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ORIGINAL RESEARCH

Esketamine at a Clinical Dose Attenuates Cerebral Ischemia/Reperfusion Injury by Inhibiting AKT Signaling Pathway to Facilitate Microglia M2 Polarization and Autophagy

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Purpose: This study aimed to assess the protective effect of a clinical dose esketamine on cerebral ischemia/reperfusion (I/R) injury and to reveal the potential mechanisms associated with microglial polarization and autophagy.

Methods: Experimental cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) in adult rats and simulated by oxygen-glucose deprivation (OGD) in BV-2 microglial cells. Neurological and sensorimotor function, cerebral infarct volume, histopathological changes, mitochondrial morphological changes, and apoptosis of ischemic brain tissues were assessed in the presence or absence of esketamine and the autophagy inducer rapamycin. The expression of biomarkers related to microglial M1 and M2 phenotypes in the ischemic brain tissues was determined by immunofluorescence staining and RT-qPCR, and the expression of proteins associated with autophagy and the AKT signaling pathway in the ischemic brain tissues was assayed by Western blotting.

Results: Esketamine alone and esketamine combined with rapamycin alleviated neurological impairment, improved sensorimotor function, decreased cerebral infarct volume, and mitigated tissue injury in the MCAO rats. Importantly, esketamine promoted microglial phenotypic transition from M1 to M2 in both the MCAO rats and the OGD-treated BV-2 microglia, induced autophagy, and inactivated AKT signaling. Furthermore, the effects of esketamine were enhanced by addition of autophagy inducer rapamycin.

Conclusion: Esketamine at a clinical dose attenuates cerebral I/R injury by inhibiting AKT signaling pathway to facilitate microglial M2 polarization and autophagy. Furthermore, esketamine combined autophagy inducer can provide an improved protection against cerebral I/R injury. Thus, this study provides new insights into the neuroprotective mechanisms of esketamine and the potential therapeutic strategies of cerebral I/R injury.

Keywords: cerebral ischemia/reperfusion injury, esketamine, microglia polarization, autophagy, ischemic stroke

Introduction

Stroke is an acute cerebrovascular disease characterized by a sudden disruption in cerebral blood flow, ranks as the second in the most common cause of global mortality.[1] As a predominant type of stroke, ischemic stroke accounts for 70–80% of cases, which seriously threatens the quality of life in the middle-aged and elderly populations.[2] Restoring blood supply remains the standard treatment strategy for ischemic stroke, but reperfusion of blood flow inevitably leads to serious tissue injury in the ischemic territory, which is called as cerebral ischemia/reperfusion (I/R) injury,[3,4] by mainly manifested as neuron necrosis in the core area of cerebral infarction.[5] The cerebral I/R injury can eventually cause

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neurological dysfunction and consciousness impairment. Hence, there is an urgent need to develop safer and more effective treatment strategies for ischemic strokes.

Accumulating evidence highlights the important role of microglia-mediated neuroinflammation in the pathogenesis of cerebral I/R injury.[6,7] As the resident immune cells in the brain, microglia are ubiquitously present in the central nervous system and have been regarded as the first line of defense against ischemic stroke.[8] When cerebral ischemia occurs, microglia can be rapidly activated and recruited to the ischemic and penumbra areas to participate in the monitoring of the microenvironment.[9,10] The activated microglia are able to phenotypically differentiate into the two phenotypes, that is, the "classically activated" M1 and "alternatively activated" M2. M1-like microglia secrete and release proinflammatory cytokines that exert deleterious effects on neuronal injury. In contrast, microglia with an M2-like phenotype produce anti-inflammatory mediators and facilitate repair and regeneration of damaged neuronal tissues.[11] Therefore, the exploration of effective candidates to inhibit M1 polarization of microglia or facilitate the phenotype shift of microglia from M1 to M2 has become a research hotspot for alternative therapies for ischemic stroke.

Esketamine is a novel non-competitive N-methyl-D-aspartate (NMDA)-receptor antagonist that has been approved by the FDA for therapy of treatment-resistant depression.[12] Increasing clinical studies have elucidated that esketamine promotes neuronal activation and neuroplasticity in the hippocampus.[13,14] In a rat model of propofol-induced brain injury and cognitive impairment, esketamine has been shown to improve the spatial learning and memory dysfunction, attenuate the neurological injury and suppress apoptosis.[15] Importantly, a recent work demonstrates that esketamine prevents postoperative emotional disorders and neurocognitive dysfunction through inhibition of microglia M1 polarization in ageing rats with preoperative sleep disturbance.[16] Autophagy is a highly conserved homeostatic process, during which autophagosomes devour and degrade damaged or aged organelles for further processing and recycling to maintain cellular homeostasis.[17] It is reported that esketamine can provide a protection against traumatic brain injury by inducing autophagy and relieving oxidative stress in the mice model.[18] Furthermore, enhanced microglial autophagy is attributable to the polarization of microglia into an anti-inflammatory state under brain ischemic conditions.[19,20] Thus, we hypothesized that esketamine could regulate microglial polarization and autophagy, thereby providing a protection against cerebral I/R injury. To validate this hypothesis, this experiment was designed to assess the effects of esketamine on the cerebral I/R injury, microglia M1/M2 polarization and autophagy in the experimental cerebral I/R injury model induced by middle cerebral artery occlusion (MCAO) and simulated by oxygen-glucose deprivation (OGD) in the BV-2 microglial cells. Given that the neuroprotection and neurotoxicity of ketamine are closely related to dose and frequency of administration,[21,22] this experiment selected a clinical dose of esketamine. Our main purpose was to determine the potential of clinical dose esketamine as a neuroprotective candidate for cerebral I/R injury, and to further reveal the contributions of microglial polarization and autophagy to the neuroprotection of esketamine.

Materials and Methods

Experimental Animals

The experimental protocol was approved by the Animal Care and Use Committee of Beijing Friendship Hospital, Capital Medical University (Number:22–1010, Date: 2023–02-08). Specific pathogen-free Sprague Dawley male rats (6 weeks old) were obtained from Beijing HFK Bioscience Co., Ltd. Rats were housed under the conditions with a 12-h light-dark cycle, temperature of 21–23 °C and a relative humidity of 60%-65%. All the animals had free access to rodent chow and water. The animals were allowed to acclimatize for seven days before the initiation of the experiment. All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of our institutes and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

BV-2 Cell Culture

The BV-2 microglia, provided by the BeNa Culture Collection (Beijing, China), were cultured in a high glucose Dulbecco's modified Eagle's medium (DMEM; Biosharp, Guangzhou, China) supplemented with 10% fetal bovine serum (FBS; Hyclone, UT, USA) under an atmosphere of 5% CO_2 at 37 °C.

Establishment of Cerebral I/R Injury Model

The in vivo model of cerebral I/R injury was established by the MCAO method, as previously reported.[23,24] After the animals were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg), a skin incision was made through the neck midline, and the right common carotid artery and the right external carotid artery were exposed. A 4–0 monofilament nylon suture coated with silicon was inserted into the right internal carotid artery through the external carotid stump and advanced by approximately 18 mm to block blood flow in the right middle cerebral artery. After a 2-h occlusion, the monofilament was removed to restore the blood flow of the right middle cerebral artery for 24-h of reperfusion. The rats with Longa scores of 1 to 3 were considered successful MCAO models and used for follow-up experiments.[23]

Animal Grouping and Interventions

Using a random number table generated by a computer, the animals were divided into four groups (n=10 per group): the control, MCAO, esketamine, and esketamine+rapamycin groups. Rats in the control group underwent the MCAO without occlusion of blood flow. Animals in the MCAO group underwent the MCAO. Animals in the esketamine group underwent the MCAO and received 1.6 mg/kg esketamine through the tail vein 1.5 h after cerebral ischemia. The rats in the esketamine+rapamycin group were intraperitoneally injected with the autophagy inducer rapamycin 1 mg/kg once a day for 3 consecutive days before MCAO surgery and then underwent the same interventions as those in the esketamine group. Rapamycin was prepared in 1 mL solution containing 2% dimethyl sulfoxide, 30% polyethylene glycol 300, 5% Tween 80 and 63% normal saline. The rats in other groups were intraperitoneally injected with normal saline with same volume for 3 consecutive days before MCAO surgery.

Neurological Deficit Assessment

After a 24-h reperfusion, the neurological deficits of animals were observed by the Longa scores as described in the previous study.[23] The Longa scores are classified into five grades (0–4 points). Grade I (0 points): normal performance without any neurological deficits; Grade II (1 point): contralateral forepaws cannot fully extend; Grade III (2 points): circling to the opposite side while walking; Grade IV (3 points): falling to the opposite side while walking; and Grade V (4 points): no spontaneous walking or loss of consciousness.

Motor Function Evaluation

After 24-h of reperfusion, the sticky label test was used to assess motor and neural function recovery in animals after cerebral I/R injury. As a training, this test was performed once a day for three consecutive days before MCAO surgery. Briefly, the animals were fitted in a clean test cage for 60s and two pieces of square viscose paper with a side length of 3 mm were pasted on the palms of the two forelimbs. The rats were quickly placed in the test cage to observe and record the time required to feel the paper and to tear it off.

Morris Water Maze (MWM) Test

The MWM test was carried out designed to evaluate the spatial learning and memory abilities of rats.[25,26] The apparatus consisted of a large circular pool (diameter, 150 cm; height, 40 cm; filled with depth of 30 cm water at 21.5 ± 1 °C). The escape platform was submerged 2 cm below the water surface. On the second day after MCAO surgery, that is, after 24-h of reperfusion, training was conducted for 5 consecutive days. In the first 5 days, the platform position was fixed, and each animal was trained four times a day. Each time, a quadrant was randomly selected as the water inlet point, and the rats were slowly placed in the water facing the pool wall and allowed to rest on the platform for 15s after reaching it in less than 60 s. If it took more than 60 s to reach the platform, the investigator led it to stay on the platform for 15 s. Escape latency and swimming distance were recorded. On the sixth day, the platform was removed, and the rat was placed in the water at the same location as before to swim freely for 60 s. The number of times the rats crossed the quadrant where the platform was located and the time spent in the target quadrant were measured and calculated. The

ANY-maze video tracking system (Shanghai Jilang Software Technology Co., LTD, China) with a CCD camera was used to analyze MWM behaviors.

Evaluation of Infarct Volume

After 24-h of reperfusion and completion of neurological assessment, the rats were euthanized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg). The brain was then rapidly removed and sliced into five coronal sections with a thickness of 2 mm from the anterior to the posterior side. These brain tissues were stained with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC; Servicebio, Wuhan, China) solution at 37 °C for 15 min. Next, 4% paraformaldehyde was used to fix and preserve brain slices overnight. The infarcted brain tissues were stained white and the non-infarcted regions were stained red. Images were photographed using an inverted microscope (Leica, Germany), and the infarct volume was calculated using the ImageJ software (National Institutes of Health, USA). The infarct area of each layer of the brain slices was calculated, and the sum of the infarct area of each brain slice was multiplied by the thickness (2 mm) to obtain the total infarct volume. The Infarct volume (%) = infarct volume/total volume×100%.[27]

Histopathological Changes of Brain

After 24-h reperfusion, histopathological changes in the ischemic area of the brain were analyzed using hematoxylin and eosin (H&E) staining. Briefly, the brain tissues were placed in 4% paraformaldehyde for 24 h. Paraffin-embedded brain sections (coronally sliced into 5 μm-thick sections) were prepared and stained with H&E. Histopathological changes in the hippocampus on the ischemic side were observed and images were taken under an inverted microscope (Leica).

