inhibition or gene deletion induced the up-regulation of TGFα and alleviated neuronal loss. In addition, we reported that TGFα released from microglia exerted anti-apoptotic effect on neurons by activating the STAT3 pathway, functioning as an essential regulator for neuronal survival against ischemia and inflammation.

GLB has been repeatedly shown to be a neuroprotective medication in various neurological diseases such as ischemic stroke, subarachnoid hemorrhage, and traumatic brain injury, mainly by its robust effect in alleviating brain edema[15–18, 22]. Recent evidence indicates that administration of GLB at the time of recanalization significantly reduced brain swelling with declined mortality and improved neurological scores while administration at 10 h after onset of ischemia could barely reduce the infarction volume howbeit capable of alleviating brain injury due to the inhibition of the activation of MMP-9 and the release of antioxidants[23]. To eliminate the confounding effect of the infarction volume, delayed administration of GLB at 10 h after ischemia/reperfusion was performed when no pronounced shrinkage of infarction volume but reduced neuronal apoptosis was observed, which was in accordance with the previous study[23]. Due to the wide protective effect of GLB in CNS, further in vitro experiments were designed to explore the anti-apoptotic effect interacting between microglia and neurons.

Selective neuronal loss (SNL), featured by neuronal apoptosis in the PIA, persists after AIS and significantly impacts the prognosis in AIS patients even with timely vascular recanalization[5]. Recent data reveals that SNL has a notable correlation with microglia activation and its regulation of neuroinflammation[5, 7], but the detailed mechanisms are still unknown. The SUR1-TRPM4 complex has been increasingly recognized as an indispensable regulatory target in various brain injuries[13, 14]. Here, we illustrated that after ischemia, unlike most SUR1 and TRPM4 co-localized with neurons in the infarct core, which might be related to its explicit mechanisms mediating cytotoxic edema[24], SUR1 and TRPM4 mostly co-localized with microglia in the PIA. So far, the functions of SUR1-TRPM4 channels in microglia are still not clear. Under normal circumstances, microglia are in a static state but can be activated when subjected to abnormal stimulation, leading to rapid proliferation and migration to the injured site, and mediating neuroinflammation and neuronal apoptosis[25]. Experimental data from both *in vivo* and *in vitro* showed that the activation of SUR1-TRPM4 channels could mediate the intracellular Ca^{2+}

homeostasis, which plays a vital role in regulating the polarization of microglia between pro-inflammatory and anti-inflammatory phenotypes[13]. Hence, as observed from the current study, blocking the SUR1-TRPM4 channels might regulate the functions of microglia to promote neuronal survival.

Via sequencing the transcriptome of *Trpm4*$^{-/-}$ and WT mice after receiving tMCAO, we uncovered that *Tgf*α was highly up-regulated in *Trpm4*$^{-/-}$ mice and exerted a direct anti-apoptotic effect after ischemia. *Tgfa* encodes TGFα protein, which is a member of the epidermal growth factor (EGF) family of proteins that activate the EGF receptor (EGFR) to regulate cellular proliferation[26, 27]. TGFα has been reported to promote neuronal survival and enhance neurogenesis and angiogenesis in models of stroke and neurodegenerative diseases[28–32]. Moreover, TGFα stimulates astrocytes to polarize into a phenotype that supports neurite outgrowth after spinal cord injury[33]. Microglia-derived TGFα also regulates astrocyte activity and limits pathogenic glial actions during experimental autoimmune encephalomyelitis[34]. In the study of Hu et al.[28], tMCAO for 3 days induced the up-regulation of TGFα in microglia and neurons, which alleviated the apoptosis of oligodendrocyte lineage cells. However, we detected a slight reduction of TGFα after tMCAO for 24 h, which may be related to the time at which microglia were detrimental at 24 h after ischemia with remarkable neuronal apoptosis. Significantly, blocking the SUR1-TRPM4 channels with GLB or *Trpm4*$^{-/-}$ both boosted the expression of TGFα and alleviated neuronal apoptosis after ischemic injury, while gene silencing of *Tgfa* weakened the protective effect of GLB and 9-Phe against neuronal loss, strongly suggest TGFα as a critical regulator downstream SUR1-TRPM4. Further studies using neuronal EGFR KO mice are warranted to validate the effects of TGFα specifically on neurons after ischemic stroke.

As a growth factor, TGFα modulates multiple intracellular signaling molecules such as Akt, ERK, and STAT3, with the latter being an essential mediator of signal transduction downstream TGFα-EGFR engagement[28]. Previous studies report that the activation of STAT3 after TGFα-EGFR engagement mediates the growth of epithelial cells and several tumor cell lines[35, 36]. In physiological conditions and after exposure to cellular stress, activation of STAT3 encourages cell survival. For example, cardiac expression of STAT3 has been shown to protect cells against ischemia in the heart, by preserving mitochondrial complex I activity, reducing reactive oxygen species production, and inhibiting caspase-3 activation[37]. Our in vitro studies revealed that STAT3 activity after OGD in SH-SY5Y cells was enhanced by recombinant TGFα treatment and inhibition of STAT3 activity partly abolished the protective effects of recombinant TGFα on neuronal survival. Hence, we speculated that the application of GLB and 9-Phe elicited the release of TGFα from microglia and exerted a considerable anti-apoptotic effect on neurons, likely by its signaling transduction with STAT3 molecule.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine kinase involved in synaptic plasticity, learning, and memory[21]. CAMKII has been known to participate in cyclic adenosine monophosphate response element-binding protein (CREB) phosphorylation[21], and the activation of the CaMKII/CREB pathway is observed in different brain injury models[39]. In microglia stimulated by LPS, inhibition of SUR1-TRPM4 using GLB led to a significant increase in phosphorylated CaMKII (pCaMKII)[13]. Consistently, in the present study, we confirmed that blocking the SUR1-TRPM4

channel with GLB or 9-Phe leads to activation of the CaMKII/CREB pathway after OGD or LPS stimulation in microglia. As a step forward, we found decreased production of TGFα after pharmacological inhibition of the CaMKII/CREB. Thus, we reasonably assumed that blocking the SUR1-TRPM4 channels may activate the CaMKII/CREB signaling pathway, subsequently increasing the production and release of TGFα from activated microglia.

