Original Article
Autophagy protects against cerebral ischemic reperfusion injury by inhibiting neuroinflammation

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Abstract: Objective: To examine the effect of autophagy on cerebral damage caused by different models and test the hypothesis that its protection mechanism acts via inhibiting expression of neuroinflammatory mediators. Methods: Autophagy was induced by rapamycin treatment. Cerebral damage was induced using models of IL-6 treatment, oxygen glucose deprivation/reoxygenation (OGD/R) *in vitro*, and middle cerebral artery occlusion (MCAO) *in vivo*. The effect and mechanism of autophagy was examined and assessed in terms of cell viability, infarction size in brain tissue, neurological score, production of inflammatory mediators IL-1β and IL-6, transcription and protein expression of autophagy markers beclin-1 and LC-3II in different experimental groups. Results: Autophagy triggered by rapamycin could protect neurons from IL-6-induced injury and astrocytes from OGD/R-induced injury *in vitro* and in rat brain tissue from MCAO *in vivo*. Autophagy significantly increased cell viability, attenuated cerebral infarction and improved neurological scores. It also inhibited production of the IL-1β and IL-6 and elevated the expression of beclin-1 and LC-3II. Conclusions: Autophagy can inhibit the inflammatory response and reduce cerebral I/R injury. There was a relationship between the extent of protection and (i) the level of the autophagic response, (ii) the stage of the cerebral I/R injury, and (iii) the time of intervention.

Keywords: Autophagy, cerebral ischemia/reperfusion, oxygen glucose deprivation, IL-1β, IL-6, neuroinflammation

Introduction

Cerebral damage caused by ischemia and reperfusion (I/R) is accompanied by a marked inflammatory response, which is initiated and aggravated by the accumulation of inflammatory cells and the expression of inflammatory mediators [1]. Thus, many researchers investigate how to block the activation of inflammatory reactions during cerebral I/R to prevent and reduce the progression of brain injury. Recently, accumulating evidence from both *in vitro* and *in vivo* study has shown that autophagy plays a critical role in inflammation by influencing and regulating inflammatory mediators [2-4]. Autophagy is a type of self-phagocytosis in eukaryotic cells which is comprised of cellular "housekeeping" processes responsible for eliminating damaged organelles, aged proteins, and unused cytoplasmic components. Under normal physiological conditions, autophagy can mobilize intracellular energy resources to meet cel-

lular and organismal demands for metabolic substrates [5]. In the presence of pathological factors (such as nutritional and growth factor deficiencies, ischemia, hypoxia, and inflammation, damaged organelles, aged proteins, and unused cytoplasmic), components are entrapped within autophagosomes, which transport the components to lysosomes via the microtubule network to promote digestion and component recycling to restore cell function and development [6-8].

Cytokines are one of the most important inflammation mediators responsible for regulating endogenous or exogenous inflammation reactions [9]. Neuroinflammation mediated by IL-1β or IL-6 worsens brain damage caused by a cerebral I/R injury. Under ischemic and hypoxic stimuli, IL-1β and IL-6 are both expressed in neurons and glial cells [10, 11]. On the contrary, inhibition of IL-1β or IL-6 was beneficial for neuroprotection. Boutin et al reported that IL-1β
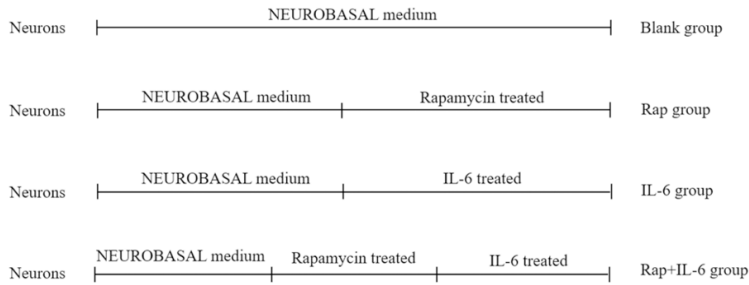
Diagram 1. Protocol used to treat neurons.

knockout mice resulted in notably reduced infarct volumes following experimental stroke [12]. Similarly, Sung et al found that IL-6 blockade led to a decreased brain injury in mice that experienced a traumatic and brief hypoxia event [13]. Therefore, inhibition of IL-1β and IL-6-mediated neuroinflammation can be protective in cerebral I/R-induced brain damage.

To date, diverse studies have focused widely on autophagy, including in the brain, heart, lung, kidney and liver [14-17]. However, the effect of autophagy in I/R injury is still controversial, with some researchers claiming that the process has protective effects, whereas others claim that it has disruptive effects [18-20]. Therefore, it remains elusive whether autophagy regulates the expression of inflammatory mediators such as IL-1β and IL-6 and attenuates cerebral I/R injury. Additionally, despite the various effects of autophagy which have been studied widely, the relationship among the level of autophagy, severity of inflammation, and outcome of cerebral I/R injury remains unclear. Thus, we investigated these issues in this study by using models of IL-6 treatment, oxygen glucose deprivation/reoxygenation (OGD/R), and middle cerebral artery occlusion (MCAO). An improved understanding of the association between autophagy and inflammation may be helpful in the development of new treatments for ischemic stroke.

Materials and methods

Cell culture: neurons and astrocytes

Primary cortical neuronal cells were isolated from 24-hour-old neonate Sprague Dawley (SD) rats and cultured as previous described [21, 22]. Briefly, within a sterile, controllable environment, brain cortical tissue was placed in cold PBS on ice. Under a microscope, we removed the meninges, blood vessels and cut

the brain into several small pieces. We transferred the cortical tissue into a new container, and 0.25% trypsin was added to digest the extracellular matrix proteins. ① The cells were plated in 24 well plates on glass coverslips and mixed with media (Neurobasal A Medium) for the neuron populations. The morphology of neurons was observed and double immunofluorescence labeling was applied to identify the expression of NeuN and MAP2 in neurons. ② The cells were plated in 24 well plates on glass coverslips and mixed with media (DMEM/F 12 Medium) for the astrocyte populations. The morphology of neurons was observed and double immunofluorescence labeling was applied to identify the expression of GAPA in astrocytes.

Treatment of neurons and assessment of viability

Neurons were cultured at 1×10^4 cells/well in a 96-well plate in a NEUROBASAL medium and, then, we carried out the procedure depicted in **Diagram 1**. We treated four groups of neurons: a control group which consisted of untreated cells (blank group); a group treated with 100 ng/ml IL-6 (IL-6 group) for 2 hours to induce inflammation; a group treated with 1 mM/L rapamycin (Rap group) for 1 hour to trigger autophagy; and a group treated with 1 mM/L rapamycin for 1 hour prior to inducing inflammation with 100 ng/ml IL-6 for 2 hours (Rap+IL-6 group). The viability of untreated and treated neurons was measured with the Cell Counting Kit-8 (CCK-8 kit), following the manufacturer's instructions (Nanjing Jiancheng Technology Co., Ltd). The CCK-8 blank well did not contain neurons, medium or CCK-8; the CCK-8 control contains medium and CCK-8, but no neurons. The absorbance was measured at 450 nm using a microplate reader. Neuronal viability (%) was calculated as follows: (OD value of experimental group - OD value of CCK-8 blank)/(OD value of CCK-8 control - OD value of CCK-8 blank).