Morphological Observation of Mitochondria

After a 24-h reperfusion, the fresh brain tissue in the ischemic area was fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in an ascending alcohol series, and embedded in epoxy resin. The ultrathin sections were stained with uranyl acetate and lead citrate. Mitochondrial morphology was observed using a transmission electron microscopy (Hitachi, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

Paraffin-embedded sections of ischemic brain tissues (5-μm thick) were conventionally dewaxed with xylene and rehydrated with gradient ethanol. After permeabilization with 20 μg/mL protein kinase K for 20 min, apoptosis was assessed using the TUNEL assay (KeyGEN Biotech, Nanjing, China) according to the manufacturer's recommendations. The cells were observed using an optical microscope (Leica). The percentage of apoptotic cells in the ischemic brain tissues was quantified using the ImageJ software (National Institutes of Health, USA).[28]

Oxygen-Glucose Deprivation (OGD) Model and Interventions

The OGD model of BV-2 cells was established to mimic cerebral I/R injury, as previously described.[29,30] The BV-2 cells were cultured in the glucose-free DMEM for 6 h, 12, and 24 h in an incubator with 94% N_2, 5% CO_2 and 1% O_2 at 37 °C. To determine the appropriate time for OGD treatment to cause microglial activation, BV-2 cells were exposed to the OGD treatment for 6, 12, and 24 h. As the percentage of Iba-1$^+$CD16$^+$ positive cells was highest when the OGD treatment lasted for 12 h, a 12-h OGD treatment was selected for the following experiment, in which BV-2 cells were pretreated with 15 mg/L esketamine with or without 100 μmol/L rapamycin for 1 h before OGD treatment for 12 h.

Cell Viability Assay

Cell viability was evaluated using a cell counting kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan). After the indicated treatments, the BV-2 cells were treated with 10 μL CCK-8 solution at 37 °C for 2 h according to the manufacturer's recommendations. The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Houston, USA).

Immunofluorescence Assay

Microglial polarization was analyzed by co-immunostaining for Iba-1 and cluster of differentiation (CD)16, or Iba-1 and CD206. Paraffin-embedded brain sections (5-μm thick) were conventionally dewaxed with xylene and rehydrated with gradient ethanol. The sections were permeabilized with 0.1% Triton X-100 (BioFROXX) for 3 min, followed by 5% bovine serum albumin (BSA; BioFROXX) for 1 h. For the immunofluorescence staining, the BV-2 cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 (BioFROXX) for 3 min. Subsequently, the cells were blocked with 5% BSA (BioFROXX) for 1 h at 37 °C. Then, the slides were exposed with Iba-1 (Servicebio, Wuhan, China), CD16 (Proteintech, Chicago, IL, USA), and CD206 (Proteintech, Chicago, IL, USA) antibodies at 4 °C overnight. After washing three times with PBS, the slides were incubated with the Cy3-labeled goat anti-rabbit IgG (Servicebio, Wuhan, China) or the FITC-labeled goat anti-mouse IgG (Servicebio, Wuhan, China) antibody for 1 h at 37 °C. The nuclei were counterstained with Hoechst 33258 in the dark. Images were obtained using an inverted fluorescence microscope (Leica).

Real-Time Quantitative PCR (RT-qPCR) Assay

Total RNA was isolated from brain tissues or cultured BV-2 cells using TRIzol reagent (Biosharp). Complementary DNA was synthesized by reverse transcription using Vazyme HiScriptIIQ RT SuperMix (Cronda, Beijing, China) according to the manufacturer's instructions. The qPCR was performed using SYBR Green PCR Master Mix (Takara, Toyobo, Japan) on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The results were expressed as the fold change from the control and analyzed using the $2^{-\Delta\Delta Ct}$ method.[31] β-actin was used as an endogenous control. Primer sequences used in this study are listed in Table 1.

Western Blotting

Total proteins were extracted from the brain tissues and BV-2 cells using RIPA lysis buffer (Biosharp) and quantified using bicinchoninic acid (BCA) protein assay kits (Biosharp) according to standard protocols. Equal amounts of protein (40 μg/lane) were loaded into each well of a 12% SDS-PAGE gel for electrophoresis. The separated proteins were transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% BSA (BioFROXX) for 1 h. Subsequently, the membranes were probed with primary antibodies against Belin1 (Proteintech, Chicago, IL, USA), LC3II/LC3I (Proteintech, Chicago, IL, USA), phospho-protein kinase B (p-AKT(Ser473)) (BIOSS, Beijing, China),

Table 1 The Primer Sequences Used in RT-qPCR

Genes	Forward (5′ to 3′)	Reverse (5′ to 3′)
In vivo experiment		
CD16 (rat)	ATTTACAGAATGGCAAAGGCAAGA	GGTAGAAAGCTGGAGGGAGACG
iNOS (rat)	TTGGAGCGAGTTGTGGATTGTT	GGTGAGGGCTTGCCTGAGTGA
CD206 (rat)	CCTGGCCGCGTTCTTTCA	GGCGTCTTGGACCCATTTCTC
Arg-1 (rat)	TTTACAAGACAGGGCTACTTTCAGG	TGATTACCTTCCCGTTTCGTTC
TNF-α (rat)	GGCCACCACGCTCTTCTGTC	TGGGCTACGGGCTTGTCACTC
IL-1β (rat)	TCCCTGAACTCAACTGTGAAATAG	GAAGCTCCACGGGCAAGA
IL-10 (rat)	ACTGCTATGTTGCCTGCTCTTAC	TGTGGGTCTGGCTGACTGG
β-actin (rat)	CACCCGCGAGTACAACCTTC	CCCATACCCACCATCACACC
In vitro experiment		
CD16 (mouse)	AATGCACACTCTGGAAGCCAA	CACTCTGCCTGTCTGCAAAAG
iNOS (mouse)	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
CD206 (mouse)	CTCTGTTCAGCTATTGGACGC	TGGCACTCCCAAACATAATTTGA
Arg-1 (mouse)	CTCCAAGCCAAAGTCCTTAGAG	GGAGCTGTCATTAGGGACATCA
TNF-α (mouse)	GGAACACGTCGTGGGATAATG	GGCAGACTTTGGATGCTTCTT
IL-1β (mouse)	TGCCACCTTTTGACAGTGATG	TTCTTGTGACCCTGAGCGAC
IL-10 (mouse)	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG
β-actin (mouse)	GTCCCTCACCCTCCCAAAAG	GCTGCCTCAACACCTCAACCC

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AKT (BIOSS, Beijing, China), and β-actin (Servicebio, Wuhan, China) at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibody for 1 h. The blotted protein bands were visualized by enhanced chemiluminescence detection system (EMD Millipore Corporation, Massachusetts, USA). The grayscale was calculated using ImageJ software (National Institutes of Health, USA). The constitutively expressed protein, β-actin, was used as a loading control.

Statistical Analysis

Data are displayed as the mean ± standard deviation (SD), and statistical tests were performed using GraphPad Prism 8.0 version (GraphPad Software Inc., USA). Comparisons between multiple groups were performed using one-way analysis of variance with post-hoc Tukey's test. Statistical significance was set at a P value of less than 0.05.

Results

Esketamine Alleviated Neurological Dysfunction and Sensorimotor Impairments by MCAO Surgery

As shown in Figure 1A, the MCAO group compared with the control group exhibited a significantly increased Longa score, which was decreased by esketamine. Furthermore, the Longa score was significantly lower in the esketamine + rapamycin group than in the esketamine group. The sticky-label test revealed that the MCAO surgery resulted in significantly increased time required for contacting and removing the sticker in the left forelimb (Figure 1B and C). However, these effects were significantly decreased by treatment with esketamine with or without rapamycin. The MWM test indicated that all tested animals displayed a progressive reduction in escape latency and swimming distance with training, suggesting that the rats learned the location of the platform had been placed (Figure 1D–F). However, escape latency and swimming distance were significantly prolonged in the MCAO group compared to those in the control group, implying that the MCAO surgery had a negative effect on neurocognitive function. In contrast, esketamine alone or in combination with rapamycin markedly shortened escape latency and swimming distance, demonstrating an improvement in MCAO-induced neurocognitive impairments. Moreover, compared with the control group, the MCAO group showed a significantly decreased number of crossings and time spent in the target quadrant, which were increased in the Esketamine and Esketamine+Rapamycin groups (Figure 1G and H). These results indicated that esketamine attenuates neurological dysfunction and sensorimotor impairments induced by MCAO surgery.

Esketamine Reduced Cerebral Infarct Volume, Injury Severity and Apoptosis by MCAO Surgery

No cerebral infarction occurred in the control group because there was no ischemic intervention. However, the MCAO surgery produced a significant infarct volume in the right brain. The infarct volume in the right brain was significantly decreased in the esketamine and esketamine + rapamycin groups compared to that in the MCAO group, but the difference was not statistically significant (Figure 2A). Similarly, the control group had normal histopathological manifestations in the brain, that is, the neuronal structures were clear and the cell membrane was intact. However, the MCAO surgery resulted in significant histopathological abnormalities in the right ischemic brain area, such as degeneration, necrosis, and neuronal arrangement disorder. Furthermore, esketamine alone or esketamine combined with rapamycin significantly attenuated the histopathological abnormalities in the right ischemic brain area induced by the MCAO surgery, particularly esketamine combined with rapamycin (Figure 2B). Transmission electron microscopy indicated that the neuronal membrane was severely damaged, the nuclear membrane was damaged, the ridges were reduced and blurred, and the number of mitochondria was obviously decreased in the ischemic brain tissues of the MCAO group, which was significantly attenuated by esketamine combined with or without rapamycin (Figure 2C). Additionally, the MCAO surgery caused a significant increase in TUNEL-positive cells in the ischemic brain tissues, but esketamine alone or esketamine combined with rapamycin significantly reduced the MCAO surgery-induced increase in TUNEL-positive cells in the ischemic brain tissues (Figure 2D). These data revealed that esketamine decreased the cerebral infarct volume, tissue injury severity, and apoptosis induced by MCAO surgery.