Conclusions

In conclusion, we characterize that blocking the SUR1-TRPM4 channels with GLB and gene deletion of *Trpm4* boosts the up-regulation and release of TGFα, which acts as an essential endogenous factor in protecting neurons from apoptosis in the PIA after stroke. Our work delivers novel insights into the mechanism of GLB-mediated neuroprotection against ischemic brain injury, favoring the clinical translation of GLB in treating ischemic stroke.

Abbreviations

SNL
Selective neuronal loss
PIA
Peri-infarct area
SUR1-TRPM4
Sulfonylurea receptor 1-transient receptor potential M4
AIS
Acute ischemic stroke
TGFα
Transforming growth factor alpha
GFAP
Glial fbrillary acidic protein
IBA1
Ionized calcium binding adapter molecule 1
NeuN
Neuronal nuclei
TUNEL
Terminal deoxynucleotidyl transferase-dUTP nick end labeling
MAP2
Microtubule-associated protein 2
DAPI
Diamidino-2-phenylindole
CaMKII
Ca^{2+}/calmodulin-dependent protein kinase II

CREB
Cyclic adenosine monophosphate response element-binding protein
MCM
Microglia conditioned medium
OGD
Oxygen-glucose deprivation
WT
Wild-type
Trpm4$^{-/-}$
Trpm4 gene deletion.

Declarations

Authors' contributions

Yihua He, Kaibin Huang and Suyue Pan designed experiments and revised the manuscript. Yuqin Peng and Xuewu Liu conducted the experiments. Yuan Chang, Jiancong Chen, Chuman Lin and Kunxue Zhang revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

All animal experiments in this study were approved by the Animal Care and Use Committee of the Nanfang Hospital, Southern Medical University (Guangzhou, China)

Consent for publication

Not Applicable.

Competing interests

The authors declare they have no competing interests.

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Figures

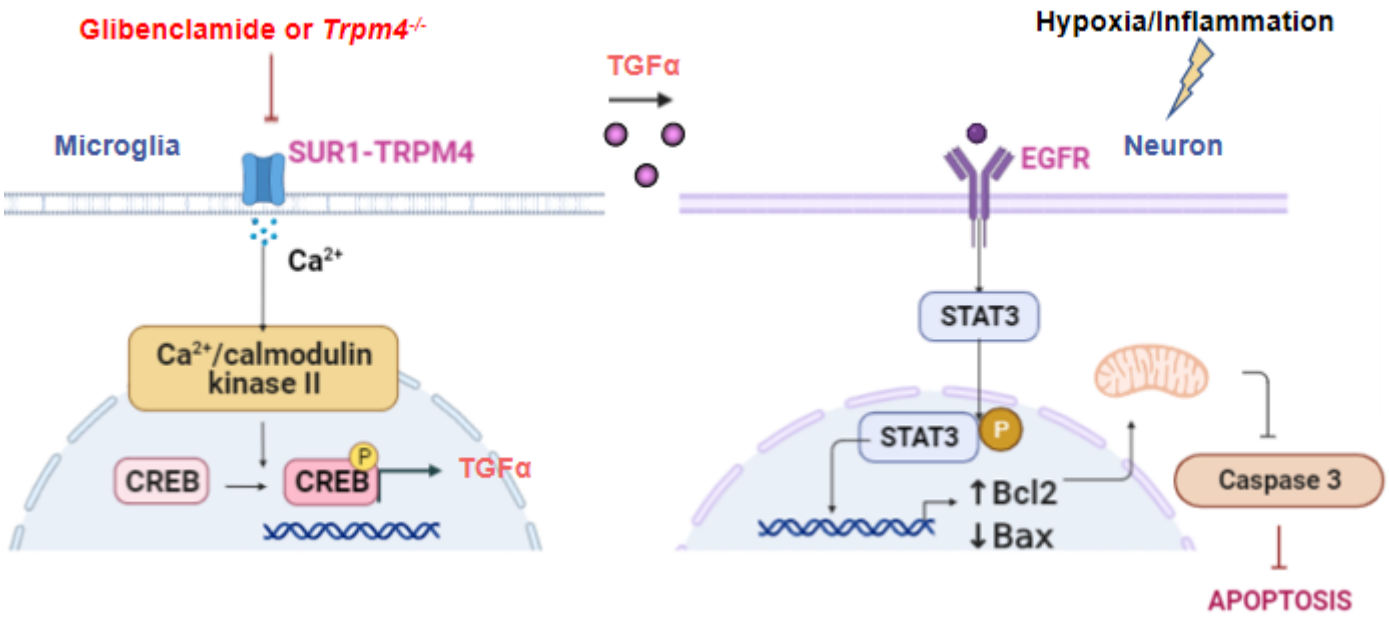

Figure 1

Illustrative image of the interaction between microglia and neurons. Blocking the SUR-TRPM4 channel of microglia by glibenclamide and gene deletion of Trpm4 induces the up-regulation and release of TGFα, alleviating the neuronal apoptosis elicited by hypoxia or inflammation.