Treatment of astrocytes

To establish the OGD model, astrocytes were cultured in glucose-free Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) and incubated in a humidified atmosphere of 5% CO_2, 1% O_2, and 94% N_2 at 37°C for 2 h [23]. Five differ-
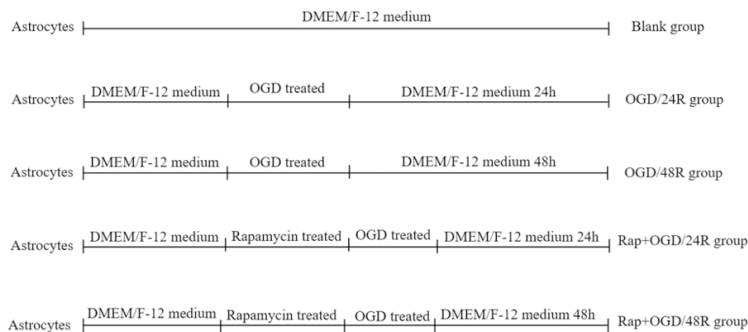
Diagram 2. Protocol used to treat astrocytes.

ent groups were created by varying treatment and exposure, depicted in **Diagram 2**: an OGD-free control group which consisted of untreated cells (blank group); the first two groups were treated with OGD and washed afterwards with phosphate buffered saline and cultured in DMEM/F-12 for reoxygenation 24 h (OGD/24R group) and 48 h (OGD/48R group) to induce reperfusion injury; and the second two groups were treated first with 10 nM/L rapamycin for 48 h prior to being treated with OGD and subsequently cultured in DMEM/F-12 for reoxygenation 24 h (Rap+OGD/24R group) and 48 h (Rap+OGD/48R group).

Establishment of middle cerebral artery occlusion model

Healthy male Sprague Dawley (SD) rats, SPF quality scale, weighing 280-320 g, n=36, provided by Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. were randomly divided into a sham group, a ischemia-reperfusion group (I/R group), and a rapamycin treated group (Rap+I/R group). The I/R group was handled to trigger MCAO as follows. Initially, the rats were intubated with tracheal intubation, ventilator assisted respiration, 1%-2% enflurane mixed with 70% N_2O and 30% O_2 inhalation anesthesia. Then, a 0.26 mm diameter filament was inserted 18-20 mm from the external carotid artery to the beginning of the middle cerebral artery of the rat to create infarction in the middle cerebral artery territory. Finally, the filament was withdrawn after 2 h of ischemia to induce reperfusion. In the Rap+I/R group, the procedure was similar, but an intracerebroventricular injection was performed with 0.8 ng rapamycin, to induce autophagy, at the beginning of reperfusion.

Assessment of neurological score

The neurological score was measured after reperfusion 48 h after the reperfusion period by an assessor who was unaware of the experimental groups. The Garcia test is a composite neurological test used to evaluating the motor and sensory deficits as described below **Table 1** [24].

Determination of cerebral infarction size

Rats in each experimental group were sacrificed by intraperitoneal injection of excessive chloral hydrate after 48 h of reperfusion. The brain was decapitated and placed in a brain slice mold, and a coronal section was made every 2 mm for a total of 6 slices. The brain slices were stained in 2% 2,3,5-triphenyltetrazolium chloride (TCC) solution prepared in 0.2 mol/L Ph7.4 PBS for 10 minutes (in the case of warm bath), and placed in a fixative solution. Photographs were taken 24 h later and the infarct volume was calculated using the Image-Pro Analysis Software 5.0 image analysis system. Cerebral infarction volume (%) = (Infarction volume/Total brain volume) × 100%.

Immunofluorescent staining

Rats in each experimental group were injected with chloral hydrate after 48 h of reperfusion and subsequently sacrificed by transcardiac perfusion with physiological saline followed by 4% paraformaldehyde dissolved in PBS (pH 7.4) to fix brain tissue. The brain sections were made by dehydration, transparency, dipping wax and embedding. The brain sections were separately incubated with antibody (GFAP 1:100 mouse anti rat; NeuN 1:100 mouse anti rat; LC-3B 1:200 rabbit anti rat; Beclin-1 1:200 rabbit anti rat; IL-1β 1:200 rabbit anti rat; IL-6 1:200 rabbit anti rat) at 4°C overnight. After washing, the sections were separately incubated with Dylight 488 labeled goat anti mouse IgG and cy3 labeled goat anti rabbit IgG for 1 hour at 37°C. The sections were given anti-fade reagent with DAPI, mounted and observed under a fluorescence microscope.

Table 1. Details of neurological score

Spontaneous activity
Normal (3)
Slightly affected (2)
Severely affected (1)
No movement (0)
Symmetry in movement of 4 limbs assessed when rat held suspended by tail
Symmetric (3)
Asymmetric (2)
Hemiplegic (1)
Forepaw outstretching assessed by bringing rat to edge of table and making it walk on forelimbs while being held by tail and observing forelimb use
Symmetric forepaws (3)
Mild asymmetry (2)
Marked asymmetry (1)
One forelimb did not move (0)
Climbing determined by placing rat on the wall of a wire cage and observing climbing and strength of attachment to wall
Climbed easily, gripped tightly (3)
One side impaired (2)
Failed to climb or tended to circle instead of climbing (1)
Body proprioception
Equal on both sides (3)
Reacted slowly to stimulus on 1 side (2)
No response on one side (1)
Response to vibrissae touch determined by brushing vibrissae on each side
Symmetric (3)
Asymmetric (2)
No response on 1 side (1)

Real time-polymerase chain reaction

Rats in each experimental group were sacrificed by intraperitoneal injection of excessive chloral hydrate after 48 h of reperfusion. The brain tissue was removed quickly. The nucleotide sequences of primers used in this experiment were designed according to the sequence described as follows: Beclin-1 (F: AAGAGTGTA-GAGAACCAGAT; R: TCGCATTGAAGACATTGG, 76 bp); LC-3B (F: AGTGATTATAGAGCGATA; R: CTAA-TTATCTTGATGAGTTC, 108 bp); IL1β (F: AACATA-AGCCAACAAGTG; R: ACAGGACAGGTATAGATTC); IL-6 (F: GAACAACTTACAAGATAACA; R: GACTCT-AACTTCTCCATTA); β-actin (F: TATGGAATCCTGT-GGCATC; R: GTGTTGGCATAGAGGTCTT). Relative abundance of mRNA was calculated after normalization to β-actin RNA. The relative quantification was determined using the comparative CT method.