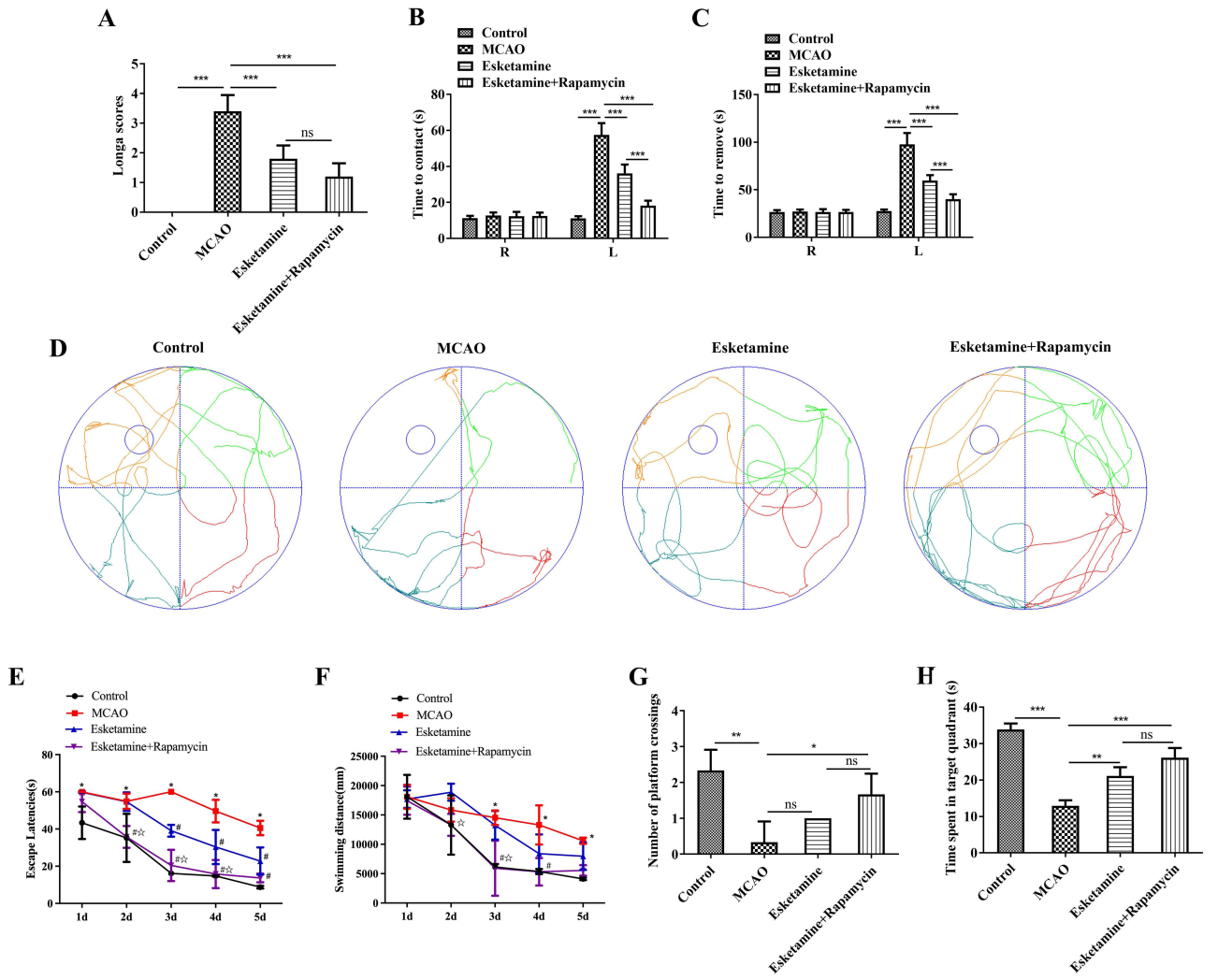

Figure 1 Esketamine alleviated neurological impairments and sensorimotor dysfunction by MCAO surgery. (**A**) Longa score (***P<0.001). (**B** and **C**) The times required to contact and to remove the sticker during the double-sided sticker removal experiment (***P<0.001). (**D**) Navigational path map of the MWM test in each group; (**E** and **F**) Mean daily escape latencies and swimming distance of rats in the MWM test (*P<0.05 vs control group; #P<0.05 vs MCAO group; *P < 0.05 vs Esketamine group). (**G** and **H**) Numbers of platform crossing and time spent in the target quadrant of rats during the probe trial (*P<0.05, **P<0.01, ***P< 0.001). n=10.
Abbreviations: MCAO, middle cerebral artery occlusion; MWM, Morris Water Maze; R, right side; L, left side; d, day.

Esketamine Promoted the Phenotypic Transition of Microglia from M1 to M2 in the MCAO Rats

Both immunofluorescence staining and RT-qPCR assays were used to measure the expression of biomarkers associated with microglial phenotypes: Iba-1^{+}CD16^{+}, inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) for M1 microglia, and Iba-1^{+}CD206^{+}, Arginase-1 (Arg-1), and interleukin-10 (IL-10) for M2 microglia. As shown in Figure 3A and B, the expression of Iba-1^{+}CD16^{+} in the ischemic brain tissues was significantly increased, and the expression of Iba-1^{+}CD206^{+} was significantly lower in the MCAO group than in the control group. However, esketamine alone or esketamine combined with rapamycin significantly restored the above expression changes caused by the MCAO surgery in both Iba-1^{+}CD16^{+} and Iba-1^{+}CD206^{+}, especially esketamine combined with rapamycin. Compared with the control group, the mRNA expression of CD16, iNOS, TNF-α, and IL-1β in the ischemic brain tissues was significantly elevated, but the mRNA expression of CD206, Arg-1, and IL-10 in the ischemic brain tissues was evidently reduced in the MCAO group. Both esketamine alone and esketamine combined with rapamycin significantly alleviated the expression changes of the above biomarkers induced by MCAO surgery, and the weakening effects of esketamine combined with rapamycin on the expression changes of these biomarkers were stronger than those of

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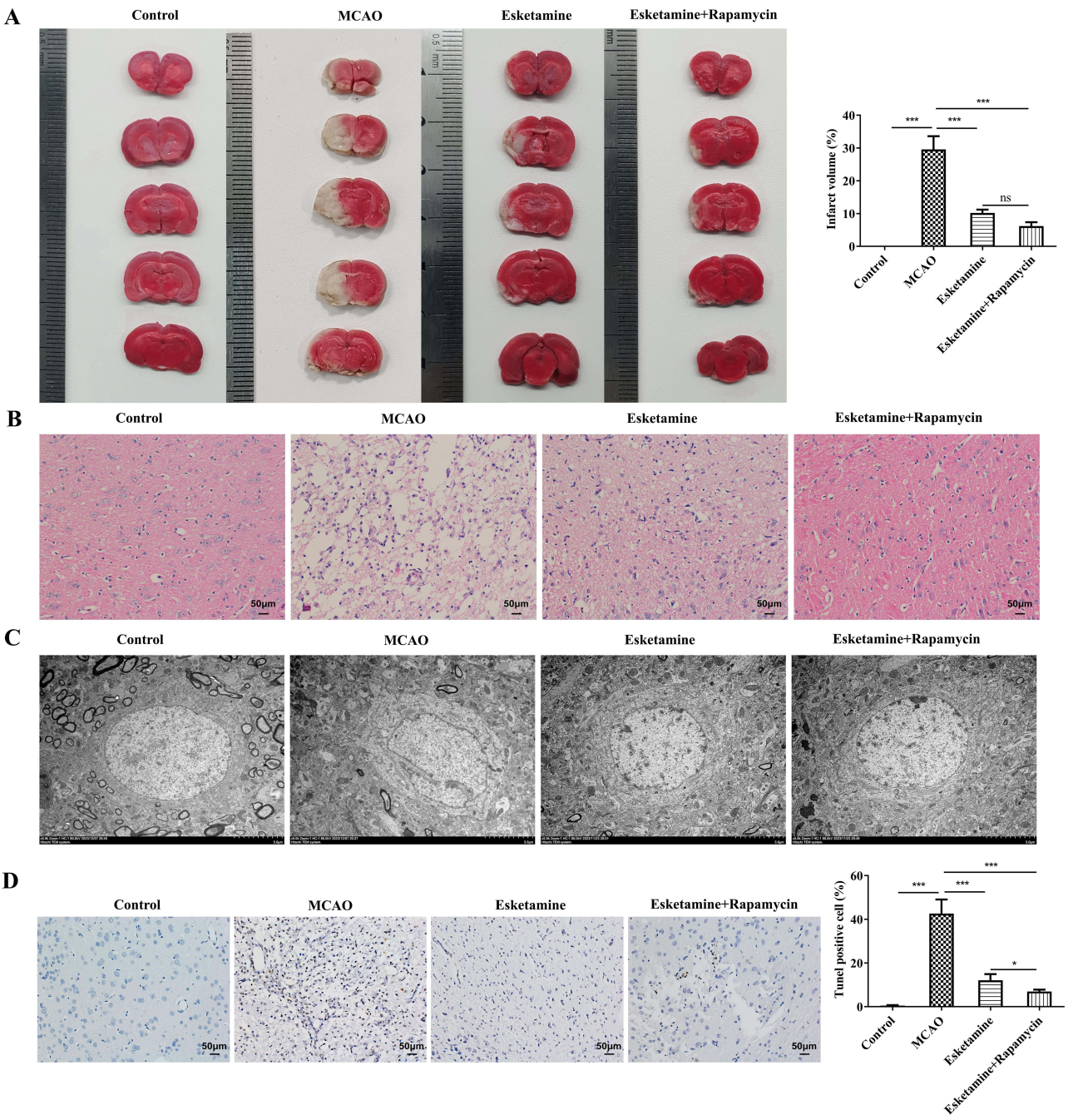

Figure 2 (**A**) Representative TTC-stained cerebral slices and the between-group comparisons of infarct volume; (**B**) The histopathological changes of hippocampus in the ischemic side with H&E staining. Scale bar = 50 μm; (**C**) Mitochondrial morphological changes using transmission electron microscope. Scale bar = 5 μm; (**D**) Representative pictures of apoptosis in the ischemic brain tissues using TUNEL staining and the between-group comparisons for the percents of TUNEL positive cells. *P<0.01; ***P<0.001. Scale bar = 50 μm. n=5.

Abbreviations: MCAO, middle cerebral artery occlusion; TTC, 2% 2, 3, 5-triphenyltetrazolium chloride; H&E, hematoxylin-eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP Nick end labeling.

esketamine alone (Figure 4A and B). These results indicate that the MCAO surgery facilitates microglial M1 phenotype expression and inhibits microglial M2 phenotype expression, whereas esketamine may reverse these changes induced by the MCAO surgery by promoting the phenotypic transition of microglia from M1 to M2.