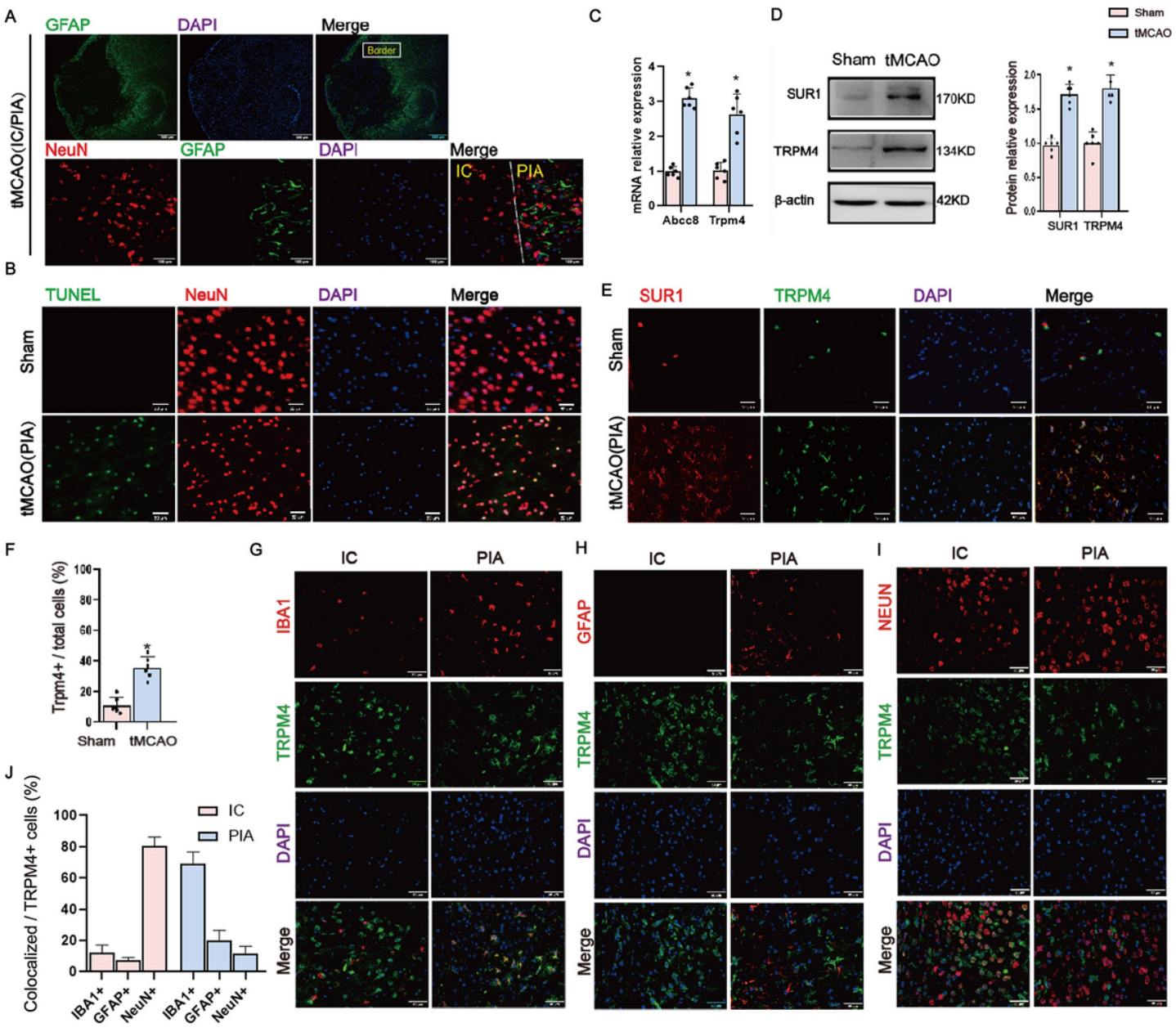

Figure 2

tMCAO induces neuronal apoptosis and the up-regulation of SUR1-TRPM4 in the PIA. (A) Double staining of NeuN and GFAP in the border area between infarct core and PIA after tMCAO; (B) Double staining of NeuN and TUNEL in the PIA after tMCAO; (C) The mRNA expression of Abcc8 and Trpm4 in the PIA detected by qPCR; (D) The protein content of SUR1 and TRPM4 in the PIA detected by WB; (E-F) Co-localized staining with SUR1 and TRPM4 antibodies in the PIA; (G) Characteristic images of colocalized staining in the IC and PIA using Iba1 and TRPM4 antibodies; (H) Characteristic images of colocalized staining in the IC and PIA using GFAP and TRPM4 antibodies; (I) Characteristic images of colocalized staining in the IC and PIA using NeuN and TRPM4 antibodies; (J) Statistical analysis of colocalized

staining in the IC and PIA using Iba-1,GFAP, NeuN and TRPM4 antibodies, respectivelys. IC: Infarct core; PIA: Peri-infarct area. *P < 0.05. N = 6 in each group.