Western blotting analysis

① The neurons and astrocytes of each experimental group were washed by PBS twice. Then, 300 μl RIPA (containing 30 μl protease inhibitor) was added to each well which was placed on ice for splitting for 10 minutes. The supernatant was collected by cell centrifugation at 12000 rpm for 10 minutes at 4°C. ② Rats in each experimental group were sacrificed by intraperitoneal injection of excessive chloral hydrate after 48 h of reperfusion. The brain tissues were removed quickly. The brain tissue/100 mg were added 500 μl RIPA (containing 50 μl protease inhibitor) and cut by sterile ophthalmic scissors on ice. Homogenization was done by an ultrasonic cell disrupter for 20 seconds and placed on ice for 20 minutes. The supernatant was collected by cell centrifugation at 12000 rpm for 10 minutes at 4°C. Protein concentration assay were performed according to the BCA kit instructions. The protein was denatured by boiling water. Then, 80 μg of protein was loaded, electrophoresed by SDS-PAGE and transferred to film. Next, 5% BSA was blocked for 2 h, and TBS solution was used to wash 3 times, 5 min each time. Cells were incubated with antibody (Beclin-1, ab210498, abcam, 1:500, LC-3B, ab192890, abcam, 1:500) overnight at 4°C and the TBS solution washed 5 times, 5 min each time. We added HRP-labeled secondary antibody (ab7090, abcam, 1:2000), incubated at 37°C

for 1 hour and washed the membrane with TBST solution 3 times, 5 min each time. The HRP substrate color development kit was used for development, and the PVDF film was scanned by a chemiluminometer. The protein band was analyzed by the targeted protein gray value/the β-actin value.

Enzyme-linked immunosorbent assays

The content from the Elisa kit was placed on a suitable work table inside a temperature controlled room or lab. We prepared the reagents and configured standard curves. Standard wells and sample wells were set separately. The standard port or a sample of 100 μl was added to each well, mixed gently by shaking and incubated for 90 minutes at 37°C. The liquid was discarded and we did not wash the wells. A total of 100 μl working solution of biotin-labeled antibody was added to each well and covered with a new plate for 1 hour at 37°C. The working solution was discarded and we washed the plate 3 times. Next, 100 μl avidin working solution of horseradish peroxidase-labeled antibody was added to each well and covered with a new plate for 30 minutes at 37°C. The avidin working solution was discarded and the plate was washed 5 times. Next, 90 μl substrate solution was added to each well in sequence and color was developed for 15 minutes at 37°C. The reaction was stopped by adding 50 μl stop solution to each well in sequence. The OD value of each well was measured at a wavelength of 450 nm using a microplate reader within 5 minutes of the termination of the reaction. We calculated IL-1β and IL-6 content in the astrocytes by standard curve.

Statistical analysis

All data were analyzed by SPSS 19.0 software and presented as mean ± SD. One-way analysis of variance was used to compare data from different groups. Post-hoc tests was performed by using Tamhane's T2. $P < 0.05$ was the criterion for significance.

Results

Rapamycin-induced autophagy inhibits the inflammatory response in neurons

The morphology of neurons in each group was observed microscopically. IL-6-induced neurons showed an altered shape, with destroyed plasma membranes and irregular network structures. The halos around several neurons disappeared. Fewer cellular protrusions were observed; of those that were present, the morphology was defective. The pretreatment of cells with rapamycin, which induced autophagy, could reverse these morphological changes (**Figure 1A**). Neurons were cultured in the presence or absence of IL-6, rapamycin, and rapamycin + IL-6, and the viability of cells was measured with CCK-8. No difference in viability was observed between the blank and Rap groups ($P > 0.05$) (**Figure 1B**). However, the viability of cells cultured in the presence of IL-6 was significantly decreased, and a significant difference in viability was noticed between the blank and IL-6 groups ($P < 0.01$) (**Figure 1B**). When rapamycin was used to induce autophagy in neurons before the addition of IL-6 into the medium, the viability of cells was increased, and a significant difference in viability was noticed between IL-6 and Rap+IL-6 groups ($P < 0.01$) (**Figure 1B**). The levels of autophagy markers, beclin-1 and LC-3ll, were measured by western blotting. The results showed that the Rap+IL-6 group showed increased levels of beclin-1 and LC-3ll compared with the IL-6 group ($P < 0.01$) (**Figure 1C-E**).

Rapamycin-induced autophagy inhibits the inflammatory response in astrocytes following glucose oxygen deprivation/reoxygenation

In the first two groups, astrocytes were cultured in glucose-free medium under conditions of OGD for 2 h, followed by complete medium and reoxygenation for 24 and 48 h. In the second two groups, astrocytes were pretreated with rapamycin and cultured under conditions of OGD, followed by reoxygenation. The morphology of astrocytes in each group was observed microscopically. In the OGD/R group, the astrocyte's shape was altered, and the plasma membranes were destroyed. In addition, the synapses were shortened, and tight junctions were destroyed. There was evidence of edema and coagulative necrosis. The pretreatment of astrocytes with rapamycin could reverse these changes (**Figure 2A**).

Previous studies have reported that astrocytes secrete inflammatory mediators, such as IL-1β and IL-6, which can also stimulate astrocytes in an autocrine manner, thereby aggravating tissue damage during cerebral I/R injury. The levels of IL-1β and IL-6 in astrocytes of each group
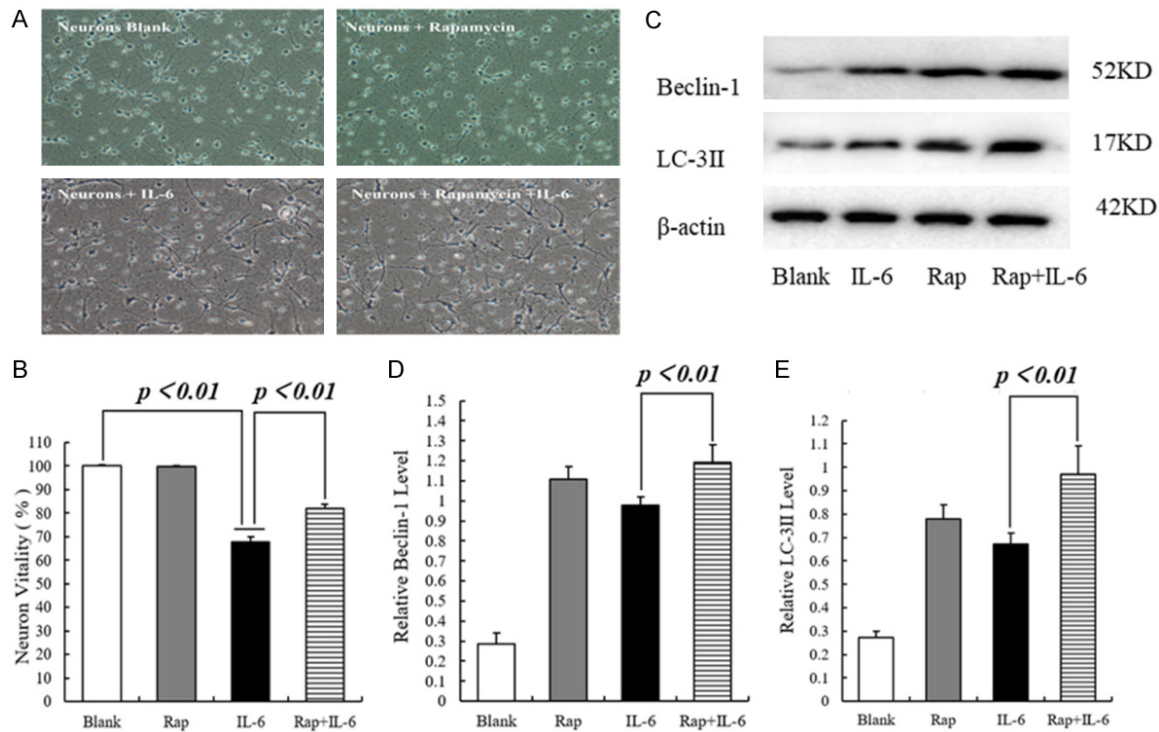
Figure 1. The effect of rapamycin-induced autophagy inhibits the inflammatory response in neurons. A. Representative morphological image of neurons, scale bar = X100. B. Statistics of neurons vitality by CCK-8. C. Representative Western blotting image of Beclin-1 and LC-3ll. D. Statistics of the expressional level of Beclin-1. E. Statistics of the expressional level of LC-3ll.