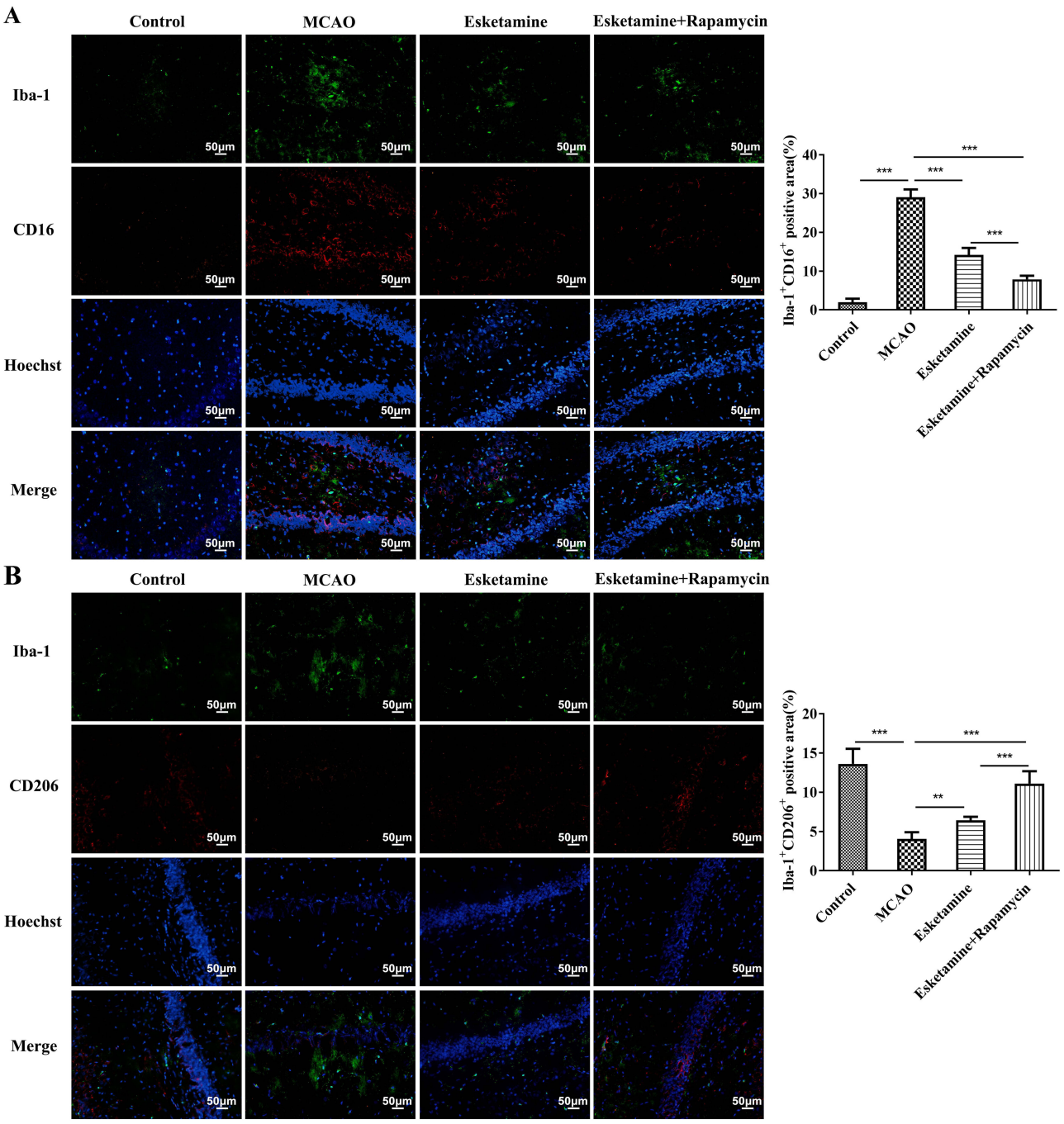

Figure 3 Esketamine promoted the phenotype transition of microglia from M1 to M2 in the MCAO rats. Representative images of double immunofluorescence staining for Iba-1$^+$CD16$^+$ (**A**) and Iba-1$^+$CD206$^+$ (**B**) in the peri-infarct regions and the between-group comparisons of their quantitative data. **P < 0.01, ***P<0.001. Scale bar = 50 μm. n=5.

Abbreviation: MCAO, middle cerebral artery occlusion.

Esketamine Enhanced Autophagy and Inhibited AKT Signaling Activation in the MCAO Rats

Western blotting was performed to determine the cerebral expression of autophagy biomarkers including Beclin1 and LC3II/LC3I. As shown in Figure 5A, the expression of Beclin1 and LC3II/LC3I in the ischemic brain tissue was significantly downregulated by the MCAO surgery. Esketamine significantly decreased the inhibitory effects of MCAO surgery on the cerebral expression of Beclin1 and LC3II/LC3I. The addition of rapamycin further enhanced the effects of

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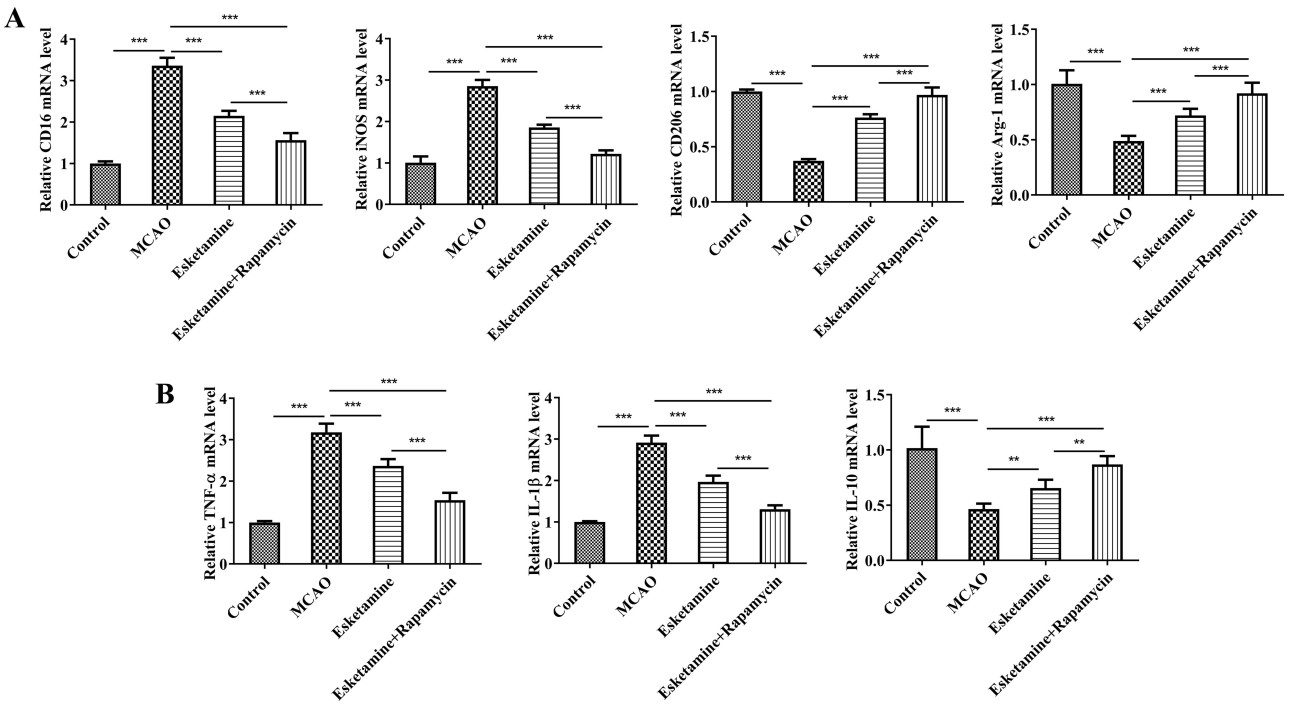

Figure 4 Esketamine promoted the expression of microglia M2 phenotype biomarkers and inhibited the expression of microglia M1 phenotype markers in the MCAO rats. (**A**) The between-group comparisons for mRNA expression of microglia M1 (CD16 and iNOS) and M2 (CD206 and Arg-1) phenotype biomarkers in the ischemic brain tissues by RT-qPCR; (**B**) The between-group comparisons for mRNA expression of TNF-α, IL-1β and IL-10 in the ischemic brain tissues by RT-qPCR. **P < 0.01, ***P < 0.001. n=5.
Abbreviations: MCAO, middle cerebral artery occlusion; iNOS, inducible nitric-oxide synthase; Arg-1, Arginase-1; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-10, interleukin-10.

esketamine on the cerebral expression of the above biomarkers, but significant between-group differences were not observed. Additionally, p-AKT expression in the ischemic brain tissues was elevated by the MCAO surgery compared to that in the control group (Figure 5B). Both esketamine alone and esketamine combined with rapamycin significantly inhibited the cerebral expression of p-AKT after MCAO surgery; however, the inhibitory effects of esketamine combined with rapamycin were more significant than those of esketamine alone. These results indicate that esketamine enhances autophagy and inhibits AKT signaling activation in the MCAO rats.

The OGD Treatment Suppressed Phenotypic Transition of Microglia from M1 to M2

The results of in vitro experiments using BV-2 microglia are shown in Figure 6A and B. The OGD treatment significantly increased the percentage of Iba-1$^+$CD16$^+$ cells and decreased that of Iba-1$^+$CD206$^+$ cells. As the percentage of Iba-1$^+$CD16$^+$-positive cells was the highest when the OGD treatment lasted for 12 h, a 12-h OGD treatment was selected for further experiments. Collectively, OGD treatment suppressed the transition of microglia from the M1 to M2 phenotype.

Esketamine Increased the Viability of OGD-Treated BV-2 Microglia and Facilitated the Phenotypic Transition of Microglia from M1 to M2

As displayed in Figure 7A, 12-h OGD treatment resulted in significantly decreased viability of BV-2 microglia compared to the control treatment. However, both esketamine alone and esketamine combined with rapamycin significantly increased the viability of OGD-treated BV-2 microglia, and the effect of esketamine combined with rapamycin was significantly stronger than that of esketamine alone (Figure 7B and C). Moreover, both the increase in the M1 microglia labeled with Iba-1$^+$CD16$^+$ and the decrease in the M2 microglia labeled with Iba-1$^+$CD206$^+$ induced by the OGD treatment were reversed by the addition of esketamine with or without rapamycin. In addition, the OGD treatment significantly elevated the mRNA expression of M1 microglia-related biomarkers (CD16 and iNOS) but reduced the mRNA expression of M2 microglia-related biomarkers (CD206 and Arg-1), upregulated TNF-α and IL-1β expression, and downregulated IL-10 expression (Figure 8A and B). Esketamine significantly inhibited the effects of OGD treatment