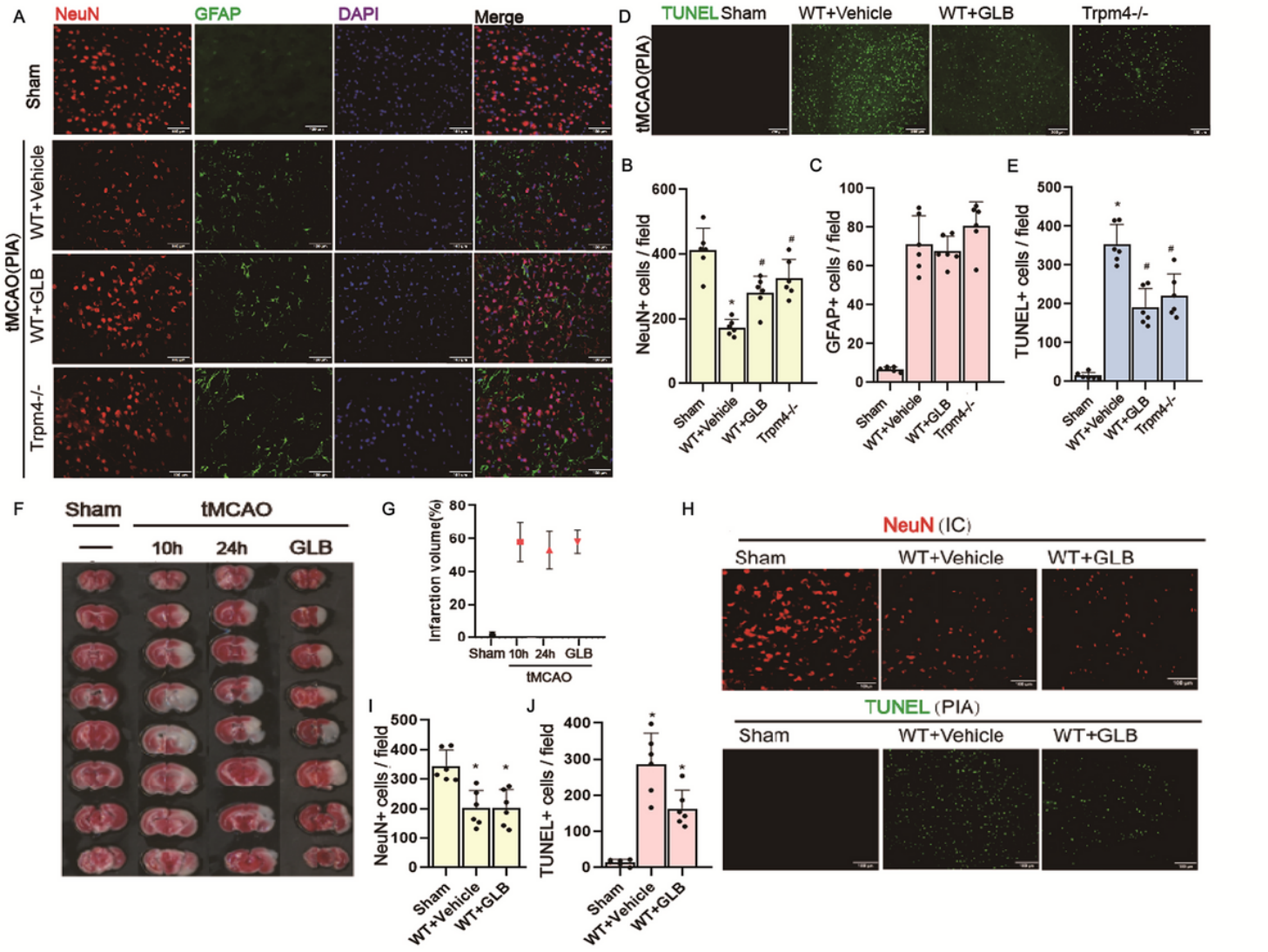

Figure 3

Effects of GLB and *Trpm4*$^{-/-}$ on infarct volume and neuronal loss after tMCAO. (A-C) Double staining and its statistically analysis of the NeuN+ and GFAP+ cells between Sham, WT+Vehicle, WT+GLB and *Trpm4*$^{-/-}$; (D-E) TUNEL staining and its statistically analysis between Sham, WT+Vehicle, WT+GLB and *Trpm4*$^{-/-}$; (F-G) Representative images and quantification of TTC-stained brain slices from each group; (H-J) Immunostaining with NeuN in the infarct core and TUNEL staining in the PIA between Sham, WT+Vehicle and WT+GLB; WT+GLB: mice were received GLB injection after ischemia for 10 h and executed at 24 h. tMCAO (10 h): mice were received vehicle injection after ischemia for 1 h and executed at 10 h. tMCAO (24 h): mice were received vehicle injection after ischemia for 1 h and executed at 24 h.

IC: Infarct core. PIA: Peri-infarct area. *P < 0.05 vs Sham group; # P < 0.05 vs WT+Vehicle group. N = 6 in each group.

