Macamide B Pretreatment Attenuates Neonatal Hypoxic-Ischemic Brain Damage of Mice Induced Apoptosis by Regulates Autophagy Via The PI3K/AKT Signaling Pathway

Xiaoxia Yang
Guangdong Pharmaceutical University https://orcid.org/0000-0003-3618-510X
Mengxia Wang
Guangdong No.2 Provincial People's Hospital
Qian Zhou
Guangdong Pharmaceutical University
Yanxian Bai
Guangdong Pharmaceutical University
Jing Liu
Guangdong Pharmaceutical University
Junhua Yang
Guangdong Pharmaceutical University
Lixia Li
Guangdong Pharmaceutical University
Guoying Li
Guangdong Pharmaceutical University/Guangdong Medical Association
Li Luo (josephluoli@hotmail.com)
Guangdong Pharmaceutical University/Guangdong Medical Association

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Abstract

Lepidium meyenii (Maca) is an annual or biennial herb from South America that is a member of the genus Lepidium L. in the family Cruciferae. This herb has antioxidant, anti-apoptotic, and enhances autophagy functions and can prevent cell death, and protect neurons from ischemic damage. Macamide B, an effective active ingredient of maca, has a neuroprotective role in neonatal hypoxic-ischemic brain damage (HIBD), and the underlying mechanism of its neuroprotective effect is not yet known. The purpose of this study is to explore the impact of macamide B on HIBD-induced autophagy and apoptosis and its potential mechanism for neuroprotection. The modified Rice-Vannucci method was used to induce HIBD on 7-day-old (P7) macamide B and vehicle-pretreated pups. TTC staining was used to evaluate the cerebral infarct volume of pups, brain water content was measured to evaluate the neurological function of pups, neurobehavioral testing was used to assess functional recovery after HIBD, TUNEL and FJC staining was used to detect cell autophagy and apoptosis, and western blot analysis was used to detect the expression levels of the pro-survival signaling pathway phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and autophagy and the apoptosis-related proteins. The results show that macamide B pretreatment can significantly decrease brain damage, improve the recovery of neural function after HIBD. At the same time, macamide B pretreatment can induce the activation of PI3K/AKT signaling pathway after HIBD, enhance autophagy, and reduce hypoxic-ischemic (HI)-induced apoptosis. In addition, 3-methyladenine (3-MA), an inhibitor of PI3K/AKT signaling pathway, significantly inhibits the increase in autophagy levels, aggravates HI-induced apoptosis, and reverses the neuroprotective effect of macamide B on HIBD. Our data indicate that macamide B pretreatment might regulate autophagy through PI3K/AKT signaling pathway, thereby reducing HIBD-induced apoptosis and exerting neuroprotective effects on neonatal HIBD. Macamide B may become a new drug for the prevention and treatment of HIBD.

Introduction

Hypoxic-ischemic brain damage (HIBD) is a brain lesion produced by hypoxic-ischemic (HI) of brain tissue due to various causes, which can lead to acute death and chronic nerve damage in infants. In developed countries, HIBD occurs in 1-8 newborns out of every 1,000 newborns. In developing countries, up to 26 newborns out of every 1,000 suffer from HIBD disease [1, 2]. Severe HIBD can lead to neurological sequelae such as mental retardation, cerebral palsy and cognitive impairment in infants, and even death. [3]. It is estimated that HIBD causes more than 1 million deaths every year worldwide [4]. To date, mild hypothermia therapy is considered the only neuroprotective treatment that can improve the outcome of neonatal HIBD patients, but only 40% of patients survive with normal nervous system development [5]. Therefore, the study of HIBD's pathological mechanism and the search for active and effective drugs for neurofunctional repair have become urgent subjects in perinatal medical research.

Lepidium meyenii (Maca) is an annual or biennial herb from South America that is a member of the genus *Lepidium L.* of the family Cruciferae. It is commonly used to treat degenerative diseases, such as neurodegenerative diseases, cardiovascular diseases, diabetes, cancer, and ageing. Maca can remove

free radicals and protect cells from developing oxidative stress [6-8]. Studies have shown that maca improves the cognitive function of middle-aged mice by up-regulating autophagy-related proteins [9]. Macaamide is the product of maca after drying treatment. It is produced by the formation of amide bonds between different amino-containing compounds and various fatty acids. Macamides are part of the unique phytochemical composition of maca, which is rich in nutrients and has remarkable medicinal effect. It has the functions such as anti-oxidation, anti-inflammatory, anti-osteoporosis, memory improvement, nerve cell protection, and nervous system regulation [9-12]. However, Macamide B (structure shown in Fig. 1a) is an effective monomer of macamide, and its potential mechanism in providing neuroprotection and exerting neuroprotective effects is not yet known.

The phenotypes of autophagy, apoptosis, oxidative stress, inflammation and necrosis are closely related to the pathological development of HIBD [13, 14]. Among these factors, accumulated data indicate that the mechanism of autophagy and apoptosis plays a fundamental role in ischemic brain damage in newborn rodents [13-15]. Currently, many studies have focused on autophagy and apoptosis as a therapeutic target to prevent neonatal HIBD [13, 16, 17]. Autophagy is a process that widely exists in eukaryotic cells, and uses the lysosomal pathway to degrade self-damaged organelles, proteins and macromolecular substances. It maintains cell survival by degrading and restoring over-expressed proteins and organs in cells. It has been considered to be related to brain ageing and various neurodegenerative diseases are related [18]. Stroke studies have shown that autophagy damage can aggravate the nerve damage induced by cerebral ischemia [19]. In the study of ischemia-reperfusion (I/R) damage, melatonin played a neuroprotective role on I/R damage by activating autophagy [20]. In addition, in focal cerebral ischemia and glucose and oxygen deprivation (OGD) models, autophagy has a neuroprotective effect on glial cells [21]. The death and survival of cells usually require the co-regulation of apoptosis and autophagy. Reducing neuronal apoptosis is a crucial process in determining neural functional recovery after HIBD. In the I/R model, the intervention of electroacupuncture significantly inhibits neuronal apoptosis induced by cerebral ischemia, thereby reducing the brain damage after I/R [22]. Acute spinal cord damage studies have found that inhibiting autophagy significantly aggravates cell apoptosis in the spinal cord damage area and hinders the recovery of nerve function after spinal cord damage [17]. As an effective active ingredient of Maca, Macamide B can prevent neonatal HIBD by regulating autophagy and reducing nerve cell apoptosis. It is worthy of further investigation.

The phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathway is one of the most crucial signaling cascade that regulate autophagy, apoptosis, proliferation, and growth of mammalian cells (Fig. 1b). It plays a vital role in mediating the pathological process of nervous system injury. In the treatment of neurodegenerative diseases, PI3K/Akt pathway is considered to be a target to promote cell survival [23]. Therefore, the neuroprotective effect of PI3K/Akt pathway in cerebral ischemia has been extensively studied. Studies have shown that Geniposide can protect perinatal HI-induced brain damage by activating the PI3K/Akt signaling pathway [24]. Ginkgetin can inhibit cell apoptosis induced by I/R by activating PI3K/Akt pathway [25]. In the rat middle cerebral artery occlusion (MCAO) model, Sodium butyrate inhibits neuronal apoptosis through GPR41/Gbc/PI3K/Akt signaling pathway, thereby reducing brain damage after MCAO and improving functional outcome [26]. Increased nerve cell apoptosis is a

common consequence of HIBD, and reducing cell apoptosis is essential to improve the neurological dysfunction caused by HIBD. As we all know, the PI3K/Akt pathway is also a meaningful way to regulate autophagy. PI3K/Akt pathway inhibitors can significantly inhibit the expression of autophagy-related proteins induced by HBCDs, suggesting that activation of PI3K/Akt pathway may promote the increase of HBCDs-induced autophagy levels [27]. It has been reported that melatonin reduces neuronal damage caused by I/R by activating the expression of autophagy-related levels [28]. In addition, the induction of mitochondrial autophagy through endoplasmic reticulum stress can prevent transient ischemic brain damage [29]. This indicates that activating the PI3K/Akt pathway to enhance autophagy may be a promising therapeutic strategy for HIBD to induce apoptosis.

PI3K/Akt signaling pathway plays a vital role in the process of cell autophagy and apoptosis [30]. A therapeutic strategy dedicated to activating the PI3K/Akt signaling pathway to enhance autophagy, thereby reducing ischemic penumbra (IP) cell apoptosis seems very promising. However, the ability of macamide B to ameliorate HIBD in neonatal mice, and whether macamide B pretreatment can regulate autophagy through the PI3K/AKT signaling pathway after HIBD, thereby reducing HIBD-induced apoptosis is unclear. Therefore, this study explored the potential of macamide B to regulate autophagy through PI3K/AKT signaling pathway to reduce HIBD in neonatal mice and inhibit cell apoptosis.

Materials And Methods

Animals

The study selected P7 C57BL/6 pups (male or female), weighing 3 to 5 g, provided by the Guangdong Medical Laboratory Animal Center (Guangzhou, Guandong). Each squirrel cage allows one female mouse to take care of its pups. Mice are reared in an environment with a temperature of 20 to 24°C and a humidity of 40% to 70%. All animal-related experiments done in this study have been approved by the Experimental Laboratory Animal Committee of Guangdong Pharmaceutical University and comply with the Chinese Council on Animal Care guidelines.

HIBD model and macamide B administration

In this experiment, we used a total of 160 P7 C57BL/6 pups. The improved Rice-Vannucci method was used to construct the HIBD model [31, 32]. Briefly, C57BL/6 pups at 7-9 days of age (P7-9) were given continuous inhalation anaesthesia (isoflurane) through a face mask, fixed on a sterile surgical drape in the supine position, and disinfected at the predetermined surgical incision location on the neck skin. A 1 cm incision was cut along the middle of the neck, the subcutaneous tissue was bluntly separated, and the unilateral common carotid artery (CCA) was separated, a coagulator was used to cut off the CCA, the skin was sutured and disinfected, and the pups were returned to the dams for feeding and recovery. After recovering for 1 h, put the pups in a 37°C water bath controlled hypoxia box, and a mixed gas of oxygen

(8%) and nitrogen (92%) was supplied. The pups were exposed to hypoxia for 4 h then returned to the dams to feed, and the model was completed.

Macamide B (Shanghai yuanye Bio-Technology Co., Ltd, China, HPLC≥98%) was dissolved in PBS solution (1.25 mg/ml). Twenty minutes before the ischemia surgery, the macamide B group mice were intraperitoneally injected with macamide B (60 mg/kg). For pups in the vehicle group, give the same volume of PBS treatment. The protocol is described in Fig. 1c.

Infarct volume measurement

After HIBD for 24 h, intraperitoneal administration of 10% chloral hydrate to the pups for anaesthesia (0.1 ml), and the brains were immediately extracted. We cut the pup's brain into four brain slices in a coronal plane (the intervals between adjacent brain slices is 2 mm). The sections were stained for 20 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, Germany), turning the brain slices from time to time to make even contact with the staining solution. Then the images of the brain slice of the pup were captured by a digital camera. The nonischemic necrotic area was red, while the ischemic necrotic tissue was white. Use ImageJ software (version 1.8.0, USA) to analyze the cerebral infarct volume of pups. The percentage of infarct volume = [(contralateral hemisphere − un-infarcted area of ipsilateral hemisphere)/contralateral hemisphere × 2] × 100% [1].