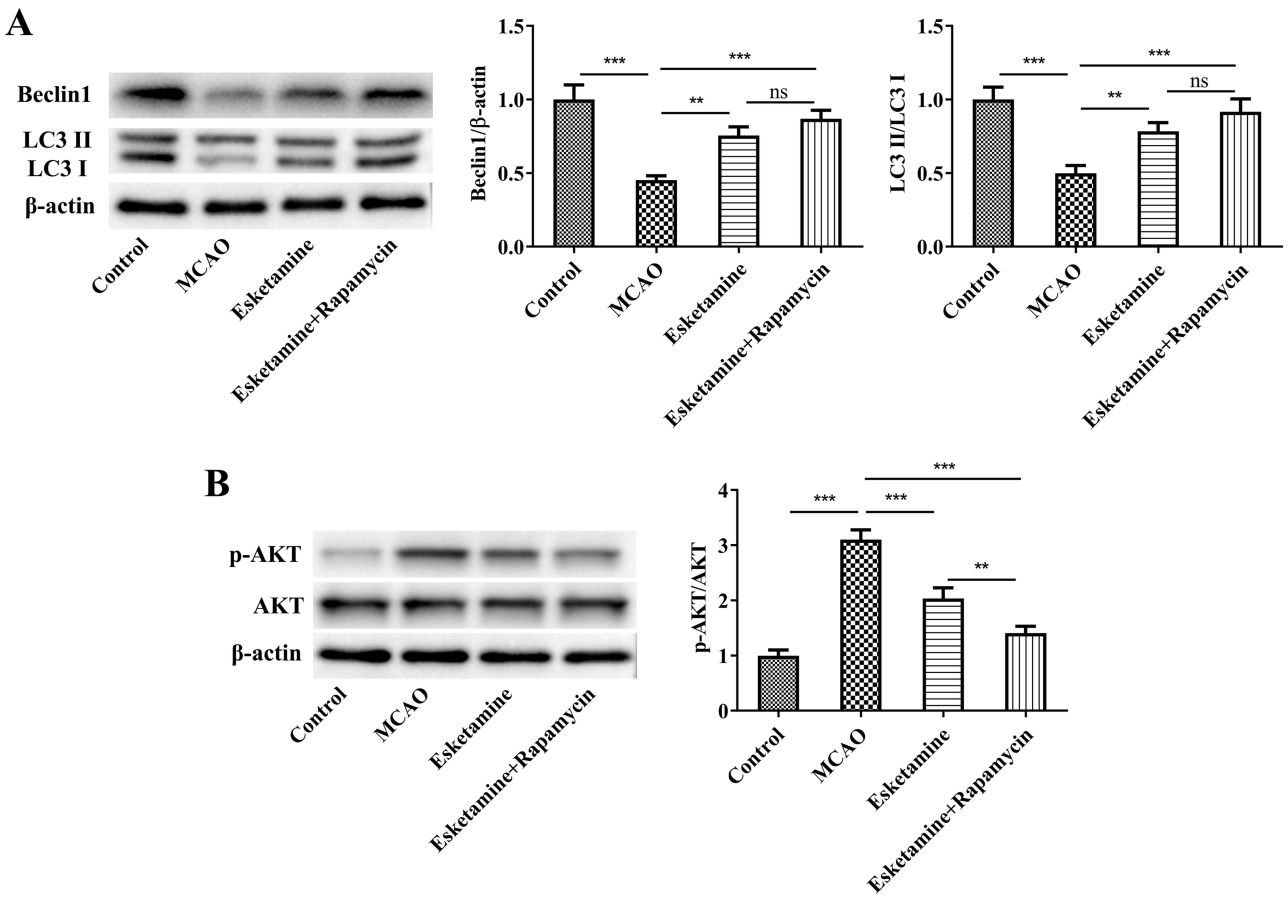

Figure 5 Esketamine enhanced autophagy and inhibited AKT signaling activation in the MCAO rats. (**A**) Representative Western blotting images for expression of autophagy-related markers in the ischemic brain tissues and the between-group comparisons of their quantitative data; (**B**) Representative Western blotting images for expression of p-AKT and AKT in the ischemic brain tissues and the between-group comparisons of their quantitative data. **P<0.01, ***P<0.001. Scale bar = 50 μm. n=5.
Abbreviations: MCAO, middle cerebral artery occlusion; AKT, serine/threonine protein kinase.

on the expression of the above-mentioned biomarkers in the BV-2 microglia, and the effects of esketamine were significantly enhanced by the addition of rapamycin. These results indicate that esketamine increases the viability of OGD-treated BV-2 microglia and facilitates the phenotypic transition of microglia from M1 to M2.

Esketamine Inhibited AKT Signaling Activation in the OGD-Treated BV-2 Microglia

The expression of p-AKT in the OGD-treated BV-2 microglia was detected using Western blotting. As shown in Figure 9A and B, the OGD treatment caused significantly elevated expression of p-AKT in BV-2 microglia compared to the control group. However, esketamine significantly attenuated the effect of OGD treatment on p-AKT expression in BV-2 microglia, and this effect was significantly enhanced by the addition of rapamycin. These results demonstrate that esketamine can inhibit AKT signaling activation in the OGD-treated BV-2 microglia.

Discussion

Ischemic stroke is one of the leading causes of disability and mortality worldwide, and has a huge emotional and economic impacts on families and societies. This study demonstrated that esketamine alleviated neurological impairments, improved sensorimotor function and cell viability, and reduced the cerebral infarct volume and injury severity in the MCAO rats and the OGD-treated BV-2 microglia. Moreover, esketamine inhibited microglial M1 polarization and AKT signaling activation induced by I/R or OGD treatment, and facilitated the phenotypic transition of microglia from M1 to M2 and autophagy. These findings suggest that esketamine provides a significant protection against cerebral injury

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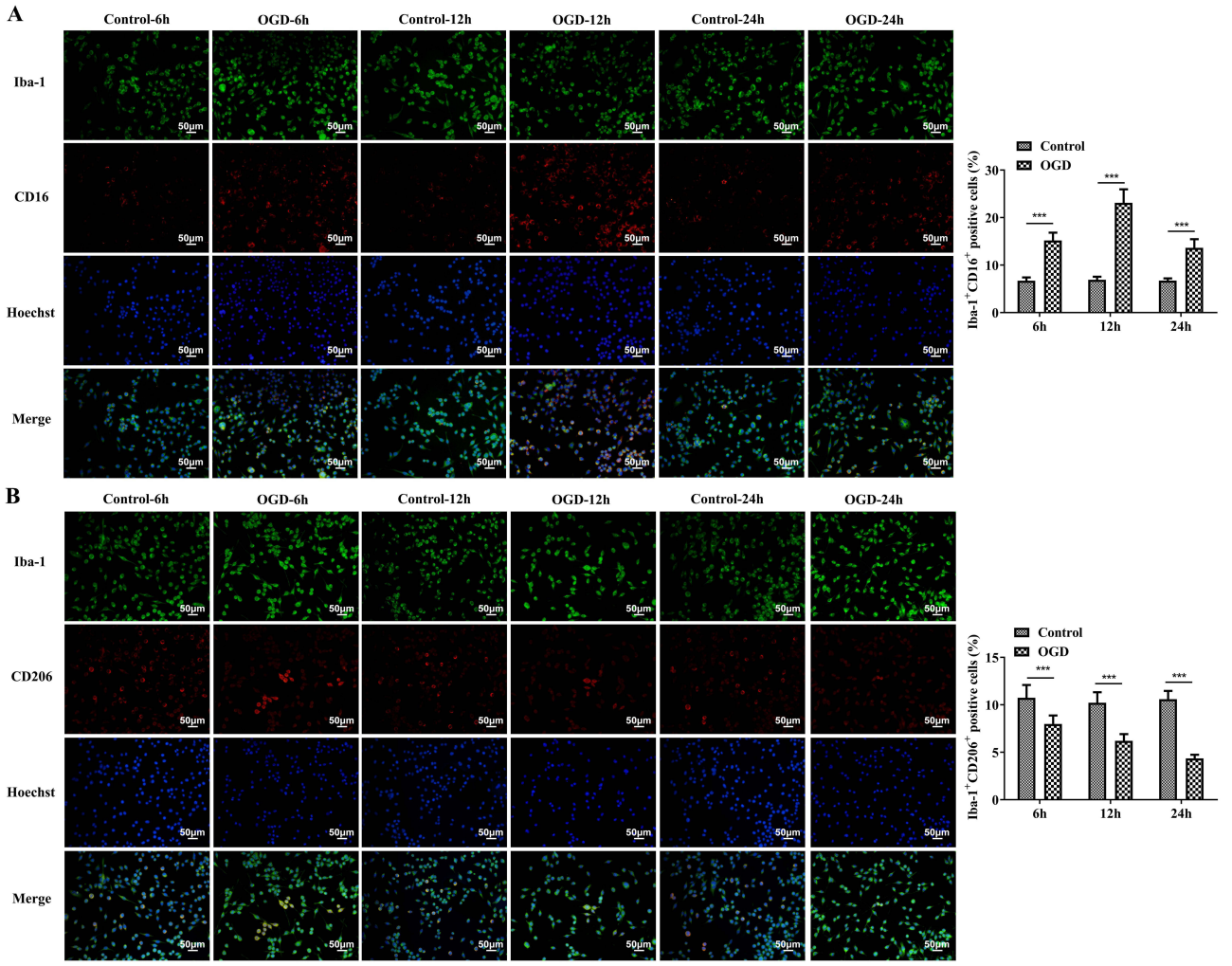

Figure 6 The OGD treatment suppressed the phenotype transition of microglia from M1 to M2. Representative images of double immunofluorescence staining for Iba-1$^+$CD16$^+$ (**A**) and Iba-1$^+$CD206$^+$ (**B**) in the BV-2 microglia exposed to the OGD treatment for 6 h, 12 h and 24 h and the between-group comparisons of their quantitative data. ***P<0.001.

Abbreviation: OGD, oxygen-glucose deprivation.

induced by I/R and OGD treatments. Thus, this experiment confirms our hypothesis and provides evidence that esketamine may be a potential therapeutic drug for ischemic stroke.

As a NMDA receptor antagonist, there is no study having determined the optimal dose of esketamine for the protection against cerebral I/R injury. Available literature indicates that the neuroprotection and neurotoxicity of another NMDA receptor antagonist ketamine are closely related to dose and frequency of administration. That is, more frequent and higher doses of ketamine may produce a significant reduction in the number of glutamate synapses caused by a long-term increased neurotoxicity.[21] In contrast, a low frequency (1–3 times/week) or small dose (10 mg/kg) of ketamine can provide significant neuroprotective effects with increased synaptic spine density and efficiency and improved synaptic plasticity, and may maintain the neuroprotective and anti-depressant effects without increasing synaptic glutamate levels and neurological toxicity.[22] Furthermore, increasing the dose of intraperitoneal esketamine injection more than 4 mg/kg does not further enhance the protection against traumatic brain injury.[17] Thus, this study was designed to determine whether esketamine at a clinical dose provided a protection against cerebral I/R injury by modulating microglial polarization and autophagy. In clinical practice, an intravenous dose of esketamine 0.5–1.0 mg/kg is recommended for anesthesia induction. Due to the differences in pharmacokinetics and pharmacodynamics between animals and human,

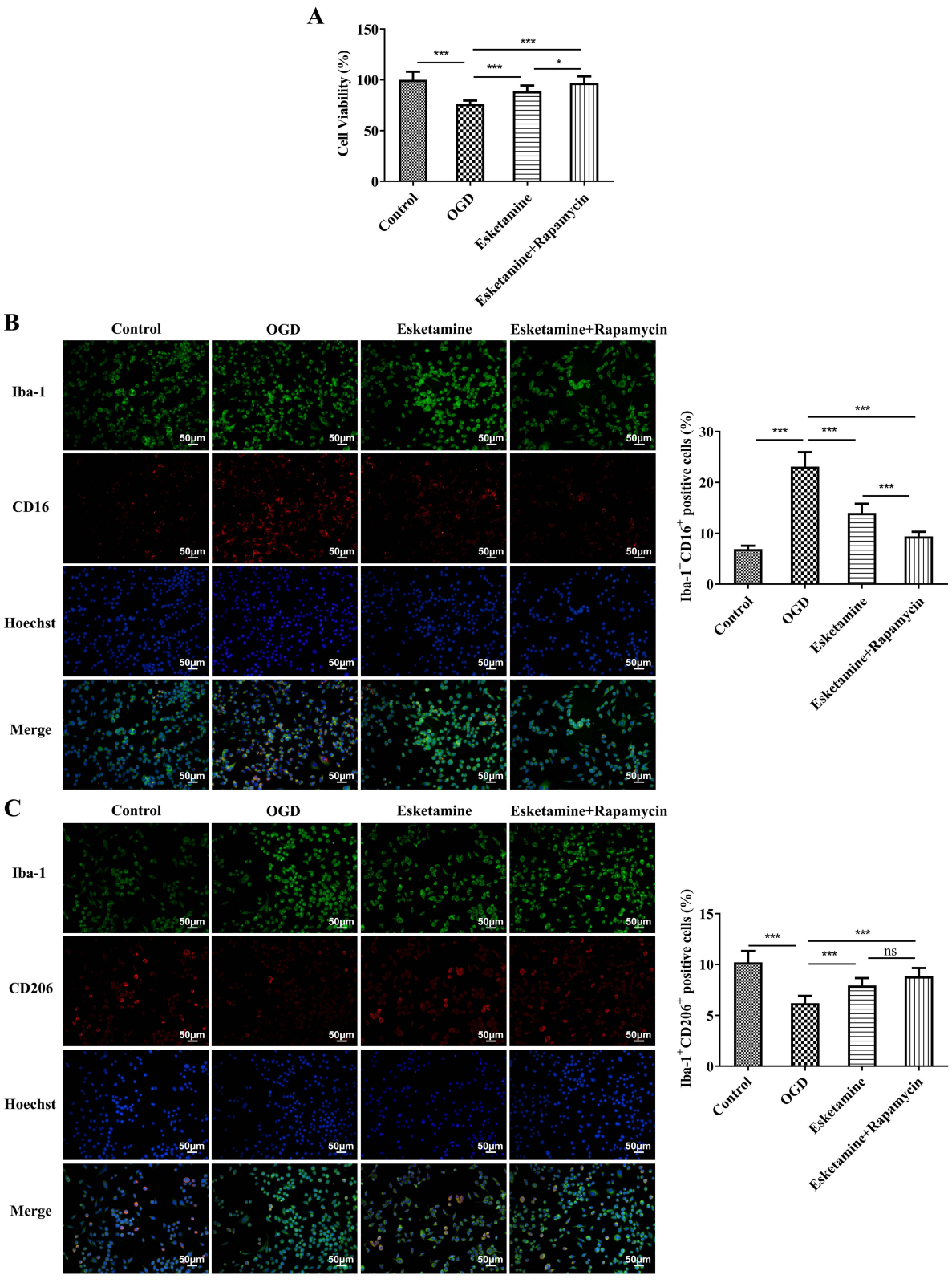

Figure 7 Esketamine increased the viability of OGD-treated BV-2 microglia and facilitated the phenotype transition of microglia from M1 to M2. (**A**) The viability of OGD-treated BV-2 microglia; (**B and C**) Representative images of double immunofluorescence staining for Iba-1$^+$CD16$^+$ and Iba-1$^+$CD206$^+$ in the OGD-treated BV-2 microglia and the between-group comparisons of their quantitative data. *P < 0.05, ***P<0.001. Scale bar = 50 μm.