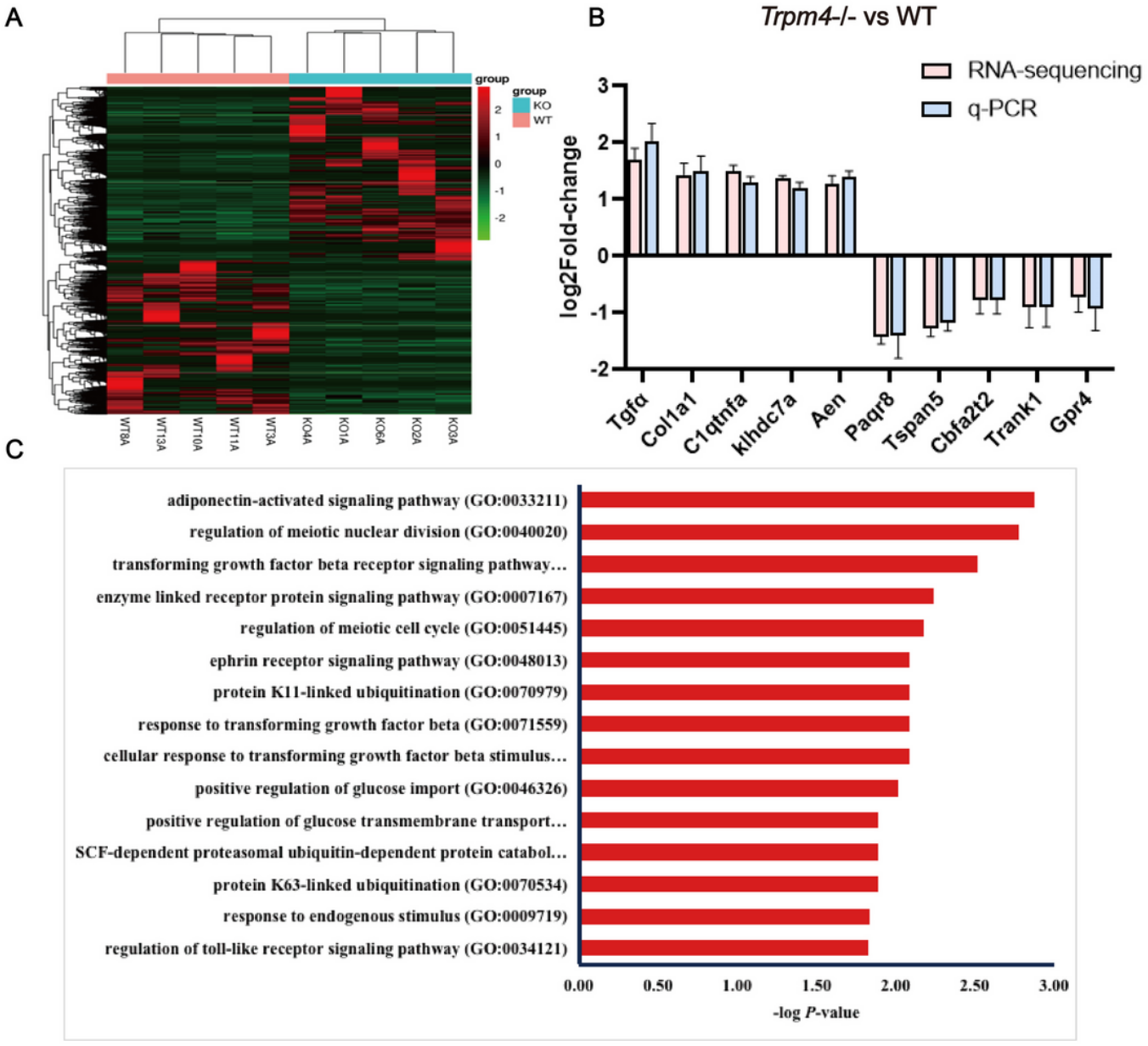

Figure 4

Differentially expressed genes and enrichment analysis between WT mice and *Trpm4*$^{-/-}$ mice after MCAO. (A) Heatmap of the RNA-sequencing results; (B) Data of RNA-sequencing and qPCR verification of the top 5 DEGs in up- and down- regulated genes; (C) GO enrichment analysis of the up-regulated genes. N = 5 in each group.

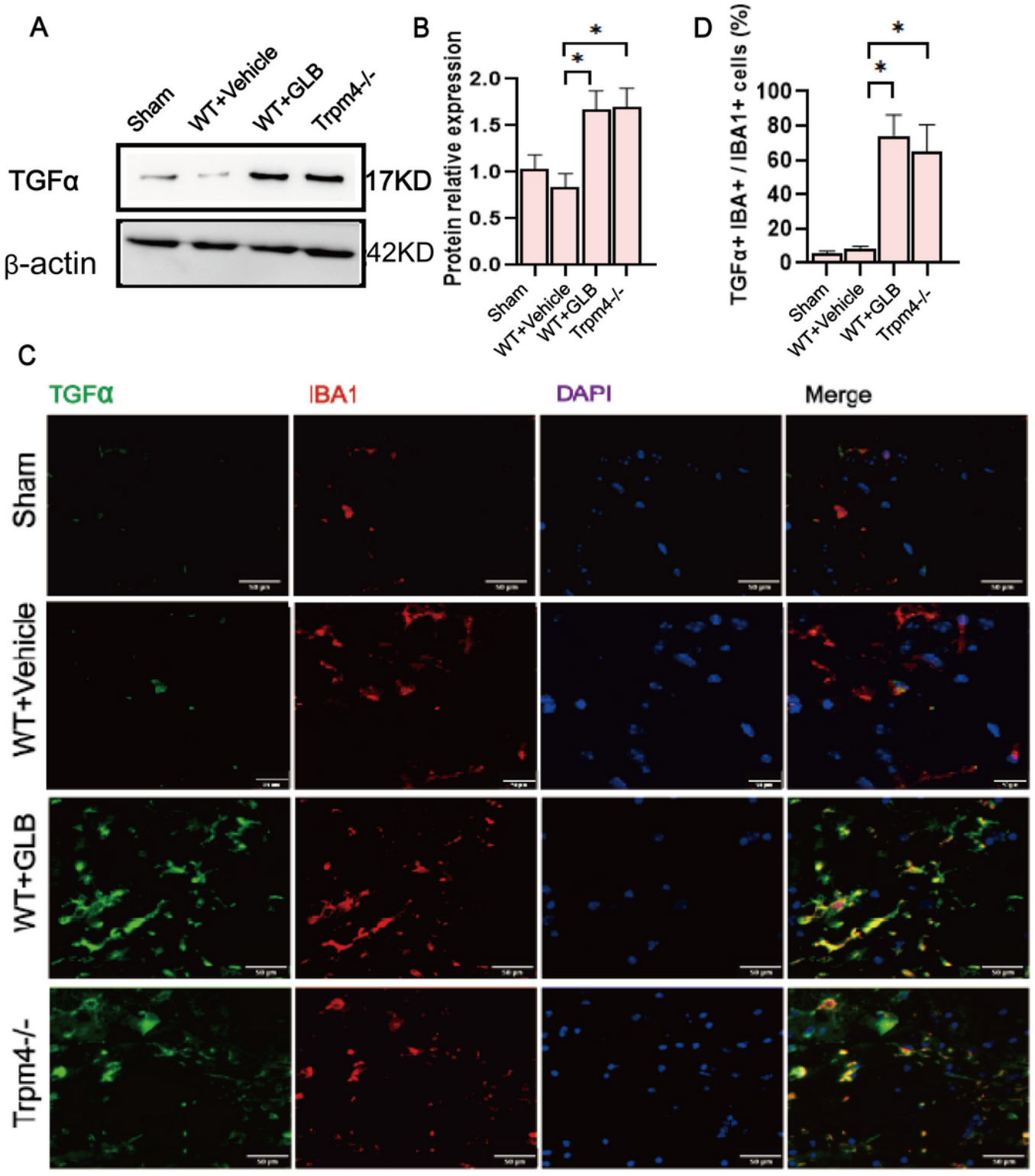

Figure 5

Application of GLB and *Trpm4*$^{-/-}$ induced the up-regulation of TGFα in microglia in the PIA. (A-B) Detection of the expression of TGFα in PIA under tMCAO condition via WB; (C-D) Co-localized staining with TGFα and Iba-1 in the PIA. * $P < 0.05$. N = 6 in each group.