Measurement of brain water content

After HIBD for 24 h, intraperitoneal administration of 10% chloral hydrate to the pups for anaesthesia (0.1 ml), and the brains were immediately extracted. The pup's brain was divided into two parts and weighed for wet weight and the cerebral hemispheres were dried in an oven at 106°C for 24 h and weighed for dry weight. Percentage of brain water content = (wet weight–dry weight)/wet weight ×100%. The percentage of brain water content = (wet weight - dry weight) / wet weight × 100%.

Neurobehavioral assessments

Neurological damage caused by HIBD can lead to sensorimotor impairments. Body weight and sensorimotor performance (righting reflex, negative geotaxis, and grip test) were tested 1, 3, and 7 days after the HI procedure in a blinded manner [33].

Righting reflex

The righting reflex is used to evaluate the recovery of the brain of pups. The pups were placed on a flat surface with one hand gently holding the head and the other hand gently holding the hind limbs; the pup was rolled onto its back and released. The time required for the mouse to return to an upright position (all

limbs on the ground) was recorded. The maximum testing time was 1 min, and times over 1 min were recorded as 1 min.

Negative geotaxis

The geotaxis test was used to diagnose vestibular or proprioceptive functions. Place the pup on a flat surface inclined at 45 degrees, with the head of the pup facing the bottom of the plane, and record the time it takes for the head and body of the pup to make a 180° turn. The maximum testing time was 1 min, and times over 1 min were recorded as 1 min.

Grip test

The pup grasped a metal wire with a diameter of 1.5 mm with both front feet. The metal wire was stretched horizontally in a test box with a width of 50 cm. The distance between the metal wire and the bottom of the box was 15 cm. The bottom was covered with cork chips. Record the time it takes for the pup to grasp the wire to release it. The minimum testing time was 20 s, and times below 20 s were recorded as 20 s.

Western blot analysis

Protein samples (15 μl/well) were separated on SDS-PAGE gel (80 V, 110 min) and transferred to 0.22 μm polyvinylidene difluoride (PVDF) membrane (290 mA, 100 min). Five percent nonfat dry milk in TBST buffer was used to block nonspecific sites for 1.5 h. Incubate the primary antibody in a refrigerator at 4℃ for 16 h. Use that following primary anti-antibody: PI3K (1:1500, Abcam, UK), p-PI3K (1:1500, Abcam, UK), AKT (1:1500, Proteintech, USA), p-Akt (1:1500, Cell Signaling Technology, USA), Beclin1 (1:750, Abcam, UK), LC3B (1;500, Proteintech, USA), p62 (1:1000, Abcam, UK), p53 (1:500, Multisciences, China), Bax (1:750, Proteintech, USA), Bcl-2 (1:750, Proteintech, USA), caspase-3 (1:600, Proteintech, USA). and cleaved caspase-3 (1:650, Proteintech, USA). β-actin (1:800; Proteintech, USA) was employed as an internal reference protein. After 16 h, the corresponding secondary antibodies (1:6000, EarthOx, USA) were incubated for 90 min. Bands were measured by an automatic chemiluminescence image analysis system (Tanon 5200, Shanghai, China). ImageJ software was used to carry out Western blot analysis, and SPSS 21.0 was applied to process the data.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining.

Because cells are prone to programmed cell death during HI [34]. Therefore, we assessed the apoptotic cells in the brain tissue of the pups by TUNEL Apoptosis Detection Kit (fluorescence) (Wanleibio, China).

On day three after HI damage, the pups were perfused into the heart, and fresh brain tissue was quickly collected in 4% paraformaldehyde (PFA) for fixation. After fixation for 24 h, the brain tissue of the pup was rinsed under running water for 16h. After washing with running water, perform gradient dehydration, paraffin embedding, tissue sectioning and other operations on the brain tissue of pups for subsequent TUNEL detection.

The slices were immersed in xylene Ⅰ and Ⅱ for 15 min each, immersed in 100% Ⅰ, 100% Ⅱ, and 95%, 90%, 80%, 70%, and 50% ethanol solutions for 5 min per solution, and washed with ddH_2O for 3×5 min. Put that slices into citrate buffer solution (0. 01 mol/L), and place them in a microwave oven at high-temperature repair for 20 min. Add 25 μl of 3% H_2O_2 buffer to each brain tissue and let stand for 12 min in the dark, followed by PBS washing for 3×5 min. Then, add 25 μl TUNEL reaction buffer to each brain tissue and incubated in a 37°C incubator in the dark for 90 min. After washing with PBS for 3×5 min, counter-stain brain tissue cell nuclei with DAPI for 5 min, then repeat the PBST wash step. After absorbing excess water with filter paper, add 25 μl of anti-fluorescence quencher to each brain tissue for mounting, immediately observe with a fluorescence microscope (Olympus BX51, Japan) , or store it at -20°C for observation within a week.

Fluoro-Jade C Staining

After HIBD for 24 h, the pups were immediately sacrificed, and brain paraffin sections were made. Fluoro-Jade C (FJC) staining (Biosensis, USA) was used to detect the degeneration of neurons in the pup's brain tissue.

The slices were immersed in xylene Ⅰ and Ⅱ for 15 min each, immersed in 100% Ⅰ and 100% Ⅱ ethanol solutions for 5 min per solution, immersed in 70% ethanol solutions for 5 min per solution, and washed with ddH_2O for 2×5 min. Transfer slides to a new Coplin jar containing ddH_2O for 2 min. Mix 9 parts of ddH_2O and 1 part of potassium permanganate solution and add to the brain tissue slices and incubate at room temperature for 10 min. Rinse slides for 2 min in ddH_2O. Mix 9 parts of ddH_2O, 1 part of FJC solution and 1 part of DAPI solution and add to the brain tissue slices and incubate for 12 min. Wash in ddH_2O 3×1 min. Put the slices in a 56°C oven and bake for 5 min. The dry slides are then cleared by brief (5 min) immersion in xylene. Mount the slides with an anti-fluorescence quencher. Immediately observe with a fluorescence microscope, or store it at -20°C for observation within a week.

Tissue immunofluorescence staining

After HIBD for 24 h, the pups were immediately sacrificed, and brain paraffin sections were made. Tissue immunofluorescence staining was used to detect the expression levels of p53, Bax, Bcl-2, caspase-3, and cleaved caspase-3 in the brain tissue of mice.

Repeat tissue dewaxing and high-temperature repair steps, and incubate with Quick Block^{TM} immunostaining blocking solution for 20 min. Incubate each brain tissue section with the primary antibody at 4°C for 16 h, and add 0.5% Triton X-100 to the corresponding primary antibody for cell rupture. The following primary antibodies were used:Beclin1 (1:200, Abcam, UK), LC3B (1;250, Proteintech, USA), p62 (1:260, Abcam, UK), p53 (1:260, Multisciences, China), Bax (1:180, Proteintech, USA), Bcl-2 (1:260, Proteintech, USA), caspase-3 (1:280, Proteintech, USA), and cleaved caspase-3 (1:280, Proteintech, USA). After 16 h, the membranes were removed from the refrigerator, allowed to return to room temperature for 30 min, and washed again with PBST for 3×10 min. Dylight 488 labelled goat anti-rabbit fluorescent secondary antibody (1:360, Sigma-Aldrich, USA) or Dylight 594 labelled goat anti-rabbit fluorescent secondary antibody (1:360, Sigma-Aldrich, USA), incubate for 2 h at room temperature in the dark. After washing with PBS for 3×5 min, counter-stain brain tissue cell nuclei with DAPI for 5 min, then repeat the PBST wash step. Mount the slides with an anti-fluorescence quencher. Immediately observe with a fluorescence microscope, or store it at -20°C for observation within a week.

Statistical analysis

All of the experiments were repeated at least three times. The data are presented as the mean ± SEM. Statistical analyses were carried out by SPSS.21.0 and GraphPad Prism (version 8.0, USA). Differences between individual groups were first compared using analysis of variance (one-way ANOVA), and then post hoc testing was analyzed with Tukey or Student-Newman-Keuls multiple comparisons. Difference between the two groups were compared using Student's t-test. A $p < 0.05$ indicated that the difference between the two groups was statistically significant.

Results

Macamide B pretreatment attenuated HIBD in newborn mice

In order to determine the optimal dose of macamide B pretreatment to treat neonatal HIBD, three doses were used for dose exploration: low dose (30 mg/kg), medium dose (60 mg/kg), and high dose (90 mg/kg). The results of TTC staining (Fig. 2a, b) showed that, compared with the vehicle group, the medium dose (60 mg/kg) of macamide B significantly reduced the percentage of infarct volume ($p<0.05$). Compared with the vehicle group, there was no significant difference in infarct volume between the low-dose (30 mg/kg) and high-dose (90 mg/kg) groups ($p>0.05$). It shows that the pretreatment of macamide B at a medium dose (60 mg/kg) can significantly reduce the cerebral infarction volume of HIBD pups.

Brain water content was used to assess brain edema damage in pups, as shown in the figure (Fig. 2c). The water content of the ipsilateral cerebral hemisphere in the vehicle group was significantly higher than that in the sham group ($p<0.0001$). Macamide B pretreatment significantly reduced the water content of the ipsilateral cerebral hemisphere in the vehicle group ($p<0.0001$). The above results indicate that

macamide B pretreatment significantly reduces HI-induced brain damage and has neuroprotective effects on HIBD in newborn mice.

Macamide B pretreatment improved neurobehavioral performance after neonatal HIBD

To explore whether macamide B can promote general health and recovery of neurological function, we conducted the following neurobehavioral tests at 1, 3, and 7 days after surgery: righting reflex, negative geotaxis, and grip tests in the sham, vehicle, and macamide B groups. Body weight is used to evaluate general health. At 1, 3, and 7 days after HIBD, macamide B pretreatment can significantly reverse the weight loss of pups after HIBD compared with the vehicle group (Fig. 3a). In the righting reflex (Fig. 3b), negative geotaxis (Fig. 3c), and grip tests (Fig. 3d), mice in the macamide B group exhibited superior performance at 1, 3, and 7 days after surgery than those in the vehicle group . These outcomes indicated that macamide B could improve neurobehavioral performance following HIBD.

Macamide B pretreatment activates PI3K-AKT signaling pathway

Whether the PI3K-AKT signaling pathway is involved in the neuroprotective mechanism of macamide B after HI damage is not yet known. Western blot was used to detect the expression levels of proteins related to the PI3K-AKT signaling pathway. Western blot experiment results showed that compared with sham, the expression levels of p-PI3K and p-AKT in the vehicle group were significantly decreased (p <0.0001, Fig. 4b, c). Compared with the vehicle group, macamide B pretreatment significantly increased the expression levels of p-PI3K and p-AKT protein (p <0.0001, Fig. 4b, c). Western blot showed that macamide B pretreatment significantly activates the PI3K-AKT signaling pathway. Macamide B has a neuroprotective effect on HIBD in neonatal mice, which may be mediated by the PI3K-AKT signaling pathway.