Abbreviation: OGD, oxygen-glucose deprivation.

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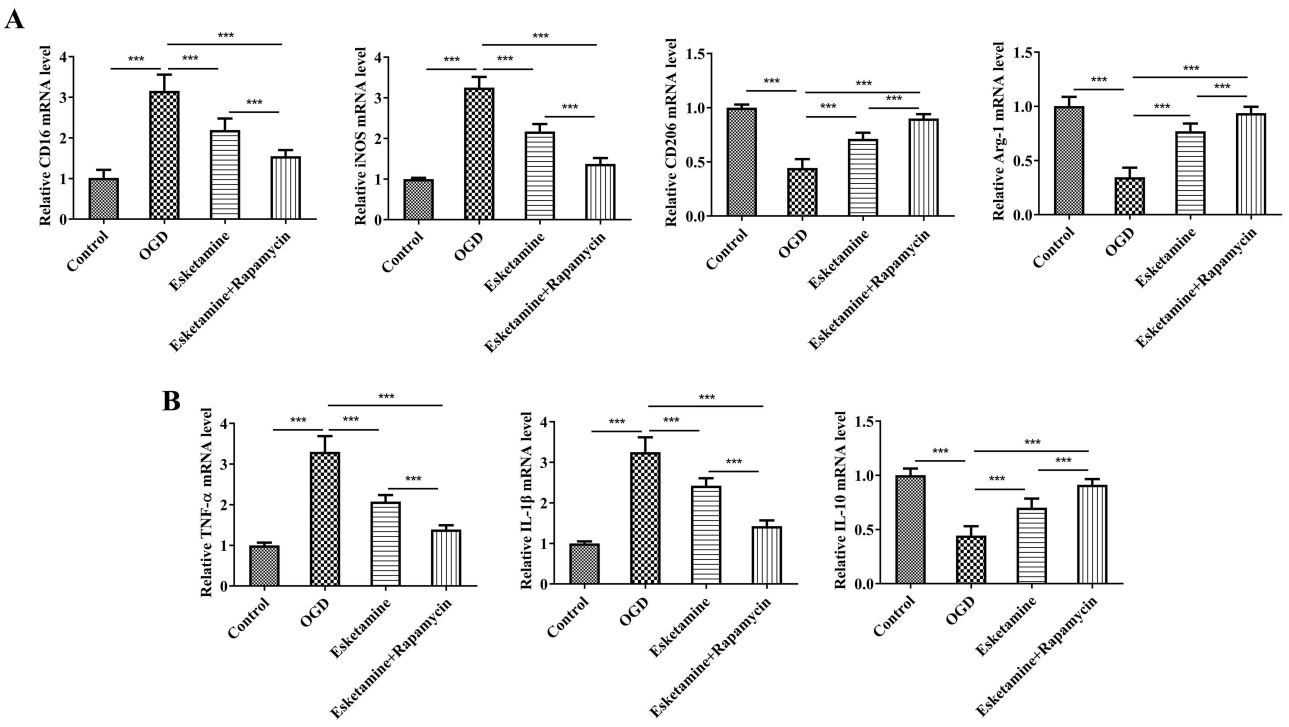

Figure 8 Esketamine promoted the expression of M2 phenotype biomarkers and inhibited the expression of M1 phenotype biomarkers in the OGD-treated BV-2 microglia. (**A**) The between-group comparisons of quantitative data for mRNA expression of microglia M1 phenotype biomarkers (CD16 and iNOS) and M2 phenotype biomarkers (CD206 and Arg-1); (**B**) The between-group comparisons of quantitative data for mRNA expression of TNF-α, IL-1β and IL-10. ***P <0.001.
Abbreviations: OGD, oxygen-glucose deprivation; iNOS, inducible nitric-oxide synthase; NOS, inducible nitric-oxide synthase; Arg-1, Arginase-1; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-10, interleukin-10.

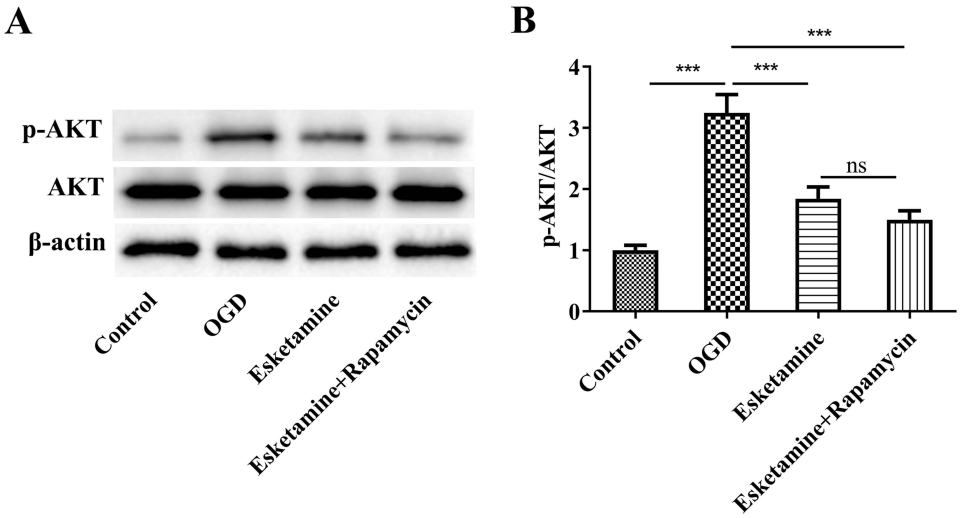

Figure 9 Esketamine inhibited AKT signaling activation in the OGD-treated BV-2 microglia. (**A**) Representative Western blotting images for expression of p-AKT and AKT; (**B**) The between-group comparisons of their quantitative data. ***P<0.001.
Abbreviations: OGD, oxygen-glucose deprivation; AKT, serine/threonine protein kinase.

the recommended conversion factor of intravenous milligram equivalent for drugs is 3.2.[32] In view of the above, 1.6 mg/kg of intravenous esketamine used in our experiment is equivalent to a clinical dose of 0.5 mg/kg.

Microglia are a distinct subtype of mononuclear macrophages that resides in the brain, which can sense mild disorders in homeostasis and be activated rapidly.[33] As the main innate immune cells of central nervous system, microglia are the

most effective regulators for the repairs and regeneration of neurological damages.[34] The dynamic responses of microglia to ischemic brain injury present a variety of phenotypes, and promoting the differentiation of microglia from M1 to M2 to attenuate the detrimental effects after ischemic stroke attack has become a topic of concern.[35] During the cerebral I/R process, M1-like phenotype microglia are characterized by the expression of classical biomarkers including CD16 and iNOS, and secret pro-inflammatory cytokines including TNF-α and IL-1β, aggravating cerebral injury.[36] On the contrary, M2-like phenotype microglia express the biomarkers including CD206 and Arg-1, and release anti-inflammatory mediators including IL-10 to facilitate the repairs and regeneration of damaged cerebral tissues.[37] Esketamine is a rapid-onset anesthetic with antidepressive effect and has been used for clinical treatment of severe treatment-resistant depression. Available evidence demonstrates that esketamine can prevent postoperative emotional disorders and neurocognitive dysfunction by inhibiting microglial M1 polarization to decrease the production and secretion of pro-inflammatory cytokines, including TNF-α and IL-1β, in aging rats with preoperative sleep disturbances.[16] Interestingly, esketamine can also improve the spatial learning and memory dysfunction as well as alleviate the neuronal injury and cell apoptosis in the propofol-induced brain injury rats.[15] Huang et al[38] reported that esketamine mitigated anxiety-like behavior of post-stroke anxiety mice by inhibiting microglial activation and decreasing production of inflammatory cytokines in the cortex. Furthermore, a recent work showed that esketamine improved sepsis-related symptoms, alleviated spatial cognitive impairments, and reduced hippocampal neuroinflammation and cell apoptosis in the mice with sepsis-associated encephalopathy.[39] In the mice model of traumatic brain injury, esketamine effectively inhibited oxidative stress and decreased the number of damaged neurons and TUNEL-positive cells in the cortex.[18] Our experiment demonstrated a significant neuroprotective effect of esketamine on cerebral I/R injury, as evidenced by alleviated neurological impairments, improved sensorimotor function, and reduced cerebral infarct volume and cell apoptosis in the MCAO rats. More importantly, esketamine shifted microglial polarization from the M1 phenotype toward the M2 phenotype, and decreased the production of inflammatory cytokines in the ischemic brain tissues. Thus, our results support the conclusion of previous studies that anti-inflammation may be a potential mechanism underlying the protective effect of esketamine against cerebral I/R injury.

Autophagy is an evolutionarily conserved process for degrading and recycling aged or misfolded proteins and damaged organelles and plays a critical role in maintaining the homeostasis and survival of cells.[40] In mammalian cells, autophagy is precisely regulated by multiple signaling pathways. Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, is crucial for the regulation of autophagy. mTOR integrates the upstream signals that inhibit autophagy via the PI3K-AKT/protein kinase B signaling pathway. AKT is activated by phosphorylation at two key regulatory sites. Ser473 phosphorylation plays a key role.[41] The activated PI3K phosphorylates plasma membrane lipids that trigger AKT via 3-phosphoinositide-dependent protein kinase 1. Activated AKT can further phosphorylate tuberous sclerosis protein 2 and obstruct its interplay with tuberous sclerosis protein 1, ultimately resulting in mTOR activation to inhibit autophagy.[40,42,43] Given that autophagy is regarded as part of an integrated pro-survival signaling pathway in ischemic or hypoxic neurons,[40] this study determined the role of autophagy in the protection of esketamine against cerebral I/R injury by using a specific mTOR inhibitor, rapamycin, and observing AKT activation.