were detected by enzyme-linked immunosorbent assays (ELISA). The OGD/R group had higher levels of IL-1β and IL-6 than the Rap+ OGD/R group ($P < 0.01$) (**Figure 2B**, **2C**). The levels of autophagy markers, beclin-1 and LC-3ll, were measured by western blot. The results showed that the Rap+OGD/R group exhibited increased levels of beclin-1 and LC-3ll compared with the OGD/R group ($P < 0.01$) (**Figure 2D-F**).

Infarction size and neurological score

Brain cross-sections were stained with TTC, and the infarction size was determined. There was no evidence of cerebral infraction in rat brains of the sham group. The extent of infarction was large in rat brains of the I/R group, with tissue death noted in the cortex, subcortical region, striatum, and hippocampus. The extent of infarction was small in rat brains of the Rap+I/R group, in which autophagy was induced with rapamycin ($P < 0.01$) (**Figure 3A**, **3B**).

Neurological scores were assigned to each group at 48 h after MCAO. No neurological deficits were observed in rats of the sham group. Poor performance in autonomic exercises, limb activities, and body sensations was noted in rats of the I/R group. Compared to the I/R group, there was a significant improvement in the neurological score in rats of the Rap+I/R group ($P < 0.05$) (**Figure 3C**).

Rapamycin-induced autophagy inhibits the expression of pro-inflammatory cytokines IL-1β and IL-6

As inflammatory mediators, IL-1β and IL-6 are secreted in large amounts during cerebral I/R injury, thereby inducing tissue damage. Compared to rat brains of the I/R group, the mRNA expression of IL-1β ($P < 0.01$) and IL-6 ($P < 0.01$) was significantly decreased in brains of the Rap+I/R group (**Figure 4A**, **4D**). Similarly, the protein expression of IL-1β ($P < 0.05$) and IL-6 ($P < 0.01$) was significantly decreased in brains of the Rap+I/R group (**Figure 4B**, **4C**, **4E**, **4F**). To examine the activation of autophagy
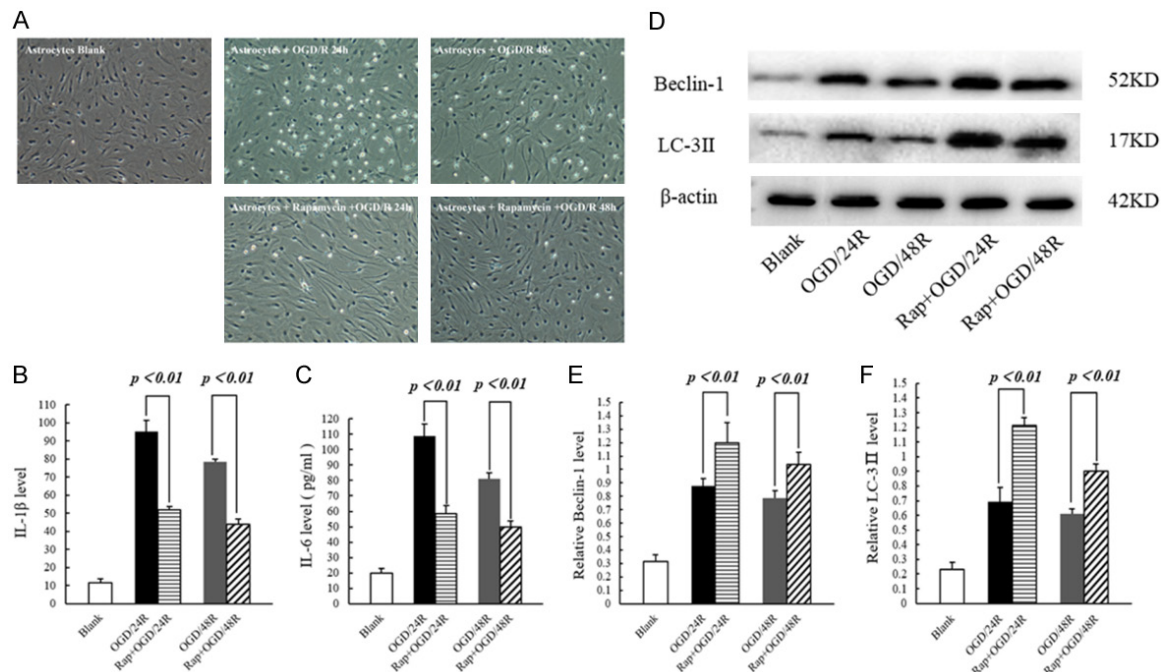
Figure 2. The effect of rapamycin-induced autophagy inhibits inflammatory responses in astrocytes. A. Representative morphological image of astrocytes, scale bar = X100. B. Statistics of the level of IL-1β. C. Statistics of the level of IL-6. D. Representative Western blotting image of Beclin-1 and LC-3ll. E. Statistics of the expressional level of Beclin-1. F. Statistics of the expressional level of LC-3ll.

markers, we used real-time PCR and western blotting to examine the mRNA and protein expression of beclin-1 and LC-3ll. Compared to the I/R group, the mRNA expression of beclin-1 (*P* < 0.01) and LC-3ll (*P* < 0.01) was significantly increased in the Rap+I/R group (**Figure 5A**, **5D**). Similarly, brains of the Rap+I/R group showed significantly decreased protein expression of beclin-1 (*P* < 0.01) and LC-3ll (*P* < 0.05) (**Figure 5B**, **5C**, **5E**, **5F**). Finally, we used dual immunofluorescent staining to observe the expression of IL-1β and IL-6, as well as beclin-1 and LC-3ll, in the rat brain. The results showed IL-1β, IL-6, beclin-1, and LC-3ll expression in neurons and astrocytes (Supplementary Figures 1, 2, 3 and 4).

Discussion

Numerous studies have reported that cerebral I/R injury involves various pathophysiological mechanisms, including free radical generation, lipid peroxidation, excitatory amino acid toxicity, mitochondrial dysfunction, and intracellular Ca^{2+} overload [25-30]. The inflammatory response caused by cerebral I/R injury, characterized by the rapid activation of microglia and astrocytes, is followed by the production of inflammatory mediators and inflammatory cells, such as neutrophils, different subtypes of T cells, and mononuclear macrophages.