Macamide B pretreatment significantly enhances the autophagy level of HIBD neonatal mice

To evaluate the effect of macamide B pretreatment on autophagy. Tissue immunofluorescence staining and western blot were used to detect the expression levels of autophagy-related proteins Beclin1 (Fig. 5a), LC3B (Fig. 5b), and p62 (Fig. 5c). Tissue immunofluorescence staining results showed that after HIBD, the number of Beclin1 and LC3B positive cells was significantly reduced, and the number of p62 positive cells was significantly increased. Macamide B pretreatment reversed this result. The results of western blot experiments showed (Fig. 6) that, compared with sham, the expression levels of Beclin1 and LC3B in the vehicle group were significantly decreased (p<0.0001), and the expression levels of p62 were significantly

increased (p<0.001). After pretreatment with macamide B, macamide B significantly up-regulated the expression levels of Beclin1 and LC3B (p<0.0001; p<0.05), and down-regulated the expression levels of p62 (p<0.01). Tissue immunofluorescence staining and western blot results indicate that macamide B pretreatment promotes autophagy, and macamide B may exert neuroprotective effects on HIBD in neonatal mice by enhancing autophagy.

Macamide B pretreatment significantly reduces HIBD-induced apoptosis

In order to detect the effect of macamide B pretreatment on cell apoptosis of HIBD neonatal mice. TUNEL and FJC staining were used to evaluate neuronal apoptosis and degeneration. Tissue immunofluorescence staining and western blot were used to detect the expression levels of apoptosis-related proteins p53, Bax, Bcl-2, caspase-3, and cleaved caspase-3.

3-MA inhibits the PI3K-AKT signaling pathway and reverses the neuroprotective effect of macamide B on HIBD in neonatal mice

3-MA is a selective inhibitor of PI3K and has an inhibitory effect on class III PI3K [35]. To determine whether macamide B exerts a neuroprotective effect on HIBD in neonatal mice through the PI3K-AKT signaling pathway. After treatment with 3-MA, an inhibitor of the PI3K-AKT signaling pathway in HIBD newborn mice, 3-MA significantly inhibited p-PI3K, p-AKT protein expression (Fig. 9c, d; p <0.0001), and blocked the activation of PI3K-AKT signaling pathway induced by macamide B pretreatment. At the same time, the results of the TTC experiment showed (Fig. 9a, b) that compared with the macamide B + vehicle 2 group, the 3-MA intervention significantly increased the cerebral infarction area of the pups（p <0.0001）; The brain water content measurement results showed (Fig. 2c) that compared with the macamide B + vehicle 2 group, 3-MA intervention significantly aggravated the brain edema damage of pups (p <0.001); In the behavioural experiment results, compared with the macamide B + vehicle 2 group, 3-MA intervention significantly aggravated the weight loss of HIBD neonatal mice at 1, 3, and 7 days after HIBD (Fig. 9c), Macamide exerts a neuroprotective effect on HIBD in newborn mice through the PI3K-AKT signaling pathway.

3-MA treatment inhibits autophagy and reverses the protective effects of Macamide B pretreatment on apoptosis

3-MA is an inhibitor of the PI3K-AKT signaling pathway, and is also a well-known autophagy inhibitor, which works by inhibiting the formation of autophagosomes [35]. In our study, immunofluorescence (Fig.

10) showed a significant increase in Beclin1 and LC3B positive cells and a significant decrease in p62 positive cells after 3-MA intervention compared to the macamide B + vehicle group. The results of western blot (Fig. 11) were consistent with the trend of immunofluorescence results. 3-MA intervention significantly inhibited the expression levels of Beclin1 and LC3B proteins (p <0.0001), and up-regulated the expression level of p62 protein (p <0.001). In apoptosis-related studies, fluorescence experiments (Fig. 12) showed that 3-MA intervention resulted in a significant increase in the number of TUNEL-positive cells, FJC-positive neurons, and p53, Bax, caspase-3, and cleaved caspase-3 cells, and a significant decrease in the expression of Bcl-2 positive cells, compared with the macamide B + vehicle group. The results of western blot experiments showed (Fig. 13) that compared with the macamide B + vehicle group, 3-MA intervention significantly increased the expression levels of p53, caspase-3 and cleaved caspase-3 (p <0.0001; p <0.001), and reduced Bcl2 / Bax ratio (p <0.0001). The above results indicate that macamide B pretreatment may enhance autophagy by activating the PI3K-AKT signaling pathway, thereby reducing HIBD-induced apoptosis.

Discussion

Neonatal HIBD is one of the common causes of neonatal death and disability. This condition usually causes sequelae such as cerebral palsy, visual impairment, and mental retardation [36, 37]. Mild hypothermia therapy is considered to be an effective method to reduce the mortality of HIBD. However, the disability and mortality rate of HIBD is still high [38]. At the same time, due to limitations in equipment, human and financial resources, and other objective reasons, mild hypothermia therapy is not yet entirely popularized in developing countries. Therefore, finding new safe and effective therapeutic drugs is the primary task of HIBD research. As a precious herb, maca has been proven to have neuroprotective effects in previous studies [9, 10]. However, the potential therapeutic impact of maca's effective monomer, macamide B, in providing neuroprotection in HIBD has remained unclear, and the key pathways and potential mechanisms of its neuroprotective effects are not yet known.

In this study, we confirmed that Macamide B has a neuroprotective effect on HIBD in newborn mice. Specifically, we showed that (1) macamide B pretreatment significantly reduces HI-induced brain damage, (2) pretreatment with macamide B can significantly improve neurobehavioral results after HIBD, (3) macamide B pretreatment significantly activates the PI3K/Akt signaling pathway, enhances autophagy, and inhibits cell apoptosis, and (4) the neuroprotective effect of macamide B may be through the PI3K/Akt signaling pathway to regulate autophagy, thereby reducing HIBD-induced apoptosis. In this study, we clarified that macamide B pretreatment protects neonatal mouse brains from HIBD and improves general conditions and neurobehavior.

The treatment and prevention of neonatal HIBD have always attracted much attention, but due to the complexity of the nervous system, treating this disease poses severe challenges. Medicinal plants can be advantageous in the drug discovery process because they have undergone indirect clinical trials during their long-term use, and their side effects and toxicity are usually known. Given the complexity of neurodegenerative diseases, natural drugs may be good candidates for targeting phenotypes such as

apoptosis, autophagy, oxidative stress, and inflammation and promote the recovery of cell death and neuronal damage in focal ischemic stroke and many neurodegenerative diseases [39, 40]. Maca is a rare medicinal and edible plant that originated in the central part of the Peruvian Andes. It has rich nutritional value and can relieve fatigue, improve sleep, and improve sexual function and exhibits antioxidant activity. Maca is called "Peruvian Ginseng" [41-43]. Studies have shown that maca has extensive neuroprotective effects both in vivo and in vitro. The cerebral infarct volume of mice with neurons damaged by H_2O_2 was treated with maca is significantly reduced in mice treated with maca, and anti-apoptosis, antioxidation, prevention of cell death, and protection of neurons from ischemia damage are its primary mechanisms of action [9]. In vitro studies showed that the viability of crayfish neurons was significantly enhanced by treatment with maca extract, which also showed the neuroprotective effects of maca [9]. Maca can be used as a neuroprotective agent alone or synergistically with other protective agents to prevent neurodegeneration and cell death in stroke and other neurodegenerative diseases [44]. Autophagy disorders are thought to be related to brain ageing and a variety of neurodegenerative diseases. Maca can promote the up-regulation of autophagy-related proteins by activating the autophagy signal in the mouse cortex and improve the cognitive function of middle-aged mice [23]. In 2000, Zheng BL and others first discovered the unique active substance macamide in maca [44]. Macamide, a type of benzylated or 3-methoxybenzylated alkanamide alkaloid, is a unique secondary metabolite in maca [44]. Purified macamide and synthetic analogues of macamide can exert neuroprotective effects by acting on the endocannabinoid system [45]. Studies have used a zebrafish model of dopaminergic neuron loss to evaluate the neuroprotective effect of macamide. It was found that macamide extract exerts a significant neuroprotective effect on zebrafish neurons [46]. Whether macamide B, a unique monomer of macamide, has a neuroprotective effect in neonatal HIBD was investigated in this study, which also included an in-depth study of the potential mechanism of its neuroprotective effect.

In this study, one of the issues we need to consider is the effectiveness of the route of administration of macamide B in the central nervous system (CNS). The prerequisite for the direct effect of drugs on the CNS is that the drug must first pass through the blood-brain barrier (BBB) from the blood and enter the extracellular fluid of the CNS to be effective. [47]. Therefore, it is necessary to confirm whether macamide B can pass through the BBB. Studies have shown that the lipophilicity of certain components of maca can promote their passage through the BBB and affect CNS function. Related reports have shown that some ingredients in maca are active in the CNS [48-50]. For some compounds, the neonatal BBB is more permeable than the adult BBB [51]. At the same time, it has also been reported that when pups are injured by HI, the normal function of the BBB will be destroyed, which will increase the permeability of the BBB. This situation also increases the possibility of macamide B successfully reaching the brain through the BBB [51]. Besides, pups have a large peritoneal surface area and strong exchange capacity, and intraperitoneal administration of macamide B enabled macamide B to reach the brain through different signal transduction pathways and thus exert its neuroprotective effect. We used the Rice-Vannucci method to establish a neonatal mouse HIBD model by ligating one side of the common carotid artery of the pups to cause hemi-brain tissue ischemia and then placing the pups in a closed hypoxic tank for 4 h. This model is currently recognized as an ideal model for the study of neonatal HIBD [52, 33]. The results

of TTC staining showed that there was a clear cerebral infarction area on the ligation side of the brain, which verified that the brain tissue damage caused by surgery and hypoxia was, as proposed by Towfghi J et al., mainly confined to the carotid artery ligation side [53], these results also indicate that the HIBD model was successfully constructed. Compared with the vehicle group, the cerebral infarct volume of pups pretreated with macamide B was significantly reduced, indicating that macamide B has a significant neuroprotective effect on HIBD in newborn mice. In this experiment, P7 pups were selected as the model mice, mainly because the brains of P7 mouse pups are similar in histological structure to the 32-34 week fetus or newborn [54].

Increasing evidence has shown that apoptosis is a crucial pathological trigger involved in neurological deficits after HIBD [55]. Compared to adult brains, apoptosis is more common in immature newborn brains [56]. Inhibition of neuronal apoptosis is strongly recommended as a therapeutic target for neuronal rescue in the neonatal HIBD paradigm [57]. Studies have found that the tumor suppressor p53 may trigger the apoptosis pathway after DNA damage, and activate caspase 3 to induce cell death by up-regulating the expression of the pro-apoptotic protein Bax [58]. In our study, macamide B pretreatment significantly reduces the expression level of p53, and has a regulatory effect on the apoptotic pathway. Apoptosis is a kind of programmed cell death that occurs through regulating genes and their products in cells. Reducing apoptosis is an essential component in the recovery of neurological function in mammals with brain damage. Caspase-3 is a critical protein in the apoptosis signaling pathway, which can induce cell apoptosis in animal models of ischemic stroke [59]. In I/R research, caspase-3 is a key protein involved in the inflammation and apoptosis of I/R damage. Both ischemia and reperfusion lead to an increase in caspase-3 activity [60]. Inhibition of caspase-3 activation can effectively prevent neonatal HIBD [61]. Bcl-2 and Bax are the crucial proteins of the Bcl-2 gene family, Bcl-2 cell apoptosis inhibitor gene, Bax can antagonize the protective effect of Bcl-2 and induce cell apoptosis [62]. Studies have shown that the ratio of Bcl-2/Bax activity levels is a crucial determinant of cell susceptibility to apoptosis, not the level of individual proteins [63]. In the I/R damage of rats, ginkgetin can restore mitochondrial membrane potential by up-regulating the activity level of Bcl-2/Bax in rats with brain damage, thereby inhibiting apoptosis induced by the caspase-3 pathway and playing a neuroprotective role in rat I/R damage [25]. In MCAO rats and the SH-SY5Y cell model induced by oxygen glucose deprivation/re-oxygenation (OGD/R), Chrysin reduces the expression levels of Bax and cleaved caspase-3 by increasing the expression level of Bcl-2, reducing the I/R damage and apoptosis of SH-SY5Y cells induced by OGD/R. [64]. In our study, macamide B pretreatment can reverse the increase in caspase-3, cleaved caspase-3, and Bax expression levels induced by HI stimulation, and up-regulate the expression levels of Bcl-2 and the number of TUNEL and FJC positive neurons. Therefore, macamide B may effectively prevent neonatal HIBD through the anti-apoptotic pathway.