In available literature, however, there is still controversy regarding the contribution of autophagy to cerebral I/R injury. Some studies have reported that autophagy may induce neuronal death in the ischemic brain tissues, and that inhibition of autophagy can protect against cerebral I/R injury. However, increasing evidence supports the neuroprotective effects of autophagy activation in ischemic brains and/or neuronal cells by degrading neuronal organelles and proteins.[36,40,43–46] The current consensus is that autophagy may be a double-edged sword for ischemic neurons.[47] That is, moderate autophagy may act as a pro-survival mechanism of ischemic neurons, while excessive autophagy can lead to neuronal death and secondary neurological injury.[43,48] Thus, precise regulation of the autophagic intensity of neurons after an ischemic attack is important to attenuate cerebral injury and improve neurological function.

As mentioned above, the intensity of autophagy activation is the key to determine the outcome of ischemic brains and/or neuronal cells.[47] In our in vivo experiment, rapamycin treatment was carried out by intraperitoneal injection. In contrast to an intracerebroventricular injection, systemic administration of rapamycin has a very poor bioavailability in the central nerve system as P-glycoprotein at the blood-brain barrier can limit the penetration of drug into the brain. Based on previous work,[48] thus, a large dose of rapamycin (1 mg/kg) was intraperitoneally administered once a day for 3

consecutive days before MCAO surgery in our experiment. It has been shown that this administration method can provide an intracerebral concentration of rapamycin to activate autophagy, improve metabolism and attenuate neuronal injury in the I/R brain area.[49,50] For in vitro experiment, 100 μmol/L rapamycin was used, according to previous work.[50]

It is reported that activation of autophagy with rapamycin can ameliorate neuronal injury and death in the neonatal rats subjected to cerebral hypoxia-ischemia.[44] Lomitapide, a microsomal triglyceride transfer protein inhibitor, can reduce the neuronal tissue loss and improve the neurological function by promoting autophagic flux, inhibiting apoptosis and microglial migration in the experimental cerebral ischemia models induced by the MCAO surgery in the adult male mice and simulated by OGD treatment in the N2a-BV2 cells.[45] By protecting mitochondrial function and enhancing microglial autophagy, moreover, sodium tanshinone IIA sulfonate can also attenuate the OGD-induced neuronal injury.[46] In addition, cellular prion protein has been shown to reduce the OGD-induced microglial damage by enhancing and prolonging autophagy activation to skew microglia toward an anti-inflammatory state.[51,52] In contrast, a previous study by Xia et al[53] demonstrates that inhibition of microglial autophagy induces M1 phenotype with increased production of TNF-α and iNOS, thereby promoting neuroinflammation. This evidence indicates that enhanced microglial autophagy can be attributed to the polarization of microglia into an anti-inflammatory state under ischemic conditions. Intriguingly, esketamine exerts a neuroprotective effect in the traumatic brain injury mice model by inducing autophagy and relieving oxidative stress.[18] In the present study, esketamine enhanced autophagy and facilitated the phenotypic transition of microglia from M1 to M2 in the MCAO rats and the OGD-treated BV-2 microglia. Furthermore, the addition of the autophagy inducer rapamycin further enhanced the protective effects of esketamine on the MCAO-induced cerebral injury and the OGD-induced microglial damage by promoting the phenotypic transition of microglia toward M2. In addition, it has been shown that esketamine can inhibit the phosphorylation of AKT, a key signaling molecule involved in the regulation of microglial autophagy and polarization in cerebral I/R injury.[54–56] This study showed that esketamine could inactivate the AKT signaling pathway in the MCAO rats and the OGD-treated BV-2 microglia. Furthermore, the autophagy inducer rapamycin inhibited p-AKT expression following MCAO and OGD treatments. Based on the findings of previous studies and our experiments, we consider that esketamine at a clinical dose may induce autophagy and promote the phenotypic transition of microglia from M1 to M2 by inhibiting the AKT signaling pathway, thereby attenuating cerebral I/R injury. Interestingly, a randomized placebo-controlled clinical trial with cross-over design demonstrates that the pretreatment with an oral low-dose rapamycin can prolong the antidepressant effect of intravenous ketamine 0.5 mg/kg, and increase the response and remission rates at 2 weeks following treatment.[57] Similarly, our results experiment also showed that the pretreatment with systemic rapamycin significantly improved the protection of esketamine against cerebral I/R injury. Thus, this experiment may provide new insights into the potential therapeutic strategies not only for cerebral I/R injury but for neuropathic pain, neurodegenerative conditions, mood disorders, and others.

Our experimental design had several limitations that require special attention. First, only a single dose of esketamine and an intervention time point were designed. Thus, the important question that this experiment cannot answer is whether the neuroprotective effect of esketamine on cerebral I/R injury is dose-dependent, and whether the implementation time of esketamine treatment can significantly affect the protective effect of esketamine on cerebral I/R injury. Second, regarding the mechanisms by which esketamine protects against cerebral I/R injury, only AKT signaling related to microglial polarization and autophagy were examined in this experiment. Available evidence indicates that various mechanisms associated with neuronal excitotoxicity, oxidative stress, calcium overload, mitochondrial dysfunction, endoplasmic reticulum stress, monocyte or macrophage polarization, and ion channels are also involved in the protection of drug interventions against cerebral I/R injury.[58–60] Evidently, this study cannot provide any evidence for the possible contributions of these factors to the neuroprotective effects of esketamine against cerebral I/R injury. Third, this experiment used only male rats to ensure consistency in research conditions. It is generally believed that there are sex differences in ischemic stroke outcomes and therapies, post-stroke remodeling, and functional recovery.[61] Furthermore, multiple levels of pathophysiology are involved in the mechanisms that affect sexual differences in ischemic stroke.[51,62] Thus, only the use of male animals to determine the protective effects and mechanisms of esketamine against cerebral I/R injury is another important limitation of this study. Fourth, this study only evaluated the role of autophagy in the neuroprotective mechanism of esketamine using an mTOR pathway inhibitor but did not perform further validation using

an mTOR pathway activator. Furthermore, this experiment did not assess the changes in autophagy at different time points after esketamine treatment, though the period and level of autophagy determine the final fate of cells.[45] Fifth, available evidence demonstrates that a high level of rapamycin in the brain can block the neuronal autophagy activation and antidepressant effect of ketamine.[57] As the level of rapamycin in brain was not determined in this experiment, we can not determine whether the beneficial interaction between esketamine and rapamycin obtained in this study is the result of ischemic brain tissues exposed to a low level of rapamycin. Last, as this is a preclinical study in animals and cultivate cells, our findings cannot be directly extrapolated into clinical practice. Therefore, more basic and clinical studies are required to address these issues.

Conclusions

In summary, this study demonstrates that esketamine at a clinical dose can protect against cerebral I/R injury by inhibiting the AKT signaling pathway to shift microglial polarization toward the M2 phenotype and induce autophagy. Furthermore, esketamine combined autophagy inducer can provide an improved protection against cerebral I/R injury. Thus, this study provides new insights into the neuroprotective mechanisms of esketamine and the potential therapeutic strategies of cerebral I/R injury.

Data Sharing Statement

The original contributions presented in this study are included in the article, and further inquiries can be directed to the corresponding authors.

Ethical Approval

All animal experiments were approved by the Animal Care and Use Committee of Beijing Friendship Hospital, Capital Medical University (Number:22-1010, Date: 2023-02-08).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests in this work.

References

1. Caprio FZ, Sorond FA. Cerebrovascular disease: primary and secondary stroke prevention. *Med Clin North Am*. 2019;103(2):295–308. doi:10.1016/j.mcna.2018.10.001
2. Zhao HL, Huang Y. Lifetime risk of stroke in the global burden of disease study. *N Engl J Med*. 2019;380(14):1377–1378. doi:10.1056/NEJMc1900607
3. Xiong Y, Wakhloo AK, Fisher M. Advances in acute ischemic stroke therapy. *Circ Res*. 2022;130(8):1230–1251. doi:10.1161/circresaha.121.319948
4. Widimsky P, Snyder K, Sulzenko J, Hopkins LN, Stetkarova I. Acute ischaemic stroke: recent advances in reperfusion treatment. *Eur Heart J*. 2023;44(14):1205–1215. doi:10.1093/eurheartj/ehac684
5. Ifergan H, Amelot A, Ismail M, Gaudron M, Cottier JP, Narata AP. Stroke-mimics in stroke-units. Evaluation after changes imposed by randomized trials. *Arq Neuropsiquiatr*. 2020;78(2):88–95. doi:10.1590/0004-282x20190154
6. Wang M, Pan W, Xu Y, Zhang J, Wan J, Jiang H. Microglia-mediated neuroinflammation: a potential target for the treatment of cardiovascular diseases. *J Inflamm Res*. 2022;15:3083–3094. doi:10.2147/jir.s350109
7. Zhang Y, Lian L, Fu R, et al. Microglia: the hub of intercellular communication in ischemic stroke. *Front Cell Neurosci*. 2022;16:889442. doi:10.3389/fncel.2022.889442