These inflammatory mediators penetrate the ischemic brain tissue and induce damage [31]. Damaged nerve cells can release large amounts of IL-1β and IL-6 [32, 33], which mediate the inflammatory response in cerebral I/R injury [34]. The levels of IL-6 and IL-1β are low in normal brain tissues, but high under ischemic and hypoxic conditions. Furthermore, IL-1β can not only promote the production of free radicals and proteolytic enzymes, which affect the metabolic activities of nerve cells and the permeability of blood vessels (causing brain edema and aggravating brain damage), but also induce the expression of IL-6, which stimulates macrophages to produce more IL-1β [35]. IL-6, a multifunctional cytokine that participates in the inflammatory response, is especially important in the acute phase of cerebral I/R injury [36]. Our experimental results of dual immunofluorescent staining confirmed that IL-1β and IL-6 are expressed in damaged brain tissue after I/R injury. Additionally, real-time PCR, western
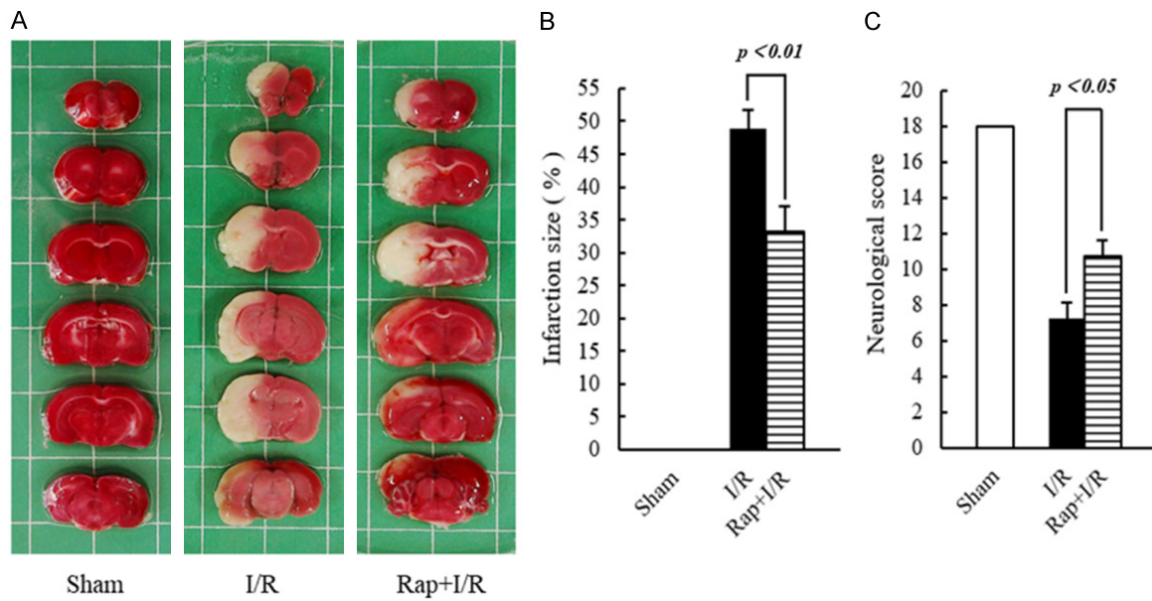
Figure 3. Infarction size and neurological score. A. Image of TTC staining. B. Statistics of infarction size. C. Statistics of neurological score.
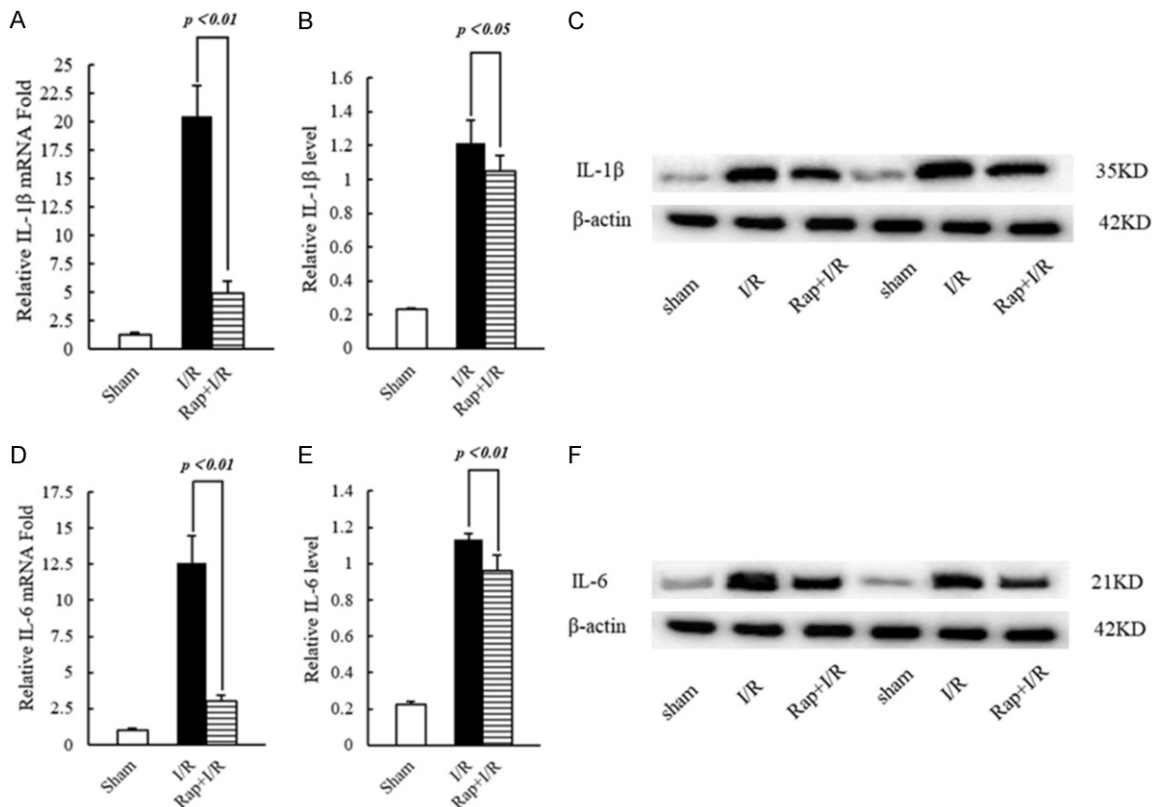