Cell death and survival in neurodegenerative diseases are usually regulated by autophagy and apoptosis. Autophagy maintains cell survival by degrading and restoring dysfunctional organelles and misfolded proteins [63]. LC3B is currently the most widely used autophagy marker protein, reflecting the number of autophagosomes. When autophagy occurs, LC3B Ⅰ is converted to LC3B Ⅱ, accompanied by the formation of autophagosomes. Beclin1 is the first autophagy-promoting protein found in mammals and regulates

autophagosome-lysosome fusion. P62 (SQSTM1) is the primary substrate for degradation during autophagy and plays a vital role in the aggregation and removal of ubiquitinated proteins. The accumulation of P62 indicates that the initiation of autophagy is decreased or the fusion of autophagosomes and lysosomes is disordered, which is negatively correlated with the level of autophagy activity [65, 66]. In the I/R model, the remote limbic postconditioning (RIPoC) mitigates I/R damage by activating autophagy [67]. Similarly, Astragaloside IV can play a neuroprotective effect on brain damage caused by ischemic stroke by promoting autophagy [68]. When HIBD occurs, autophagy and apoptosis are often inseparable. The signal network between autophagy and apoptosis is staggered, coherent, and complex, and they affect each other. Studies have shown that mild hypothermia reduces microglia activation after traumatic brain damage by inhibiting autophagy and promoting apoptosis, indicating that apoptosis of autophagy is interrelated [69]. It has been reported that activated autophagy can alleviate I/R damage by inhibiting the apoptosis cascade of ischemic stroke [70]. All these studies show that enhancing autophagy and inhibiting apoptosis has a neuroprotective effect on brain damage. However, it has also been reported that autophagy is harmful to I/R damage, and inhibition of autophagy activation may reduce I/R damage [71]. This controversy may be caused by different ischemia time, animal model, animal strain, administration time, and injection time of inhibitor agonist. Xiaowei Sun et al. found that Eugenol played a neuroprotective role in I/R damage by promoting the increase of Beclin1 level and LC3II/I ratio induced by MCAO or OGD/R, and the decrease of p62 level [72]. Consistent with the research of Xiaowei Sun et al., in this study, we proved that enhancing autophagy is beneficial to HIBD. Macamide B pretreatment significantly promoted the increased of Beclin1 and LC3B expression level and the decrease of p62 level induced by HIBD, reduced brain damage induced by HI, and improved neurological deficit. However, a selective inhibitor of PI3K, also known as the autophagy inhibitor 3-MA, reversed this finding and exacerbated apoptosis. Therefore, macamide B may reduce the apoptosis induced by HIBD by enhancing autophagy, and play a neuroprotective role on HIBD in newborn mice.

PI3K/AKT is a vital pro-survival signaling pathway, involved in many critical cellular processes, such as apoptosis, autophagy, and proliferation, and is considered to be an important regulator of autophagy and apoptosis. Studies have shown that this signaling pathway can protect neurons from damage from different brain diseases [73, 74]. p-Akt can be observed in both adults and neonates soon after cerebral ischemia, and IP is particularly significant [75]. Studies have shown that activation of PI3K/AKT and its downstream pathways can inhibit neuronal apoptosis [76]. When a brain damage occurs, pAkt can inhibit cell apoptosis, and the increase of pAkt protein has a neuroprotective effect on HIBD [77, 78]. Xiaohui Tan et al. found that luteolin can reduce neurotoxicity by inhibiting PI3K/Akt pathway-mediated p53 accumulation and p53-triggered apoptotic pathway, and exert neuroprotective effects on rat brain damage [79]. Consistent with the study by Xiaohui Tan et al., in this study, pretreatment with macamide B inhibited the PI3K/Akt pathway through 3-MA, resulting in a large accumulation of p53, thereby aggravating the apoptosis triggered by p53, leading to caspase-3, cleaved caspase -3 and Bax expression levels increased, and Bcl-2 expression levels decreased. On the contrary, the PI3K/Akt pathway activated by macamide B pretreatment can antagonize the apoptosis induced by HIBD and play a neuroprotective effect. Recent studies have shown that autophagy has a neuroprotective effect, and

autophagy can alleviate the traumatic brain injection (TBI) by inhibiting mitochondrial apoptosis pathway or neuroinflammation in the rat brain damage model [80]. In addition, Sevoflurane post-conditioning promotes autophagy by activating the PI3K/AKT signaling pathway, thereby attenuating TBI-induced neuronal apoptosis [81]. In the study of spinal cord damage, Melatonin enhances autophagy by regulating the PI3K/AKT signaling pathway and reduces cell apoptosis [17]. In this study, we found that Macamide B pretreatment can activate the PI3K/AKT signaling pathway, enhance the expression level of autophagy, and reduce HIBD-induced apoptosis, After intraperitoneal administration of PI3K inhibitor 3-MA, 3-MA significantly inhibited the activation of PI3K/AKT signaling pathway and blocked the formation of autophagosomes, resulting in a significant decrease in LC3B and Beclin1 expression levels, and a significant increase in p62 expression levels, Thereby aggravating the apoptosis induced by HIBD (Fig. 14). We confirmed that macamide B pretreatment might regulate autophagy through PI3K/AKT signaling pathway to reduce apoptosis induced by HIBD.

In summary, macamide B pretreatment can effectively treat or prevent HIBD in newborn mice, and its effect may be achieved by regulating autophagy through the PI3K/AKT signaling pathway, thereby reducing HIBD-induced apoptosis. Macamide B may be a potential drug candidate for effective prevention and treatment of neonatal HIBD.

Declarations

Author Contributions Li Luo and Mengxia Wang established the animal models and providing technical and writing guidance throughout the process ; Xiaoxia Yang contributed in experimental operations, statistical analysis, and manuscript writing; Qian Zhou assisted in the completion of western blot, behavioral tests, immunofluorescence, etc.; Yanxian Bai, Jing Liu, Junhua Yang, and Guoying Li provided technical guidance. All authors read and approved the final manuscript.

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Consent to participate Not applicable to this study

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figures

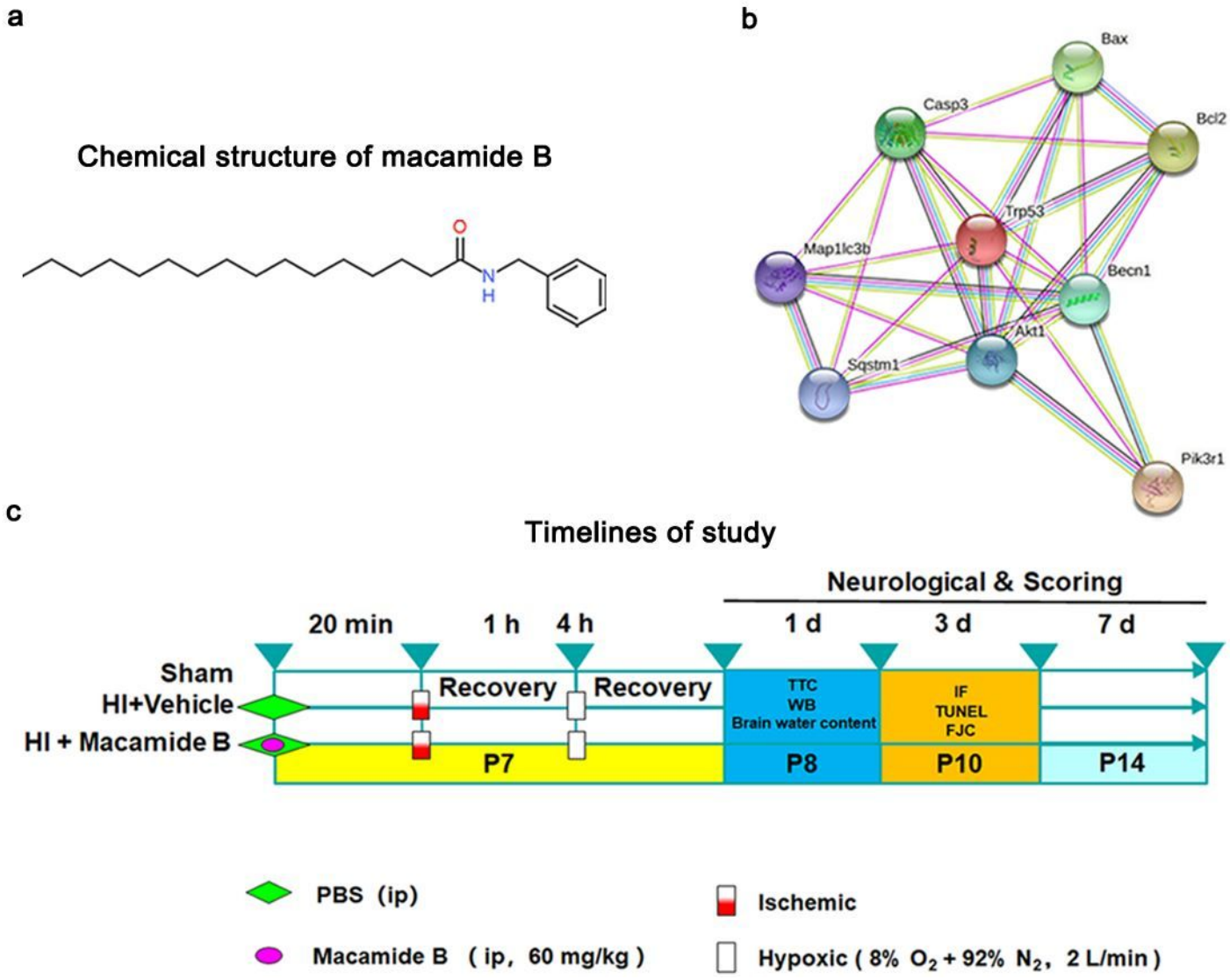

Figure 1

Molecular structure of macamide B and timelines of study. a Molecular structure of macamide B. b Protein-protein interaction network of DEGs. c Timelines of study.