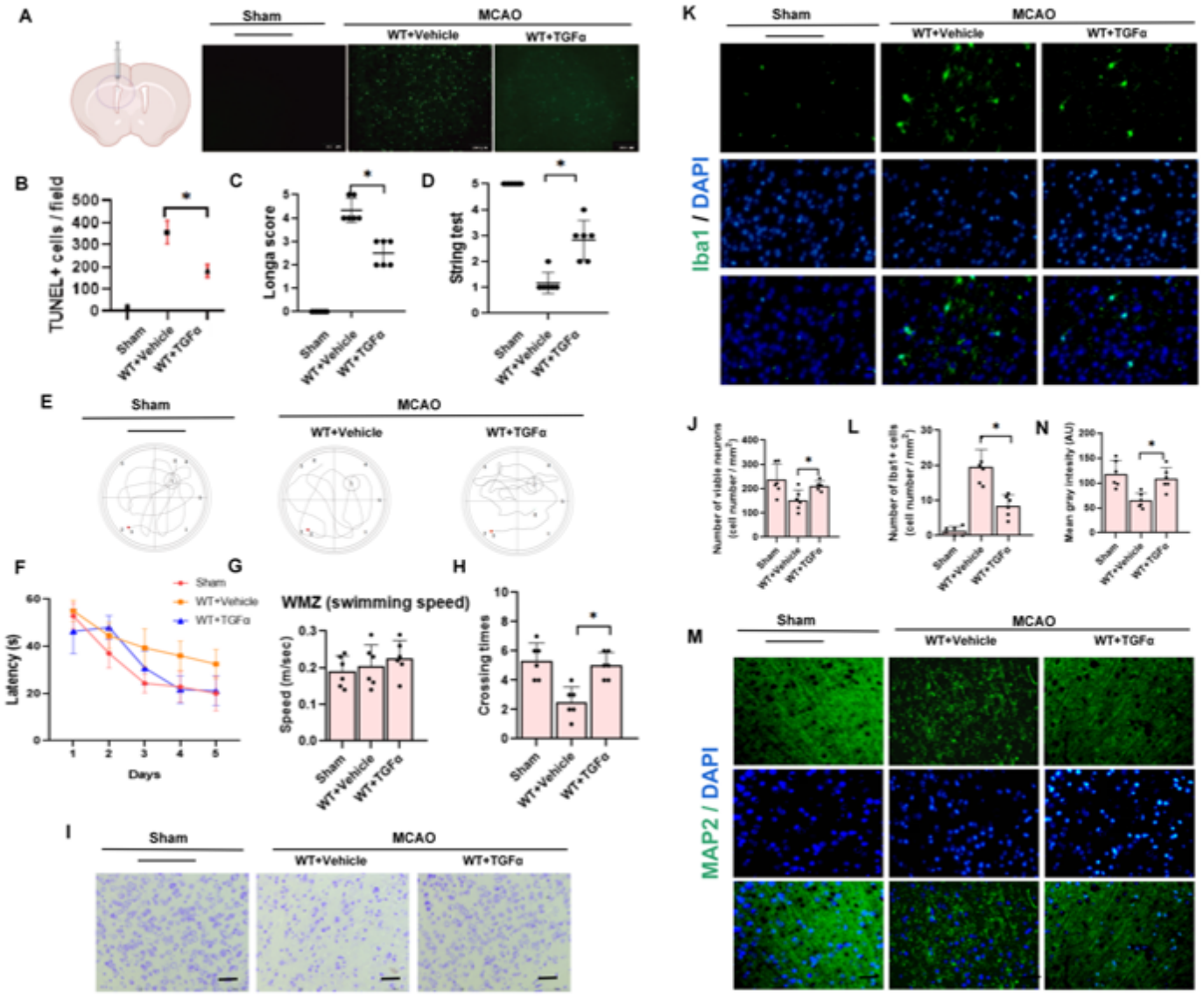

Figure 6

Intracerebroventricular administration of recombinant TGFα alleviated neuronal apoptosis and improve neurological outcome after tMCAO. (A-B) TUNEL staining after intracerebroventricular injection of TGFα in the under tMCAO condition; (C-D) Longa score and string test after MCAO for 24 h; (E-H) Swimming speed and times of the mice crossing the platform assessed by Morris water maze between different groups; (I-J) Viable neurons in the PIA assessed by Nissl staining after MCAO for 12 days; (K-L) Iba1 immunostaining in the PIA between different groups after MCAO for 12 days; (M-N) MAP2 immunostaining in the PIA between different groups after MCAO for 12 days. * $P < 0.05$. N = 6 in each group.