Figure 4. The transcription and expression of IL-1β and IL-6 in rat brain tissue. A. Statistics of the transcription level of IL-1β. B. Statistics of the protein expression level of IL-1β. C. Representative Western blotting image of IL-1β. D. Statistics of the transcription level of IL-6. E. Statistics of the protein expression level of IL-6. F. Representative Western blotting image of IL-6.
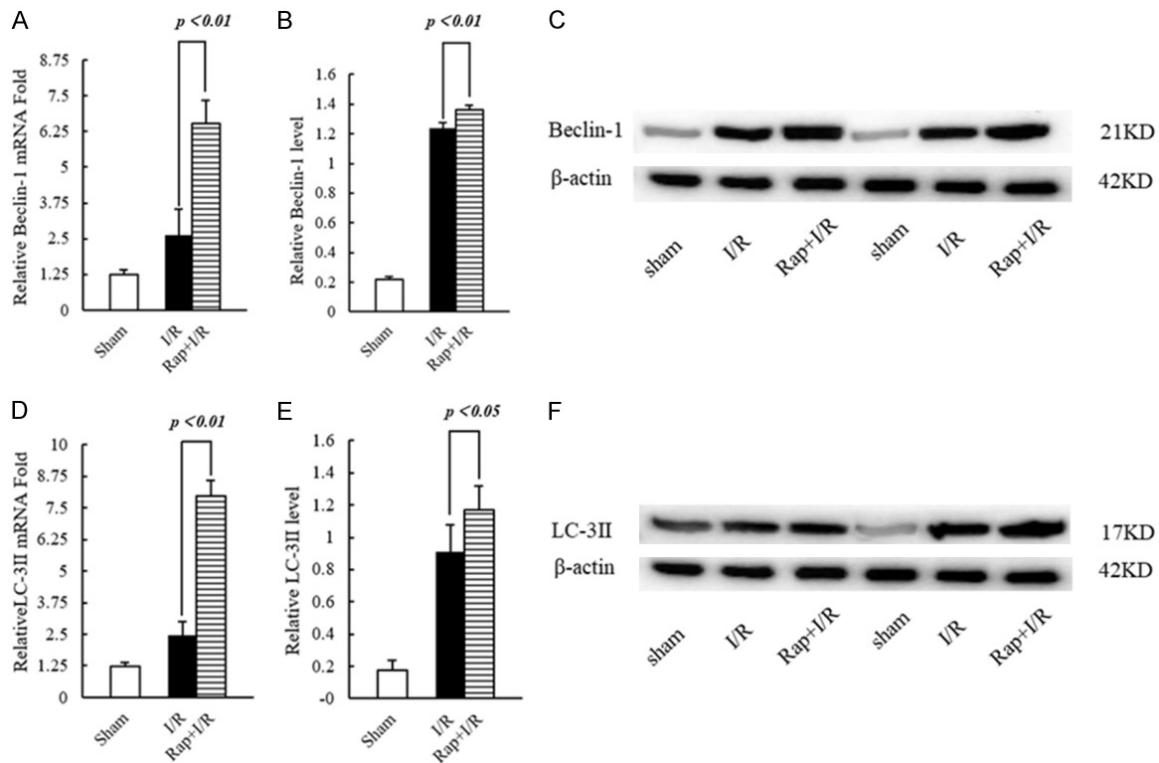
Figure 5. The transcription and expression of beclin-1 and LC-3ll in rat brain tissue. A. Statistics of the transcription level of beclin-1. B. Statistics of the protein expression level of beclin-1. C. Representative Western blotting image of beclin-1. D. Statistics of the transcription level of LC-3ll. E. Statistics of the protein expression level of LC-3ll. F. Representative Western blotting image of LC-3ll.

blotting, and ELISA showed that the levels of IL-1β and IL-6 increased in neurons and astrocytes too. During inflammation, neurons and astrocytes showed morphological changes and decreased neuron viability *in vitro*. Significant infarction and a decline in neurological function were also observed *in vivo*.

We used rapamycin to induce autophagy in both neurons, astrocytes and rat brain tissue to inhibit inflammation, Beclin-1 and LC3II were examined as key markers for autophagy. Our results showed that autophagy triggered by rapamycin could protect neurons from IL-6-induced injury and astrocytes from OGD/R-induced injury *in vitro* and the rat brain tissue from I/R injury *in vivo* had a reduced inflammatory response, characterized by increased viability of neurons, decreased infarction size in the brain, recovery of neurological function, decreased expression of IL-1β and IL-6, and increased expression of beclin-1 and LC-3ll. The protective mechanism of autophagy on neurons, astrocytes, and the brain tissue indicates that rapamycin could effectively inhibit

the inflammatory response caused by cerebral I/R injury.

Under pathological conditions, autophagy's effect is critical for neuronal function [37]. However, the current literature in this matter is contradictory [38]. Shi and colleagues reported that activity of autophagy had a protective role in ischemic-hypoxic injury [39]. On the other hand, Zhou and colleagues indicated that autophagy could contribute to the apoptosis process [40]. We believe that these contradictory outcomes must be understood taking into consideration the level of autophagy per se: considerably high or low autophagy extent might be harmful. For instance, the work of Sun and colleagues [41] showed that an excessive level of autophagy promoted cell death and, hence, a decrease in autophagy proved beneficial for brain tissue. In our study, we found out that autophagy markers beclin-1 and LC3ll in neurons can be detected and indicated the activity of autophagy in the IL-6 group, but neuronal viability decreased. It has been considered that an insufficient level of autophagy in

the IL-6 group may be also harmful, but neurons treated with rapamycin prior to IL-6 treatment in the Rap+IL-6 group had a higher beclin-1 and LC3ll expression, increased neuron viability and improved neuronal shape, as compared to the IL-6 group, even though rapamycin increases autophagy levels. Thus, we believe that both extremes, either an insufficient or an excessive degree of autophagy, can be harmful for the neurons; therefore, moderate autophagy may protect neurons from death.

We also observed that the effects of autophagy on astrocytes were linked with the extent of ischemic and hypoxic events. Our results showed that the levels of beclin-1 and LC-3ll were higher in the Rap+OGD/24R group than those in the Rap+OGD/48R group. However, the levels of IL-1β and IL-6 were lower in the Rap+OGD/48R group than those in the Rap+OGD/24R group, contrary to our expectations. The inflammatory response caused by a cerebral I/R injury is a gradually escalating pathological process, which reaches its peak of IL-1β and IL-6 production at 24 hours after starting reperfusion [42, 43]; hence, this suggests that the protective effects of autophagy may be counteracted by the peak of inflammatory response. Therefore, our results showed the ability of autophagy to suppress the inflammatory response was limited and was influenced by the severity of inflammation on cerebral I/R injury.

Cerebral I/R injury is an acute and long-lasting process that proceeds from ischemia to reperfusion, the protective effects of autophagy related to the rapamycin administration time are also relevant. Wu and colleagues reported that rapamycin treatment at 30 minutes and 12 hours prior to cerebral ischemia exerts a protective effects on cerebral I/R injury in rats [44]. Similarly, Lv and colleagues reported that rapamycin treatment 2 days prior to cerebral ischemia also downregulated I/R injury related inflammatory response [45]. However, in clinical practice, it is difficult to predict when a stroke will occur and treatments can only take place once ischemia or reperfusion has occurred. We think that the value of autophagy could be obtained at the beginning of the reperfusion. In our study, we induced autophagy in rats through an intracerebroventricular injection of rapamycin at the beginning of the reper-

fusion period and observed a reduction in the infarction size, a recovery of the neuronal function, and a decreased expression of IL-1β and IL-6, which essentially maintained neuronal function and protected brain tissue. Our results also suggest that the induced autophagy at the beginning of reperfusion could also effectively inhibit the inflammatory response, thereby protecting the brain from cerebral I/R injury.