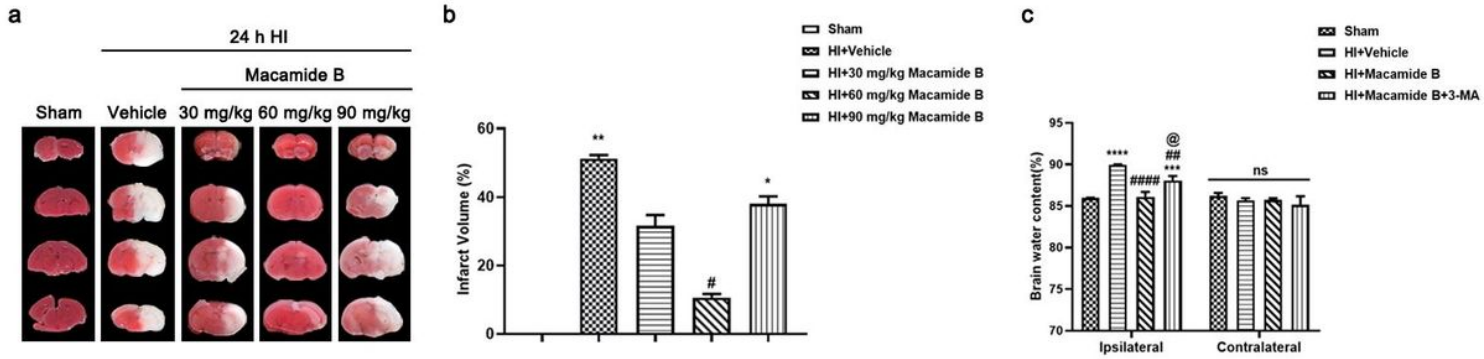

Figure 2

Effect of macamide B pretreatment on HIBD in newborn mice. a Representative pictures of coronal brain sections stained with TTC 24 h after HIBD. b Quantitative analysis results of cerebral infarct volume in pups. Compared with pretreatment with the vehicle, pretreatment with the medium dose (60mg/kg) of macamide B significantly reduced the infarct volume. c Quantitative analysis results of brain water content. The water content of the ipsilateral cerebral hemisphere of the vehicle group was significantly higher than that of the sham group, and the brain water content of the macamide B group was significantly lower than that of the vehicle group. *p < 0.05 vs. sham group; **p < 0.001 vs. sham group; ***p = 0.0003 vs. sham group; ****p < 0.0001 vs. sham group; #p < 0.05 vs. vehicle group; ##p = 0.001 vs. vehicle group; ####p < 0.0001 vs. vehicle group; @p < 0.01 vs. macamide B group. n = 6 for each group.

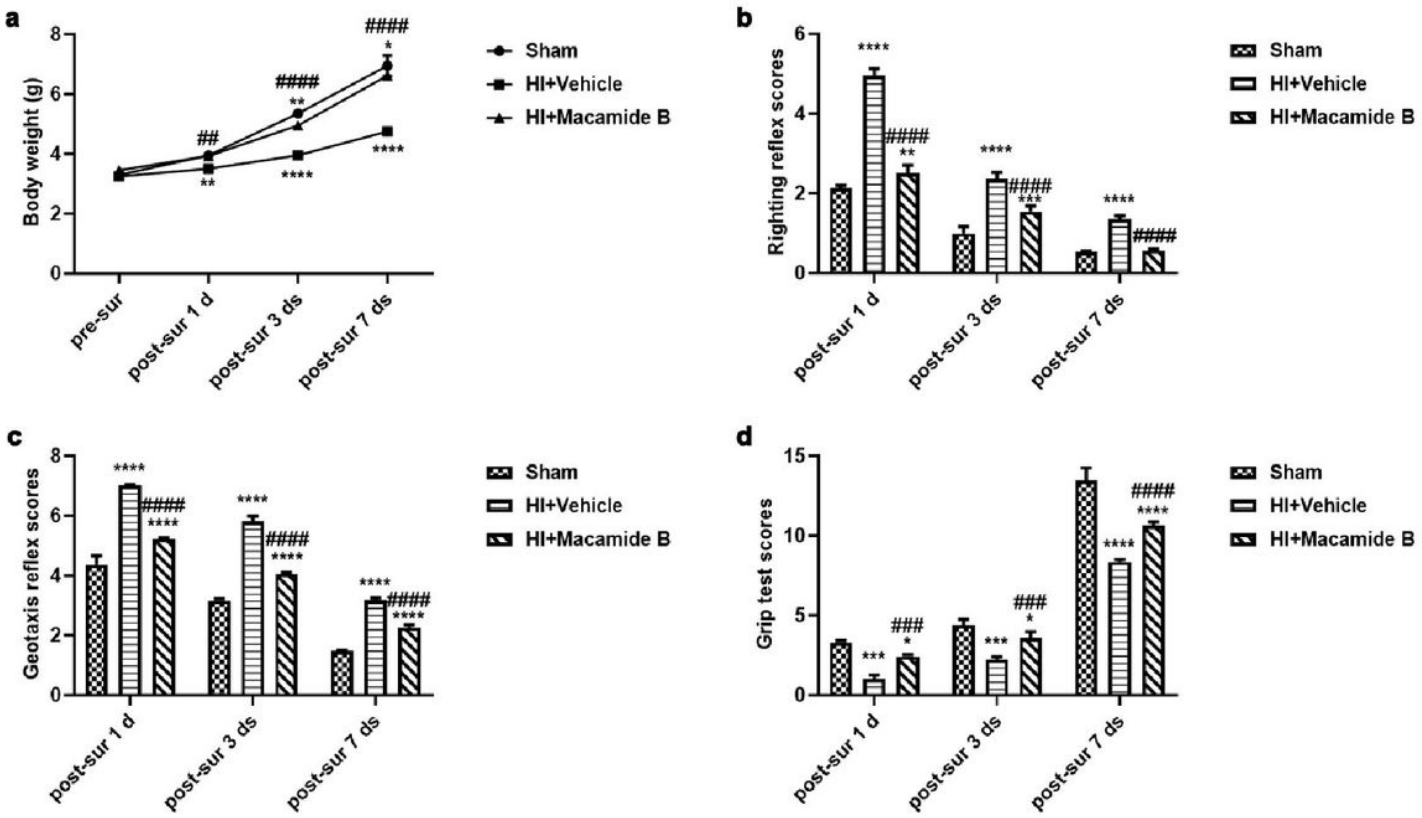

Figure 3

Macamide B pretreatment improved the neurological function and reversed body weight loss of mice after HIBD. a Mice in the macamide B group had significantly higher body weights than mice in the vehicle group at 1, 3, and 7 days post-surgery. The macamide B pretreatment group exhibited notably improved neurobehavioral outcomes of the b righting reflex, c negative geotaxis, and d grip test at 1, 3, and 7 days postsurgery compared to the vehicle group. (*p < 0.05, **p < 0.01). n = 8 for each group.

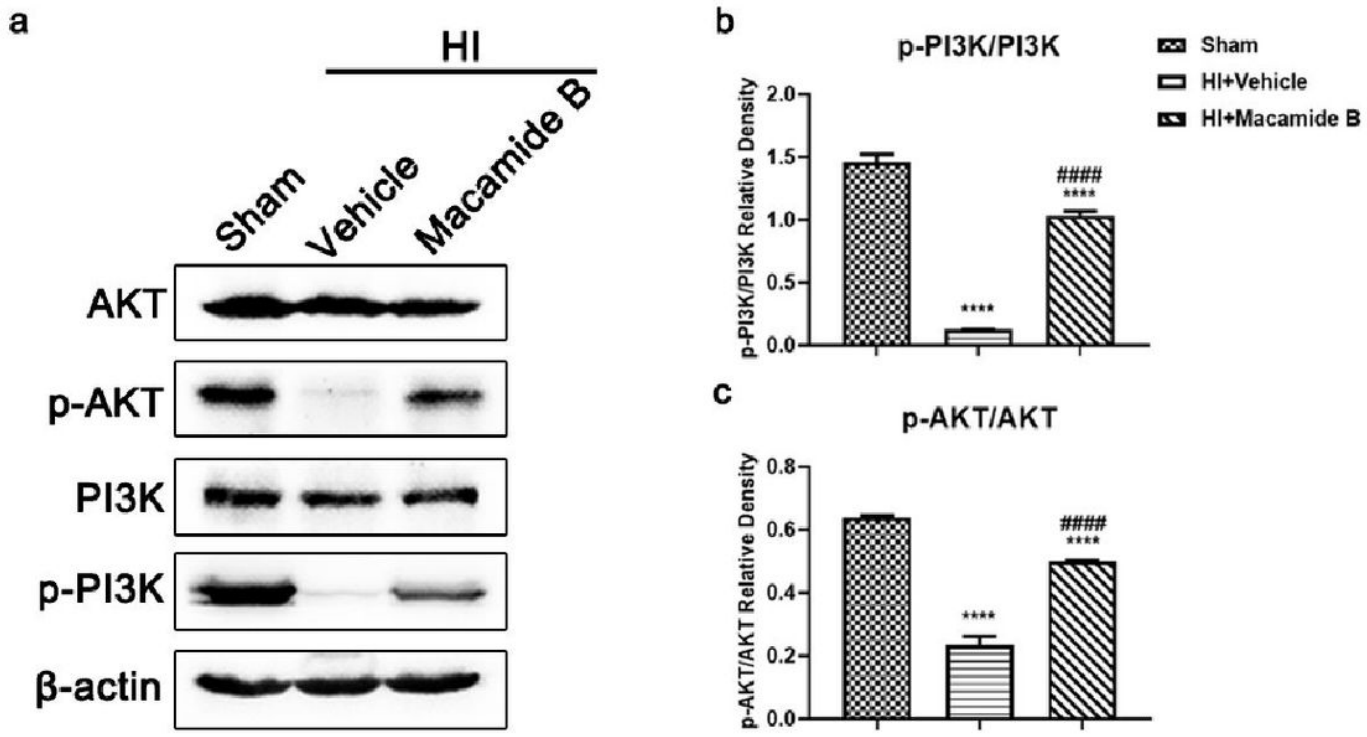

Figure 4

The effect of macamide B pretreatment on PI3K-AKT signaling pathway in HIBD neonatal mice. a Representative western blots and quantification data of b p-PI3K and c p-Akt protein in the ipsilateral cerebral hemisphere were detected 24 h after HIBD. Macamide B pretreatment significantly restored p-PI3K and p-Akt protein expression in HI brains. ****p < 0.0001 vs. sham group; ####p < 0.0001 vs. vehicle group. n = 6 for each group.