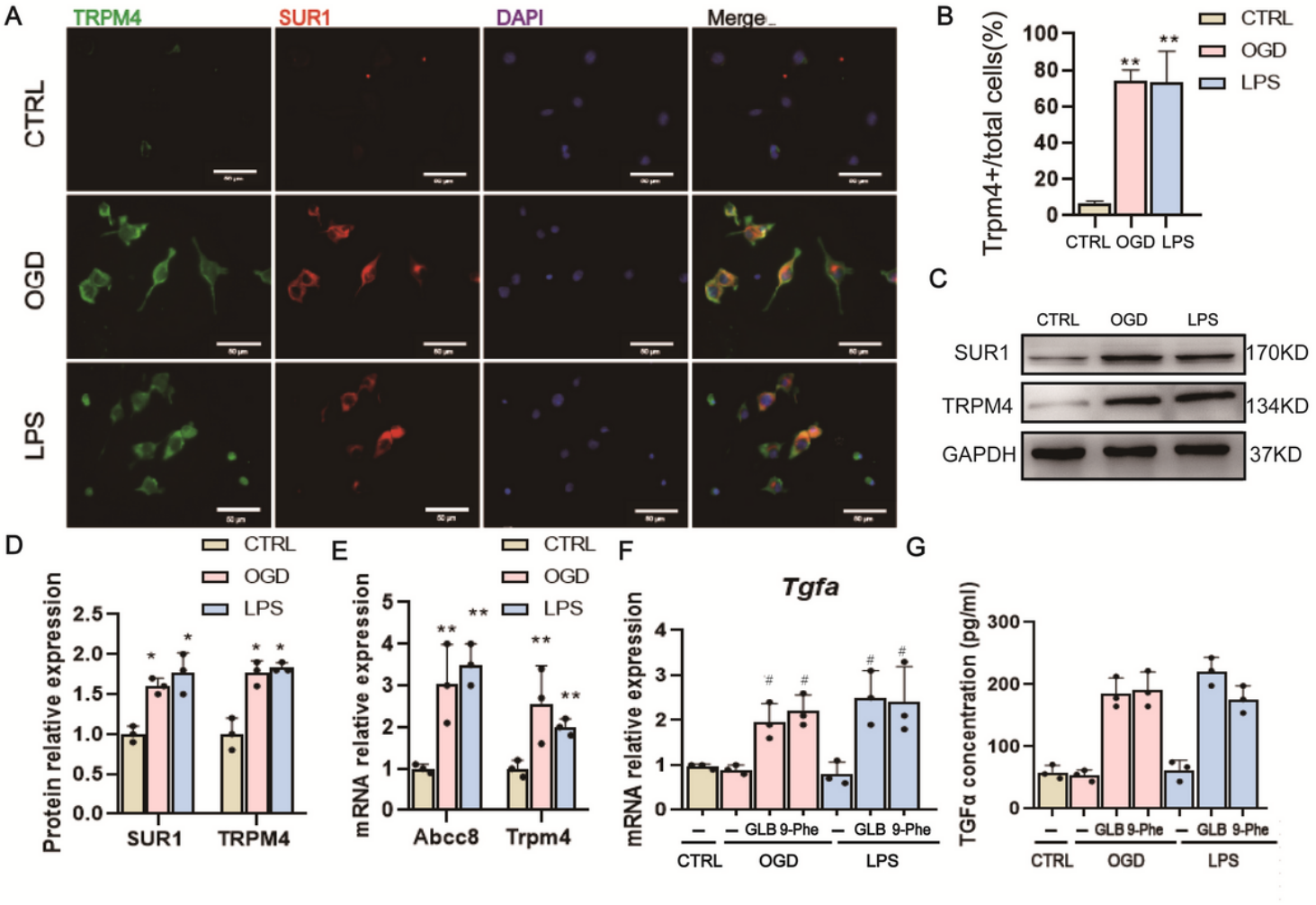

Figure 7

Expression of SUR1 and TRPM4 after OGD and LPS for 24 h in BV-2 microglia. (A-B) Immunostaining with SUR1 and TRPM4 and its statistical analysis after OGD and LPS stimulation in BV-2 microglia; (C-D) Protein content of SUR1 and TRPM4 after OGD and LPS stimulation in BV-2 microglia detected by WB; (E) mRNA expression of Abcc8 and Trpm4 detected by qPCR. (F) mRNA expression of TGFα after modeling for 24 h in BV-2 microglia detected by qPCR; (G) Concentration of TGFα in culture medium after modeling in BV-2 microglia detected by ELISA. ** $P < 0.01$ *$P < 0.05$ versus CTRL group. $^{\#}P < 0.05$ versus OGD or LPS group. N = 3 in each group.