Conclusion

Autophagy can inhibit the inflammatory response caused by IL-6 treated, OGD and cerebral I/R injury, as well as protect neurons, astrocytes and rat brain tissue. There is a relationship between the extent of protection and the level of the autophagic response, the stage of the cerebral ischemic event, and the time of intervention. The relationship between autophagy and the inflammatory response is complex. An effective understanding of this relationship is essential for reducing cerebral I/R injury. Further studies are needed to address (i) how autophagy can be adjusted to moderate levels in various models, and (ii) how the balance between autophagy and the inflammatory response can be maintained. Meanwhile, we plan to study the best time to induce the autophagy during other time points after the cerebral I/R episode.

Disclosure of conflict of interest

None.

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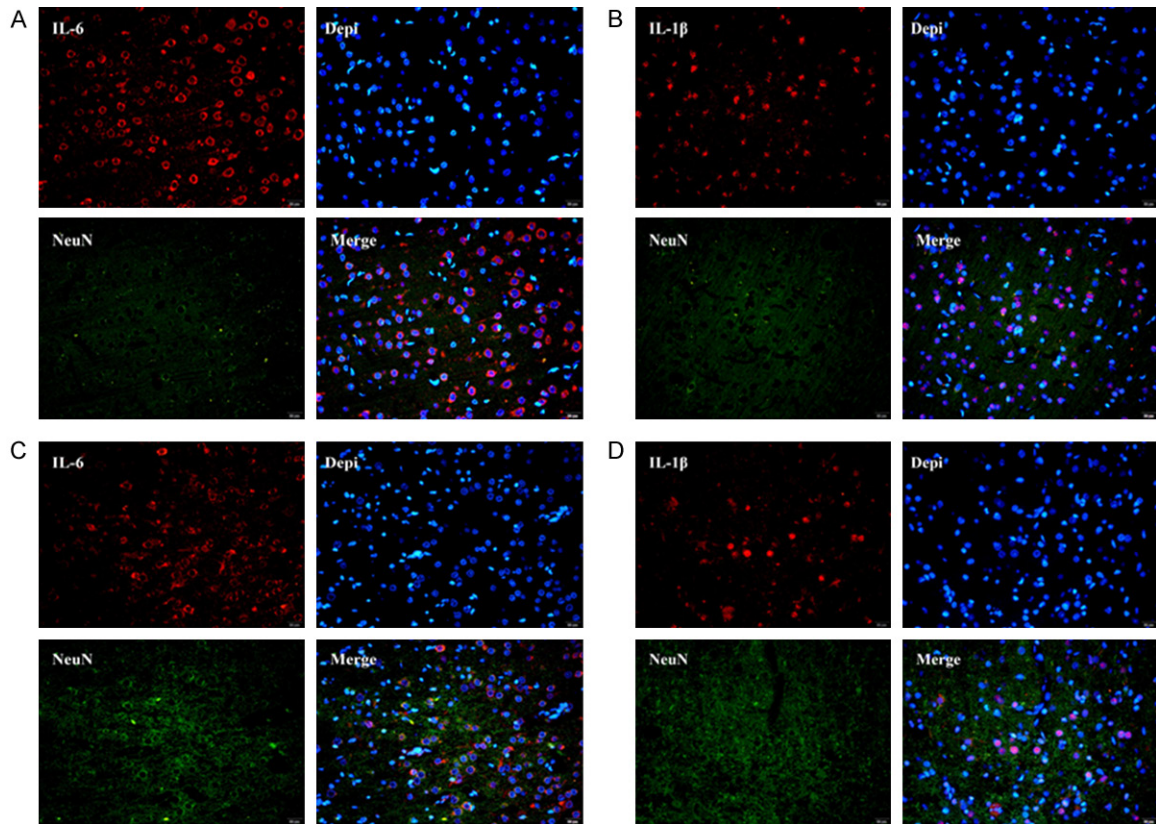
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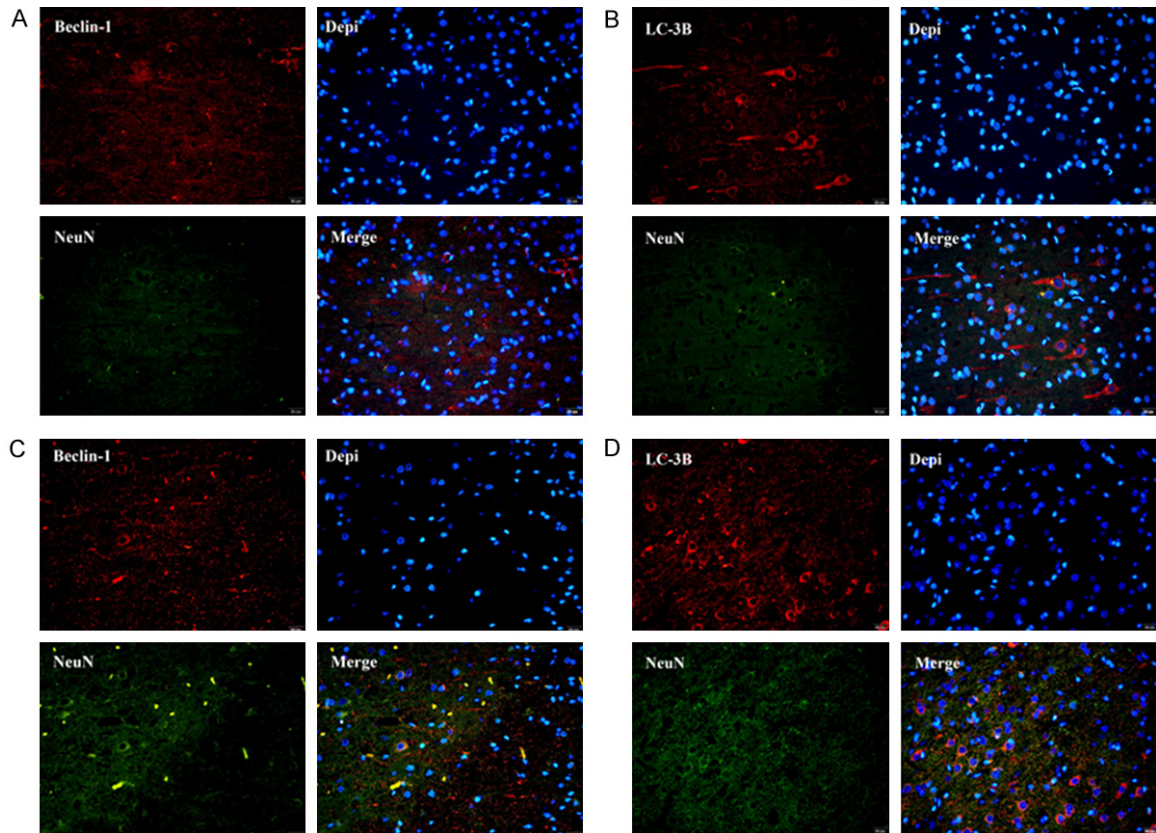
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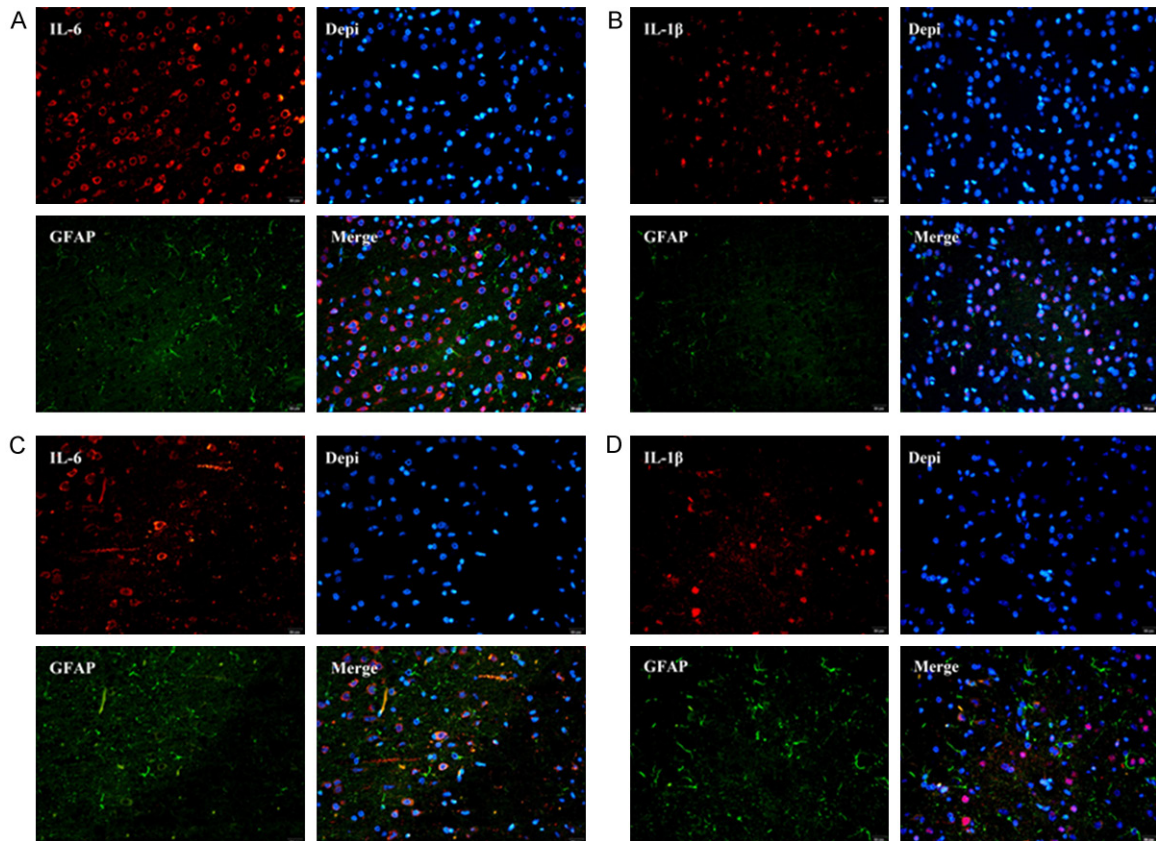