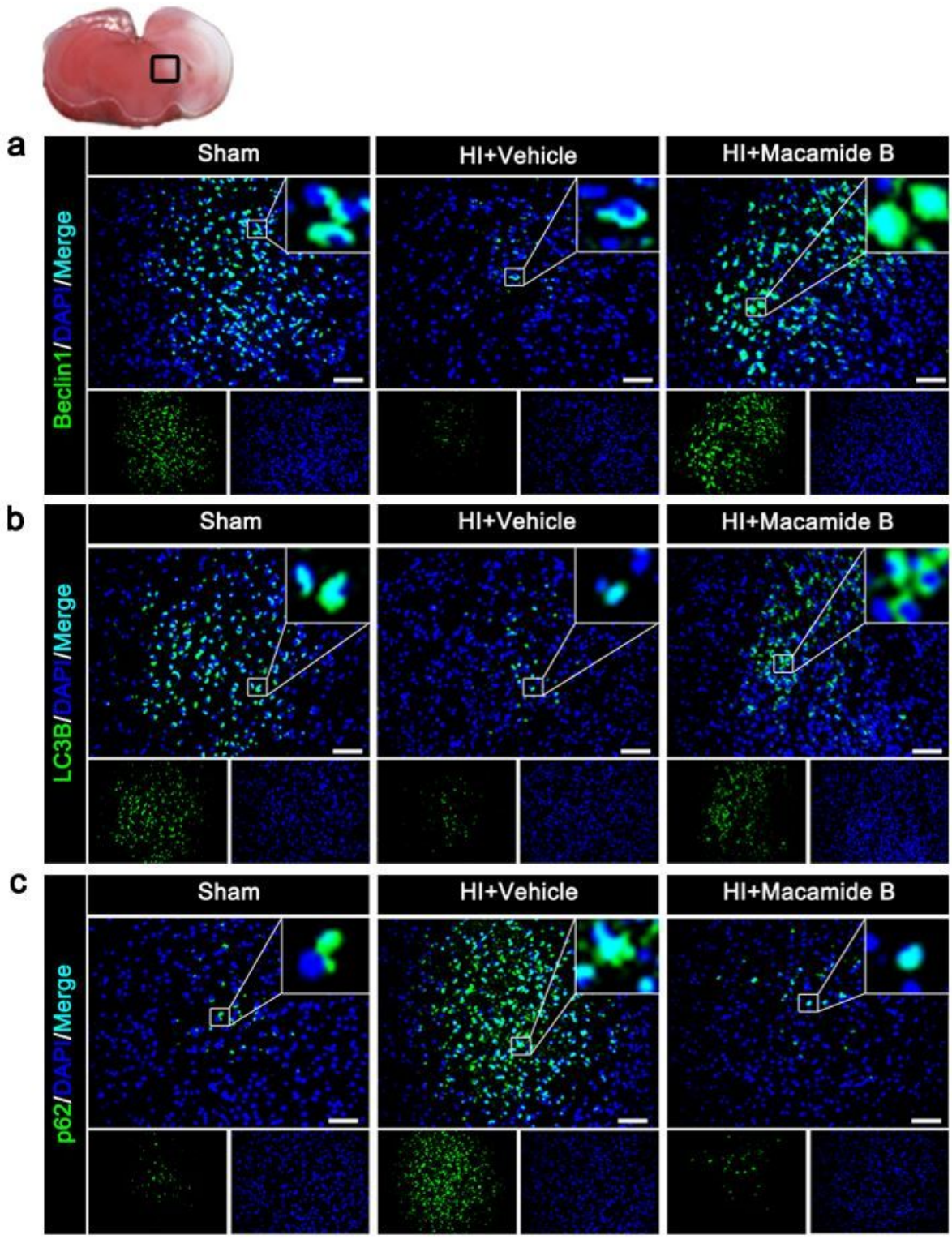

Figure 5

Representative microphotographs of a Beclin1 (green fluorescence), b LC3B (green fluorescence), c p62 (green fluorescence) positive cells in the ipsilateral cortex of HIBD neonatal mice. The blue fluorescence represents DAPI, and merge is the merged image of the target cell and the corresponding nucleus. After HIBD in newborn mice, the number of Beclin1 and LC3B positive cells decreased significantly, and the number of p62 positive cells increased significantly. Macamide B pretreatment significantly improved this

result. Macamide B significantly increased the number of Beclin1 and LC3B positive cells and inhibited the increase in the number of p62 positive cells. The brain slice in the upper left corner shows the position of immunofluorescence staining (small black box). n = 8 for each group. Scale bar = 100 μm.

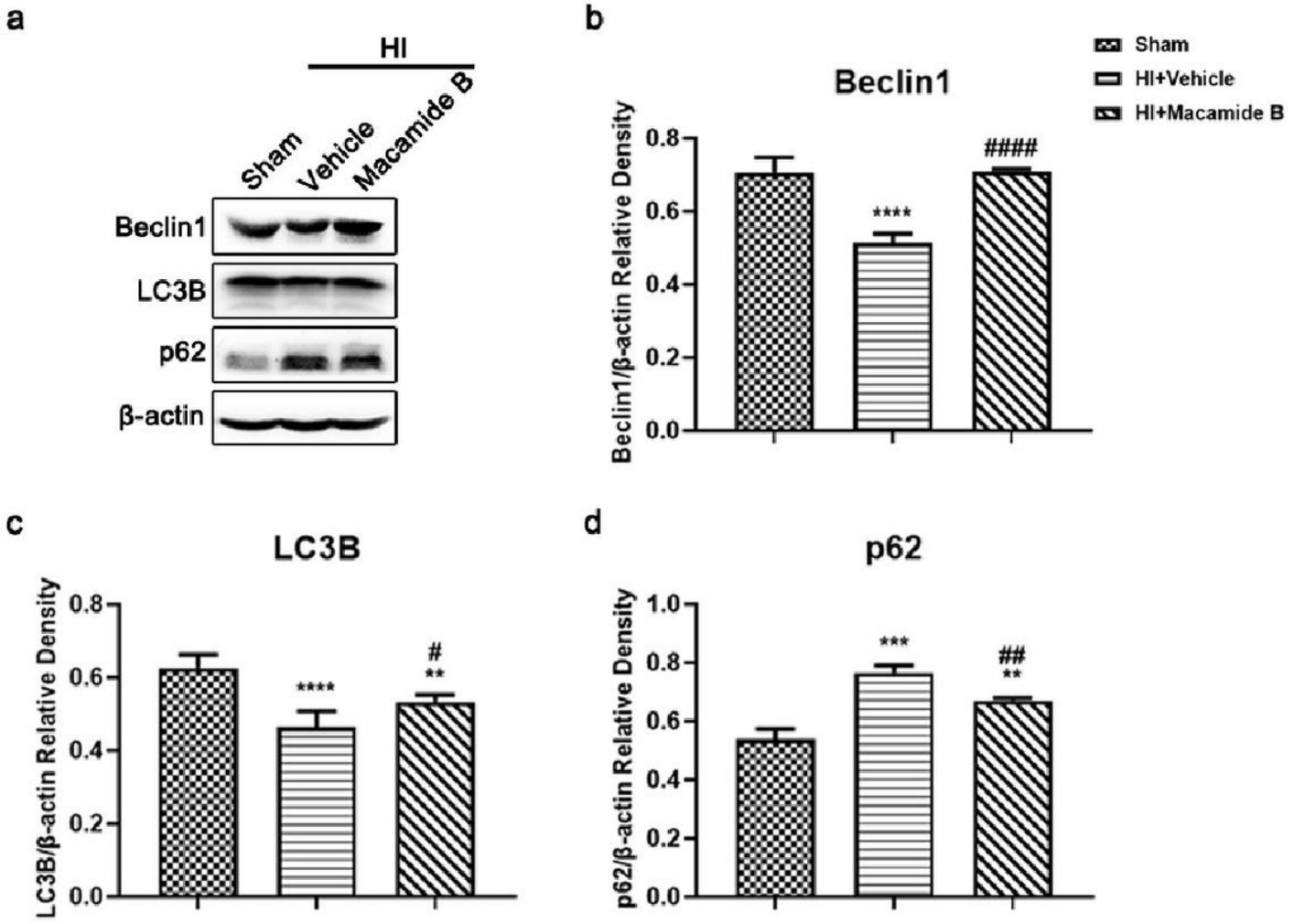

Figure 6

The effect of macamide B pretreatment on autophagy in HIBD neonatal mice. a Representative western blots and quantification data of b Beclin1, c LC3B and d p62 protein in the ipsilateral cerebral hemisphere were detected 24 h after HIBD. Macamide B pretreatment significantly up-regulates the expression levels of Beclin1 and LC3B in HIBD pups, and down-regulates the expression levels of p62. ****p < 0.0001 vs. sham group; ***p < 0.0001 vs. sham group; **p < 0.01 vs. sham group; ####p < 0.0001 vs. vehicle group; #p < 0.05 vs. vehicle group; ##p < 0.01 vs. vehicle group. n = 6 for each group.

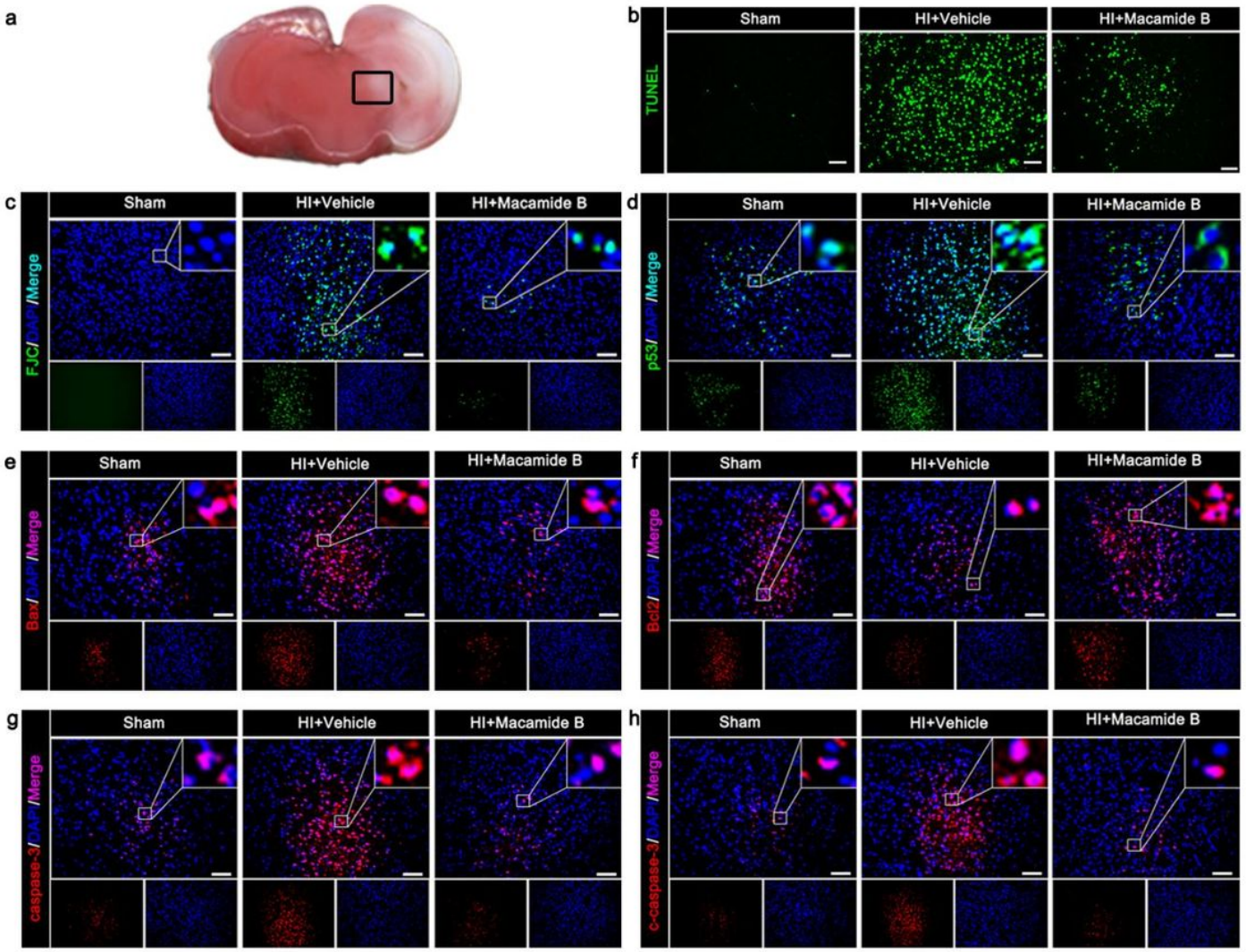

Figure 7

Typical micrographs of b TUNEL-positive cells (green), c FJC-positive neurons (green), and d p53 (green), e Bax (red), f Bcl-2 (red), g caspase-3 (red), and h cleaved caspase-3 (red) positive cells in the ipsilateral cortex of HIBD newborn mice. Blue fluorescence represented DAPI, and merge was the combination of the target cell and the corresponding nucleus. After HIBD in newborn mice, the number of TUNEL positive cells and FJC positive neurons increased significantly. Macamide B pretreatment significantly reversed this result, and significantly reduced the number of TUNEL positive cells and FJC positive neurons, which indicated that macamide B pretreatment could inhibit apoptosis. After neonatal mice HIBD treatment, the number of apoptotic related proteins p53, Bax, caspase-3, and cleaved caspase-3 positive cells was significantly increased, and the number of Bcl-2 positive cells was significantly decreased. Macamide B pretreatment significantly improved this result and played an anti-apoptotic effect on neonatal mice HIBD. i panel indicates the location of staining (small black box). n = 8 for each group. Scale bar = 100 μm.