8. Anttila JE, Whitaker KW, Wires ES, Harvey BK, Airavaara M. Role of microglia in ischemic focal stroke and recovery: focus on toll-like receptors. *Prog Neuropsychopharmacol Biol Psychiatry*. 2017;79(Pt A):3–14. doi:10.1016/j.pnpbp.2016.07.003
9. Kanazawa M, Ninomiya I, Hatakeyama M, Takahashi T, Shimohata T. Microglia and monocytes/macrophages polarization reveal novel therapeutic mechanism against stroke. *Int J Mol Sci*. 2017;18(10):2135. doi:10.3390/ijms18102135
10. Jiang CT, Wu WF, Deng YH, Ge JW. Modulators of microglia activation and polarization in ischemic stroke (Review). *Mol Med Rep*. 2020;21 (5):2006–2018. doi:10.3892/mmr.2020.11003
11. Ma Y, Wang J, Wang Y, Yang GY. The biphasic function of microglia in ischemic stroke. *Prog Neurobiol*. 2017;157:247–272. doi:10.1016/j.pneurobio.2016.01.005
12. Kaur U, Pathak BK, Singh A, Chakrabarti SS. Esketamine: a glimmer of hope in treatment-resistant depression. *Eur Arch Psychiatry Clin Neurosci*. 2021;271(3):417–429. doi:10.1007/s00406-019-01084-z
13. Höflich A, Hahn A, Küblböck M, et al. Ketamine-dependent neuronal activation in healthy volunteers. *Brain Struct Funct*. 2017;222(3):1533–1542. doi:10.1007/s00429-016-1291-0
14. Höflich A, Kraus C, Pfeiffer RM, et al. Translating the immediate effects of S-Ketamine using hippocampal subfield analysis in healthy subjects-results of a randomized controlled trial. *Transl Psychiatry*. 2021;11(1):200. doi:10.1038/s41398-021-01318-6
15. Xu G, Wang Y, Chen Z, Zhang Y, Zhang X, Zhang G. Esketamine improves propofol-induced brain injury and cognitive impairment in rats. *Transl Neurosci*. 2022;13(1):430–439. doi:10.1515/tnsci-2022-0251
16. Wen Y, Xu J, Shen J, et al. Esketamine prevents postoperative emotional and cognitive dysfunction by suppressing microglial M1 polarization and regulating the BDNF-TrkB pathway in ageing rats with preoperative sleep disturbance. *Mol Neurobiol*. 2024;61(8):5680–5698. doi:10.1007/s12035-023-03860-4
17. Tang Y, Liu Y, Zhou H, et al. Esketamine is neuroprotective against traumatic brain injury through its modulation of autophagy and oxidative stress via AMPK/mTOR-dependent TFEB nuclear translocation. *Exp Neurol*. 2023;366:114436. doi:10.1016/j.expneurol.2023.114436
18. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell*. 2011;147(4):728–741. doi:10.1016/j.cell.2011.10.026
19. Zang J, Wu Y, Su X, et al. Inhibition of PDE1-B by vinpocetine regulates microglial exosomes and polarization through enhancing autophagic flux for neuroprotection against ischemic stroke. *Front Cell Dev Biol*. 2020;8:616590. doi:10.3389/fcell.2020.616590
20. Zhou X, Zhou J, Li X, Guo C, Fang T, Chen Z. GSK-3β inhibitors suppressed neuroinflammation in rat cortex by activating autophagy in ischemic brain injury. *Biochem Biophys Res Commun*. 2011;411(2):271–275. doi:10.1016/j.bbrc.2011.06.117
21. Krystal JH, Kaye AP, Jefferson S, et al. Ketamine and the neurobiology of depression: toward next-generation rapid-acting antidepressant treatments. *Proc Natl Acad Sci U S A*. 2023;120(49):e2305772120. doi:10.1073/pnas.2305772120
22. Li N, Lee B, Liu RJ, et al. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science*. 2010;329 (5994):959–964. doi:10.1126/science.1190287
23. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*. 1989;20(1):84–91. doi:10.1161/01.str.20.1.84
24. Shi M, Chen J, Liu T, et al. Protective effects of remimazolam on cerebral ischemia/reperfusion injury in rats by inhibiting of NLRP3 inflammasome-dependent pyroptosis. *Drug Des Devel Ther*. 2022;16:413–423. doi:10.2147/dddt.s344240
25. Morris RG, Garrud P, Rawlins JN, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. *Nature*. 1982;297(5868):681–683. doi:10.1038/297681a0
26. Xu L, Gao Y, Hu M, et al. Edaravone dexborneol protects cerebral ischemia reperfusion injury through activating Nrf2/HO-1 signaling pathway in mice. *Fundam Clin Pharmacol*. 2022;36(5):790–800. doi:10.1111/fcp.12782
27. Zhao J, Ma M, Li L, Fang G. Oxysophoridine protects against cerebral ischemia/reperfusion injury via inhibition of TLR4/p38MAPK-mediated ferroptosis. *Mol Med Rep*. 2023;27(2):44. doi:10.3892/mmr.2023.12931
28. Duan Q, Wu J. Dihydroartemisinin ameliorates cerebral I/R injury in rats via regulating VWF and autophagy-mediated SIRT1/FOXO1 pathway. *Open Med*. 2023;18(1):20230698. doi:10.1515/med-2023-0698
29. Yang H, Chen Z, Gao W. EHMT2 affects microglia polarization and aggravates neuronal damage and inflammatory response via regulating HMOX1. *Transl Neurosci*. 2023;14(1):20220276. doi:10.1515/tnsci-2022-0276
30. Ma X, Jiao J, Aierken M, Sun H, Chen L. Hypoxia inducible factor-1α through ROS/NLRP3 pathway regulates the mechanism of acute ischemic stroke microglia scorching mechanism. *Biologics*. 2023;17:167–180. doi:10.2147/btt.s444714
31. Livak KJ, Schmittgen TD Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods*. 2001;25(4):402–408. doi:10.1006/meth.2001.1262
32. Xu SY, Bian RL, Chen X. *Methodology of Pharmacological Experiments*. 3rd ed. Beijing: People's Medical Publishing House; 2003:202–203.
33. Qiu M, Xu E, Zhan L. Epigenetic regulations of microglia/macrophage polarization in ischemic stroke. *Front Mol Neurosci*. 2021;14:697416. doi:10.3389/fnmol.2021.697416
34. Xiong XY, Liu L, Yang QW. Functions and mechanisms of microglia/macrophages in neuroinflammation and neurogenesis after stroke. *Prog Neurobiol*. 2016;142:23–44. doi:10.1016/j.pneurobio.2016.05.001
35. Xue Y, Nie D, Wang LJ, et al. Microglial polarization: novel therapeutic strategy against ischemic stroke. *Aging Dis*. 2021;12(2):466–479. doi:10.14336/ad.2020.0701
36. Lyu J, Xie D, Bhatia TN, Leak RK, Hu X, Jiang X. Microglial/Macrophage polarization and function in brain injury and repair after stroke. *CNS Neurosci Ther*. 2021;27(5):515–527. doi:10.1111/cns.13620
37. Mo Y, Xu W, Fu K, et al. The dual function of microglial polarization and its treatment targets in ischemic stroke. *Front Neurol*. 2022;13:921705. doi:10.3389/fneur.2022.921705
38. Huang A, Chen Y, Wang S, et al. Esketamine ameliorates post-stroke anxiety by modulating microglial HDAC3/NF-κB/COX1 inflammatory signaling in ischemic cortex. *Eur J Pharmacol*. 2023;947:175667. doi:10.1016/j.ejphar.2023.175667
39. Wang CM, Zhang Y, Yang YS, Lin S, He HF. Effect of esketamine pretreatment on acute sepsis-associated encephalopathy. *Exp Neurol*. 2024;372:114646. doi:10.1016/j.expneurol.2023.114646
40. Ahsan A, Liu M, Zheng Y, et al. Natural compounds modulate the autophagy with potential implication of stroke. *Acta Pharm Sin B*. 2021;11 (7):1708–1720. doi:10.1016/j.apsb.2020.10.018

41. McKenna M, Balasuriya N, Zhong S, Li SS, O'Donoghue P. Phospho-form specific substrates of protein kinase B (AKT1). *Front Bioeng Biotechnol*. 2021;8:619252. doi:10.3389/fbioe.2020.619252
42. Lushnikova I, Kostiuchenko O, Kowalczyk M, Skibo G. mTOR/α-ketoglutarate signaling: impact on brain cell homeostasis under ischemic conditions. *Front Cell Neurosci*. 2023;17:1132114. doi:10.3389/fncel.2023.1132114
43. Hou K, Xu D, Li F, Chen S, Li Y. The progress of neuronal autophagy in cerebral ischemia stroke: mechanisms, roles and research methods. *J Neurol Sci*. 2019;400:72–82. doi:10.1016/j.jns.2019.03.015
44. Carloni S, Girelli S, Scopa C, Buonocore G, Longini M, Balduini W. Activation of autophagy and Akt/CREB signaling play an equivalent role in the neuroprotective effect of rapamycin in neonatal hypoxia-ischemia. *Autophagy*. 2010;6(3):366–377. doi:10.4161/auto.6.3.11261
45. Zheng Y, Hu Y, Han Z, et al. Lomitapide ameliorates middle cerebral artery occlusion-induced cerebral ischemia/reperfusion injury by promoting neuronal autophagy and inhibiting microglial migration. *CNS Neurosci Ther*. 2022;28(12):2183–2194. doi:10.1111/cns.13961
46. Ma Z, Wu Y, Xu J, et al. Sodium tanshinone IIA sulfonate ameliorates oxygen-glucose deprivation/reoxygenation-induced neuronal injury via protection of mitochondria and promotion of autophagy. *Neurochem Res*. 2023;48(11):3378–3390. doi:10.1007/s11064-023-03985-x
47. Shi Q, Cheng Q, Chen C. The role of autophagy in the pathogenesis of ischemic stroke. *Curr Neuropharmacol*. 2021;19(5):629–640. doi:10.2174/1570159x18666200729101913
48. Wu Y, Zhang RB, Liang SL, Zhu YH, Xu B. Effects of rapamycin and 3-MA on motor behavior and autophagy related protein LC3 in PD model mice. *Chin J Behav Med Brain Sci*. 2016;25(11):961–965. doi:10.3760/cma.j.issn.1674-6554.2016.11.001
49. Chauhan A, Sharma U, Jagannathan NR, Reeta KH, Gupta YK. Rapamycin protects against middle cerebral artery occlusion induced focal cerebral ischemia in rats. *Behav Brain Res*. 2011;225(2):603–609. doi:10.1016/j.bbr.2011.08.035
50. Shao M, Shen Y, Sun H, Meng D, Huo W, Qi X. Protectiveness of artesunate given prior ischemic cerebral infarction is mediated by increased autophagy. *Front Neurol*. 2018;9:634. doi:10.3389/fneur.2018.00634
51. Wang P, Shao BZ, Deng Z, Chen S, Yue Z, Miao CY. Autophagy in ischemic stroke. *Prog Neurobiol*. 2018;163–164:98–117. doi:10.1016/j.pneurobio.2018.01.001
52. Shao J, Yin X, Lang Y, et al. Cellular prion protein attenuates OGD/R-induced damage by skewing microglia toward an anti-inflammatory state via enhanced and prolonged activation of autophagy. *Mol Neurobiol*. 2023;60(3):1297–1316. doi:10.1007/s12035-022-03099-5
53. Xia CY, Zhang S, Chu SF, et al. Autophagic flux regulates microglial phenotype according to the time of oxygen-glucose deprivation/reperfusion. *Int Immunopharmacol*. 2016;39:140–148. doi:10.1016/j.intimp.2016.06.030
54. Wang H, Liu Q, Zhang X. C1q/tumor necrosis factor-related protein-1 attenuates microglia autophagy and inflammatory response by regulating the Akt/mTOR pathway. *Life Sci*. 2020;256:117992. doi:10.1016/j.lfs.2020.117992
55. Liu P, Zhang K, Tong C, Liu T, Zheng J. Progesterone alleviates esketamine-induced hypomyelination via PI3K/Akt signaling pathway in the developing rat brain. *Biotechnol Genet Eng Rev*. 2023;1–16. doi:10.1080/02648725.2023.2193058
56. Hu K, Gao Y, Chu S, Chen N. Review of the effects and mechanisms of microglial autophagy in ischemic stroke. *Int Immunopharmacol*. 2022;108:108761. doi:10.1016/j.intimp.2022.108761
57. Abdallah CG, Averill LA, Gueorguieva R, et al. Modulation of the antidepressant effects of ketamine by the mTORC1 inhibitor rapamycin. *Neuropsychopharmacology*. 2020;45(6):990–997. doi:10.1038/s41386-020-0644-9
58. You Q, Lan XB, Liu N, et al. Neuroprotective strategies for neonatal hypoxic-ischemic brain damage: current status and challenges. *Eur J Pharmacol*. 2023;957:176003. doi:10.1016/j.ejphar.2023.176003
59. Yang Q, Huang Q, Hu Z, Tang X. Potential neuroprotective treatment of stroke: targeting excitotoxicity, oxidative stress, and inflammation. *Front Neurosci*. 2019;13:1036. doi:10.3389/fnins.2019.01036
60. Lin W, Zhao XY, Cheng JW, et al. Signaling pathways in brain ischemia: mechanisms and therapeutic implications. *Pharmacol Ther*. 2023;251:108541. doi:10.1016/j.pharmthera.2023.108541
61. Tang T, Hu L, Liu Y, et al. Sex-associated differences in neurovascular dysfunction during ischemic stroke. *Front Mol Neurosci*. 2022;15:860959. doi:10.3389/fnmol.2022.860959
62. Kim T, Chelluboina B, Chokkalla AK, Vemuganti R. Age and sex differences in the pathophysiology of acute CNS injury. *Neurochem Int*. 2019;127:22–28. doi:10.1016/j.neuint.2019.01.012