Supplemntary Figure 1. IL-6 and IL-1β immunofluorescent staining in neurons. (A) Image of IL-6 expression in I/R group; (B) Image of IL-1β expression in I/R group; (C) Image of IL-6 expression in Rap+I/R group; (D) Image of IL-1β expression in Rap+I/R group.
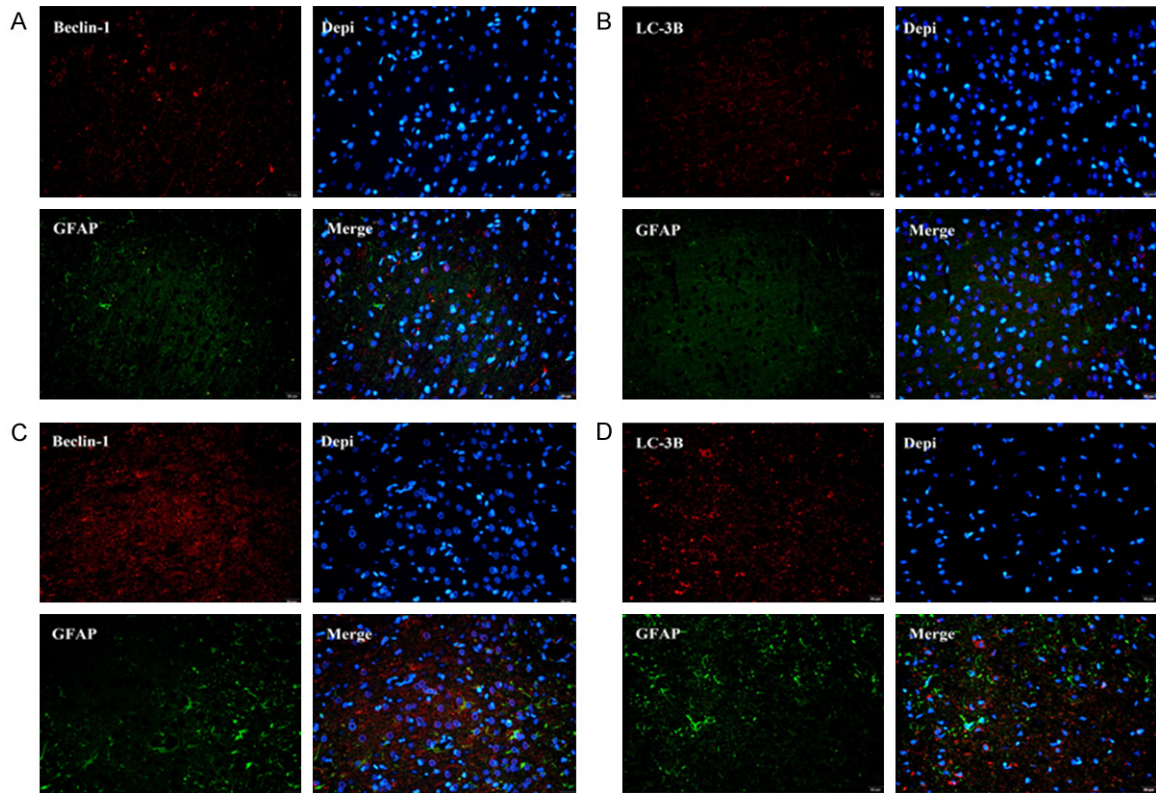
Supplemntary Figure 2. Beclin-1 and LC-3β immunofluorescent staining in neurons. (A) Image of Beclin-1 expression in I/R group; (B) Image of LC-3B expression in I/R group; (C) Image of Beclin-1 expression in Rap+I/R group; (D) Image of LC-3B expression in Rap+I/R group.

Supplemntary Figure 3. IL-6 and IL-1β immunofluorescent staining in astrocytes. (A) Image of IL-6 expression in I/R group; (B) Image of IL-1β expression in I/R group; (C) Image of IL-6 expression in Rap+I/R group; (D) Image of IL-1β expression in Rap+I/R group.

Supplemntary Figure 4. Beclin-1 and LC-3β immunofluorescent staining in astrocytes. (A) Image of Beclin-1 expression in I/R group; (B) Image of LC-3B expression in I/R group; (C) Image of Beclin-1 expression in Rap+I/R group; (D) Image of LC-3B expression in Rap+I/R group.