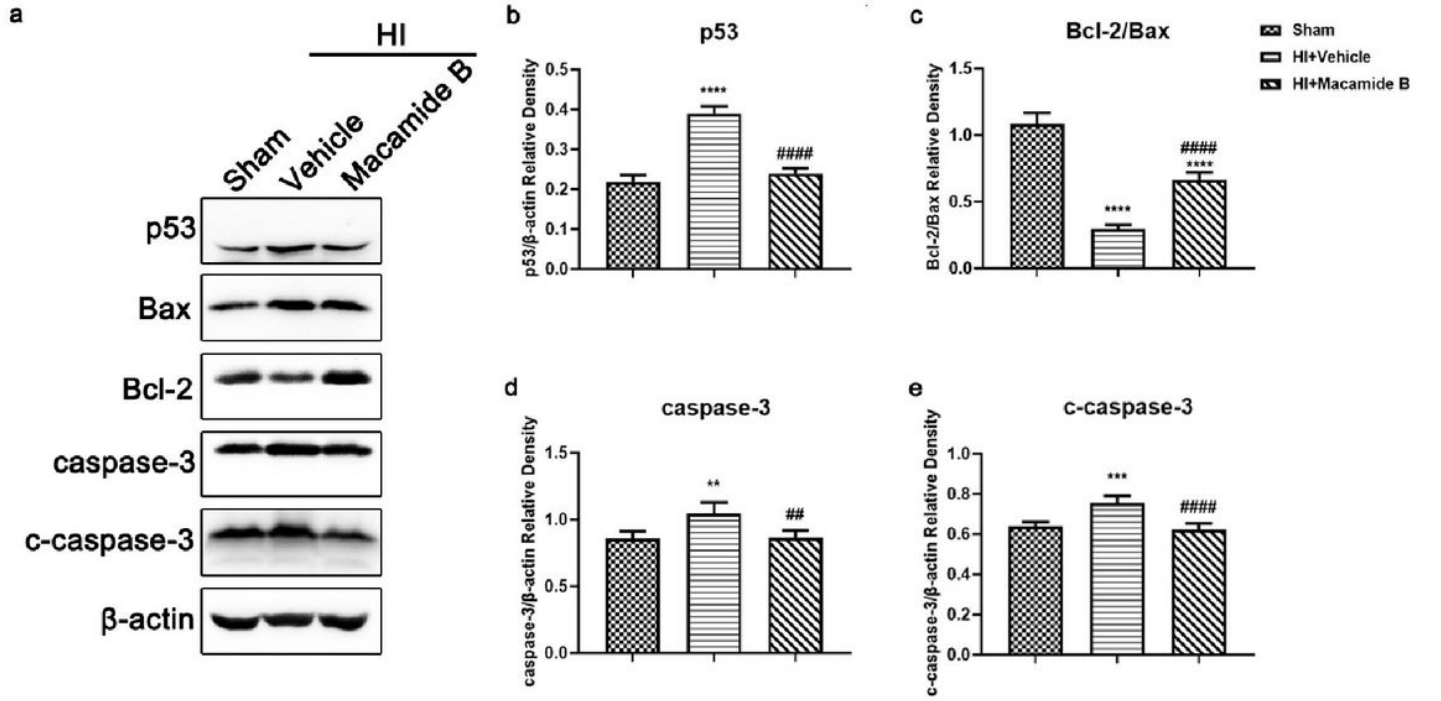

Figure 8

The effect of macamide B pretreatment on cell apoptosis in HIBD neonatal mice. a Representative western blots and quantification data of b p53, c Bcl-2 / d Bax, e caspase-3, and f cleaved caspase-3 in the ipsilateral cerebral hemisphere were detected 24 h after HIBD. Macamide B pretreatment significantly down-regulated p53, caspase-3, and cleaved caspase-3 protein expressions and up-regulated the Bcl2/Bax ratio when compared with the vehicle group. Western blot results showed that macamide B pretreatment significantly reduced the apoptosis of HIBD neonatal mice. ****p < 0.0001 vs. sham group; **p < 0.01 vs. sham group; ***p < 0.0001 vs. sham group; ####p < 0.0001 vs. vehicle group; ##p < 0.05 vs. vehicle group. n = 6 for each group.

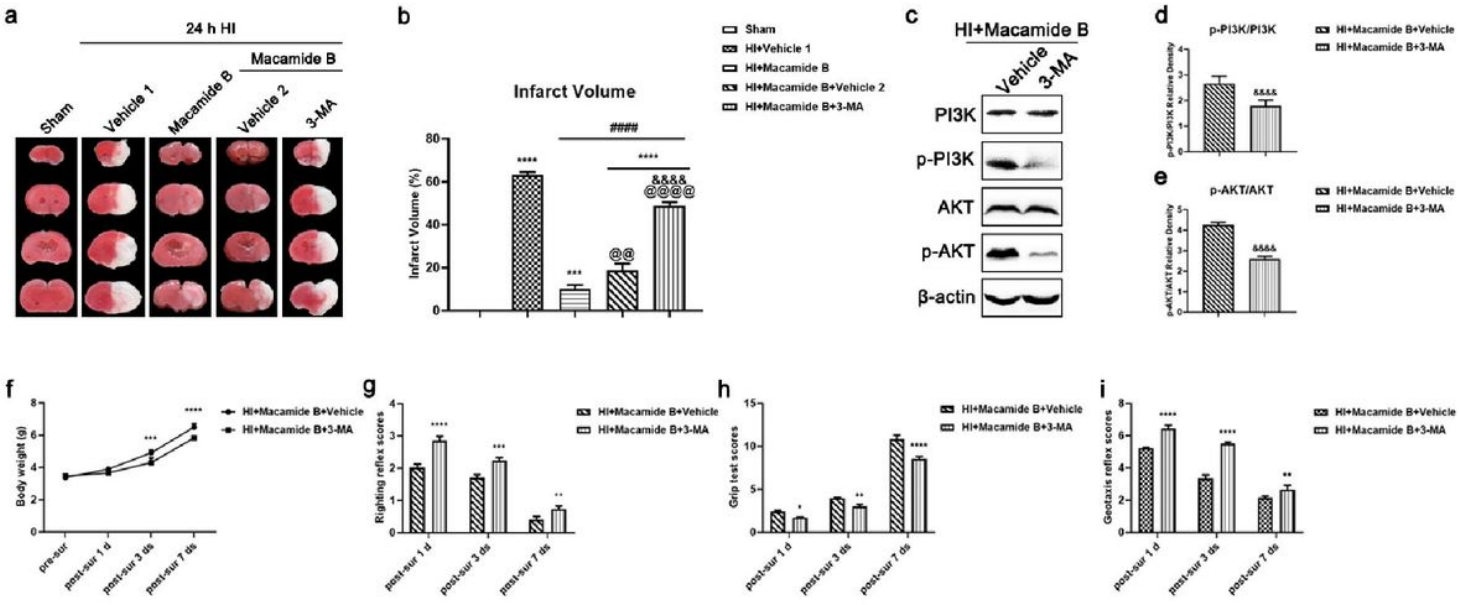

Figure 9

The effect of 3-MA on PI3K-AKT signaling pathway and HIBD neonatal mice pretreated with macamide B. a Representative pictures of coronal brain sections stained with TTC 24 h after HIBD. b Quantitative analysis results of cerebral infarct volume in pups. Vehicle 1 is the vehicle of macamide B, vehicle 2 is the vehicle of 3-MA. Compared with the vehicle 2 group, the cerebral infarct area of the pups treated with 3-MA increased significantly. n = 6 for each group. c Representative western blots and quantification data of d p-PI3K and e p-Akt protein in the ipsilateral cerebral hemisphere were detected 24 h after HIBD. Macamide B pretreatment significantly inhibited the expression of p-PI3K and p-Akt proteins in the HI brain. n = 6 for each group. On postoperative days 1, 3, and 7, 3-MA reversed macamide B-pretreated HIBD pups' increased f body weights and exacerbated neurobehavioral damage in the g righting reflex, h negative geotaxis, and i grip tests compared to the macamide B + vehicle 2 groups. ****p < 0.0001 vs. sham group; ***p < 0.01 vs. sham group; ####p < 0.0001 vs. vehicle1 group; @@p < 0.01 vs. macamide B group; @@@@p < 0.0001 vs. macamide B group; &&&&p < 0.0001 vs. macamide B + vehicle 2 group.