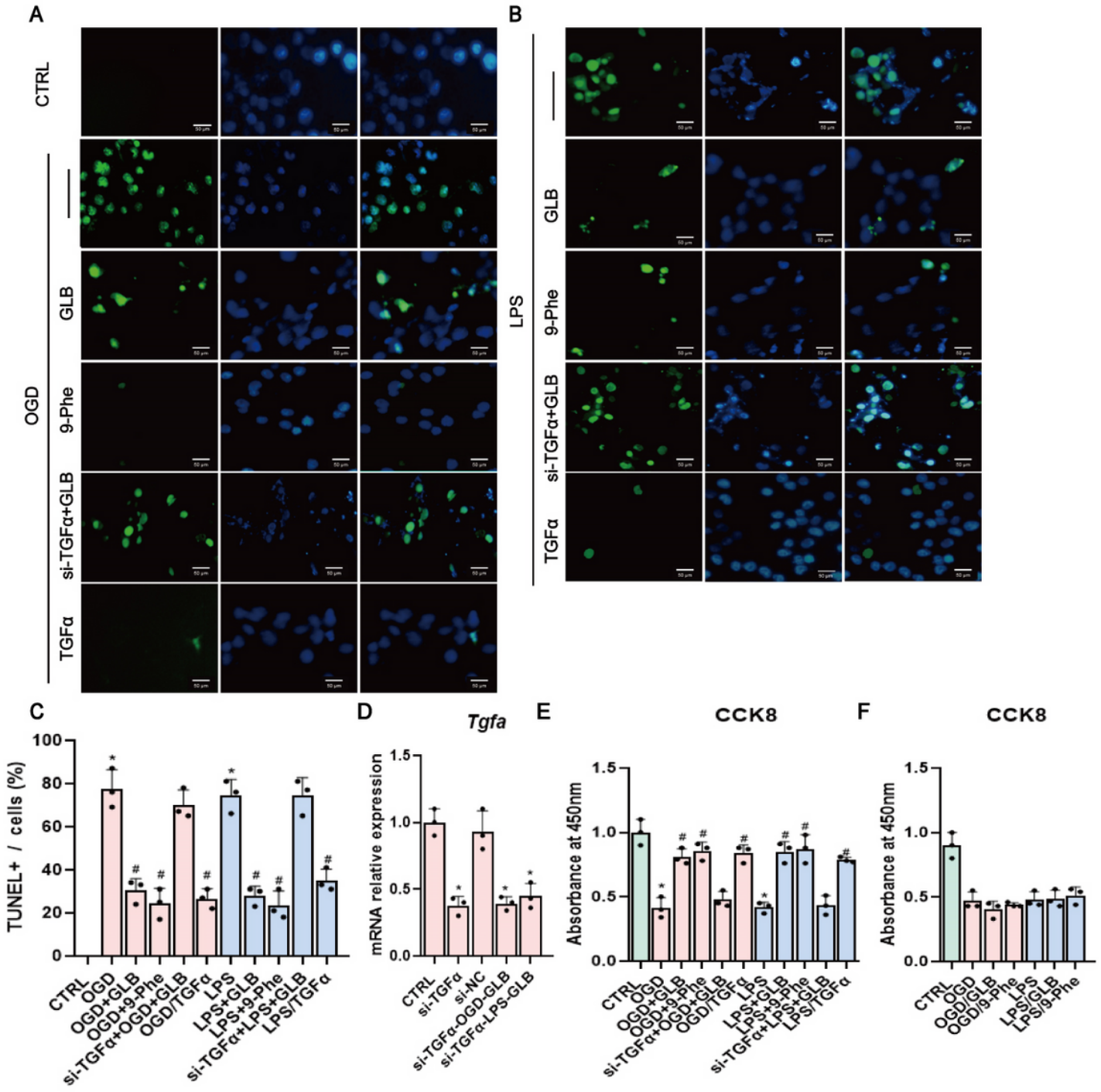

Figure 8

Detection of the survival of SH-SY5Y cultured by different MCM. (A) Detection of the apoptosis of SH-SY5Y cultured by OGD-derived MCM via TUNEL staining; (B) Detection of the apoptosis of SH-SY5Y cultured by LPS-derived MCM via TUNEL staining; (C) Statistical analysis of TUNEL staining; (D) Test of the efficiency of the siTGFα via qPCR; (E) Comparison of the survival of SH-SY5Y cultured by different MCMs via CCK8; (F) Comparison of the survival of SH-SY5Y cultured by MCM plus GLB and 9-Phe via CCK8. *P < 0.05 versus CTRL group; #P < 0.05 versus OGD or LPS group. N = 3 in each group.

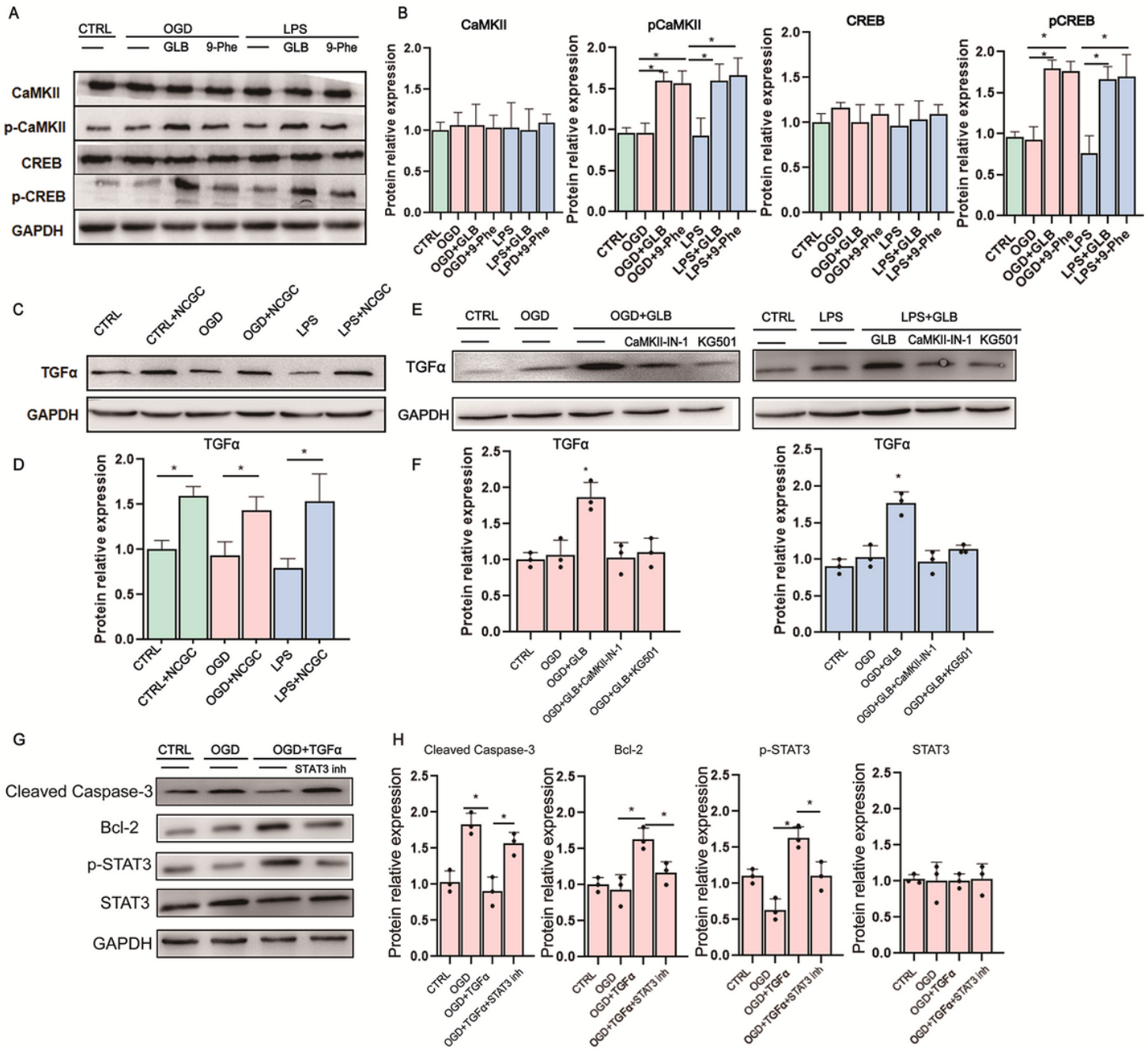

Figure 9

Expression of CaMKII/CREB signaling pathway after OGD/LPS stimulation for 24 h. (A-B) The expression of CaMKII/CREB and pCaMKII/pCREB detected by WB; (C-D) The expression of TGFα after using CREB agonists detected by WB; (E-F) The expression of TGFα after using CaMKII/CREB inhibitors detected by WB; (G-H) The protein content of STAT3 pathway in SH-SY5Y cells after MCM treatment. NCGC: 100 nM NCGC00067819, an agonist of CREB. CaMKII-IN-1: 100 nM, an inhibitor of CaMKII. KG501: 10 μM, an inhibitor of CREB. STAT3 inh: STAT3 inhibitor VI, 15 μM. * $P < 0.05$. N = 3 in each group.

Supplementary Files

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