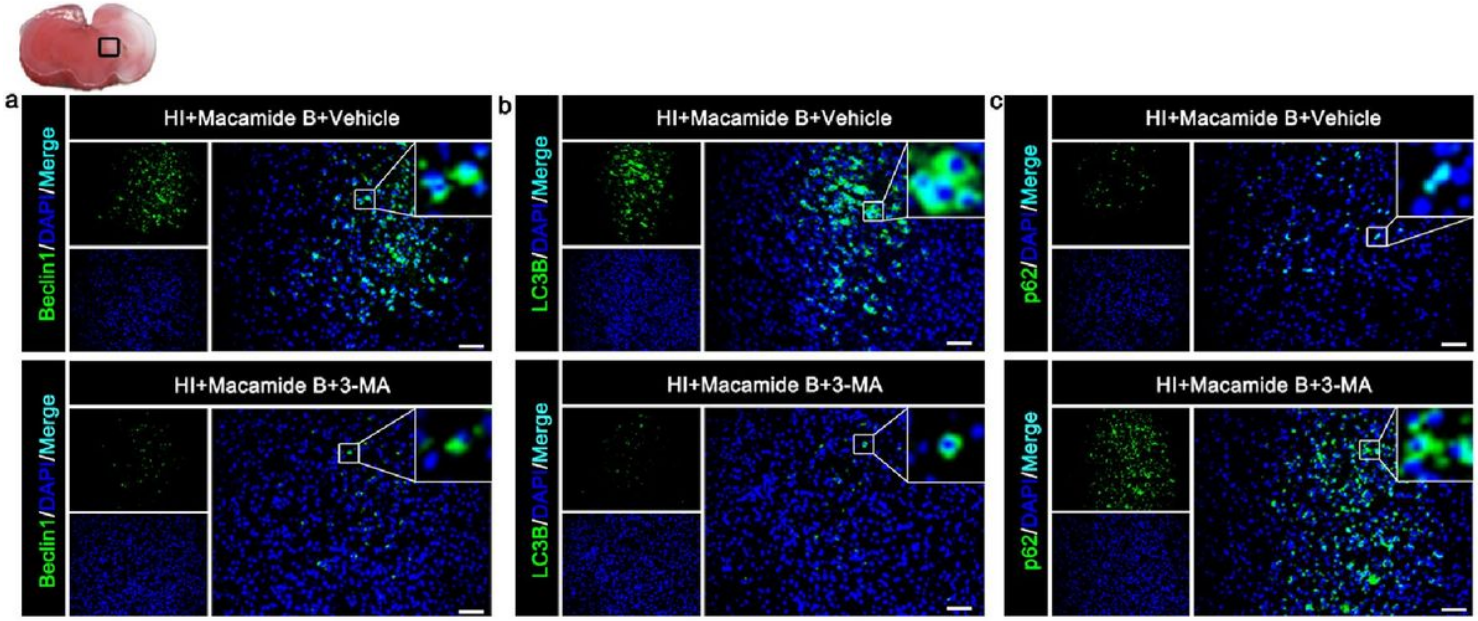

Figure 10

Representative micrographs of a Beclin 1 (green fluorescence), b LC3B (green fluorescence), and c p62 (green fluorescence) positive cells in the ipsilateral cortex of HIBD neonatal mice showed the effect of 3-MA intervention on autophagy in HIBD neonatal mice. Blue fluorescence represented DAPI, and merge was the combination of the target cell and the corresponding nucleus. The vehicle is the vehicle of 3-MA. Compared with macamide B + vehicle control group, the number of a Beclin1 and b LC3B positive cells in the 3-MA intervention group increased significantly, while the number of c p62 positive cells decreased significantly. The brain slice in the upper left corner shows the position of immunofluorescence staining (small black box). n = 8 for each group. Scale bar = 100 μm

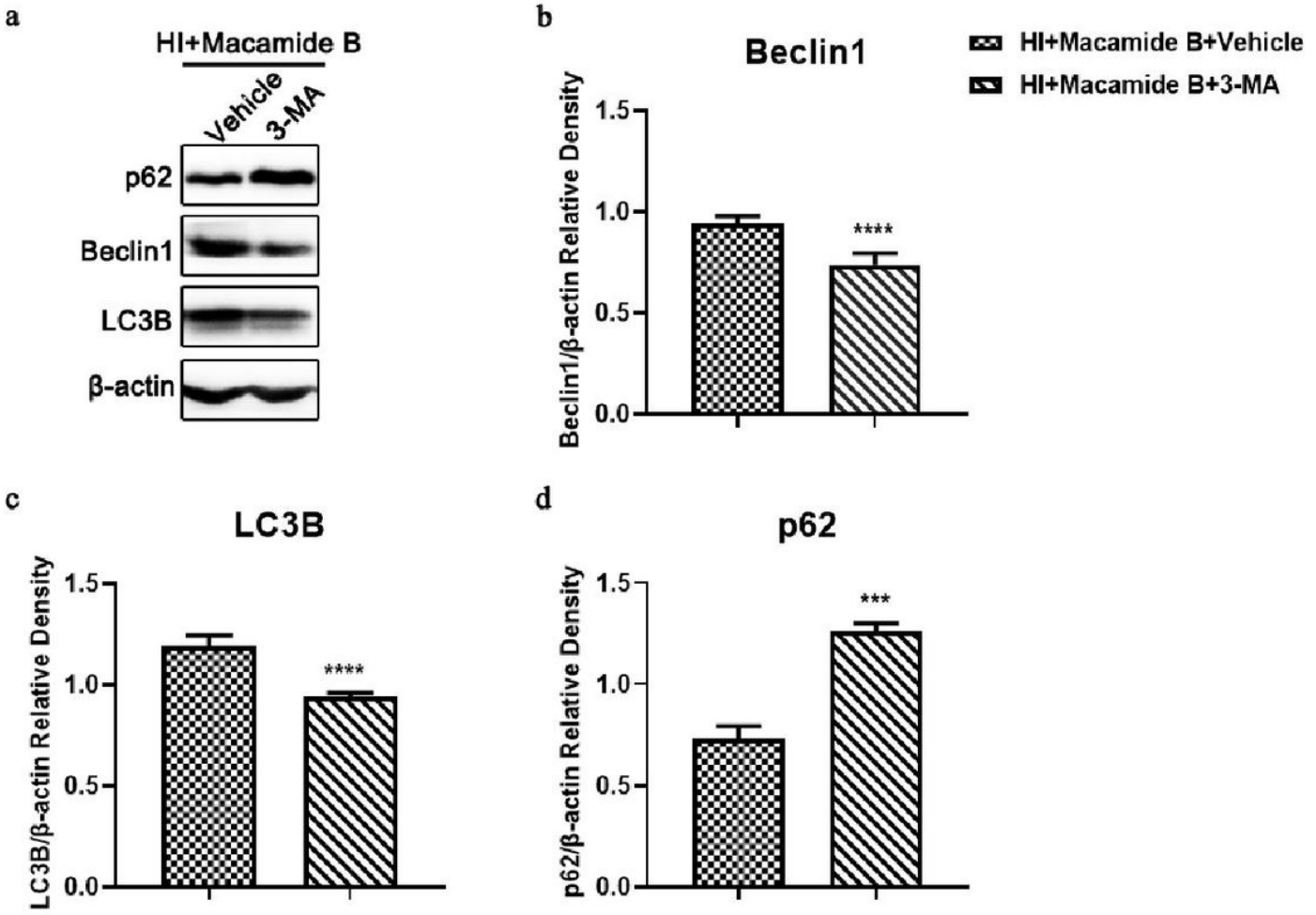

Figure 11

a Representative western blots and quantification data of b Beclin1, c LC3B, and d p62 protein in the ipsilateral cerebral hemisphere were detected 24 h after HIBD. It shows the effect of 3-MA intervention on autophagy-related proteins in HIBD neonatal mice pretreated with macamide B. Vehicle is the vehicle of 3-MA. Compared with the macamide B + vehicle control group, the Beclin1 and LC3B protein expression levels in the 3-MA intervention group decreased significantly, and the p62 protein expression level increased significantly. ****$p < 0.0001$ vs. sham group; ***$p < 0.0001$ vs. sham group. n = 6 for each group.

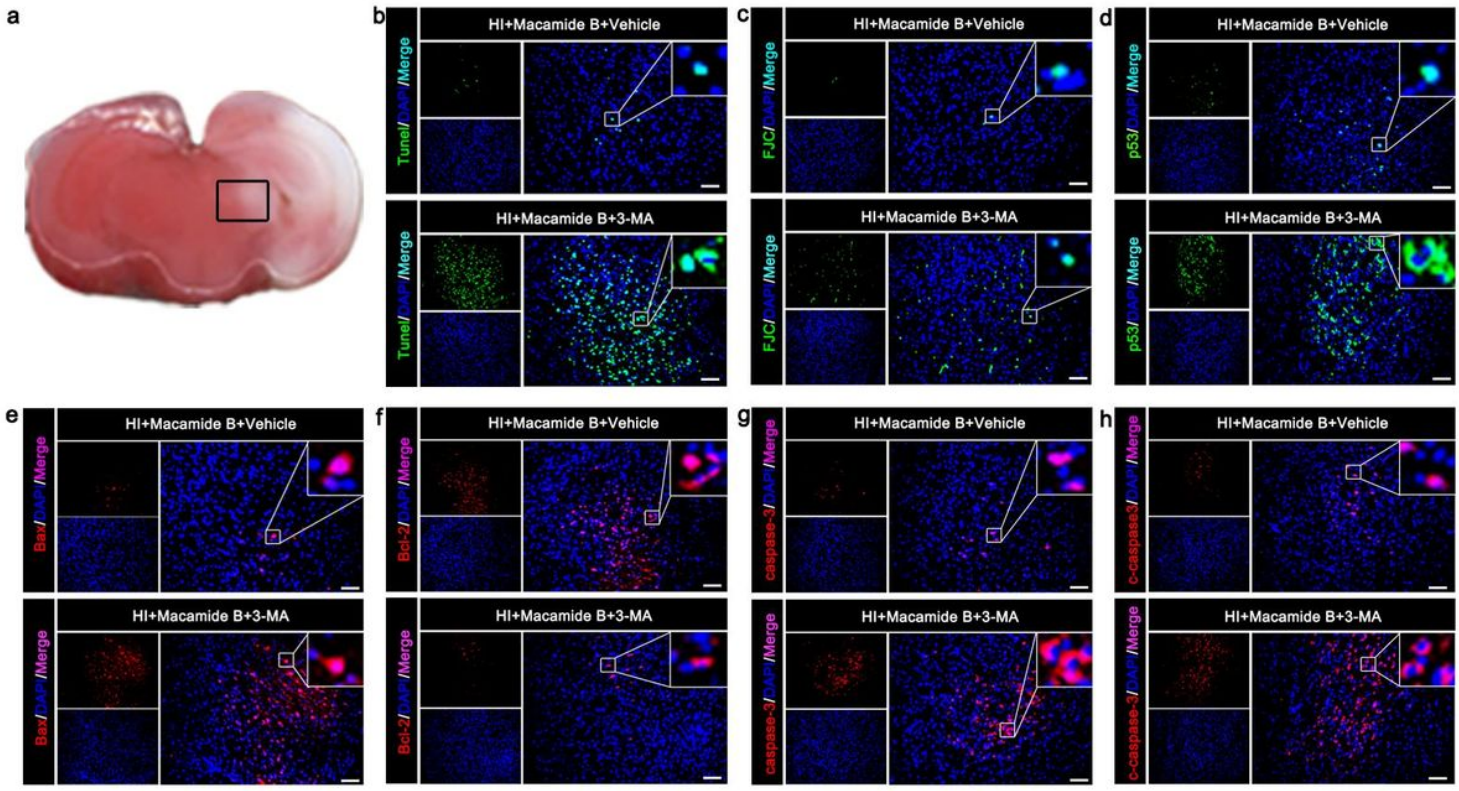

Figure 12

Representative micrographs of b TUNEL-positive cells (green), c FJC-positive cells (green), and d p53 (green), e Bax (red), f Bcl-2 (red), g caspase-3 (red), and h cleaved caspase-3 (red) positive cells in the ipsilateral cortex of HIBD newborn mice showed the effect of 3-MA intervention on apoptosis in HIBD newborn mice. Blue fluorescence represented DAPI, and merge was the combination of the target cell and the corresponding nucleus. The vehicle is the vehicle of 3-MA. Compared with the macamide B + vehicle group, the number of p53, Bax, caspase-3, and cleaved caspase-3 positive cells in the 3-MA intervention group increased significantly, while the number of Bcl-2 positive cells decreased significantly. a The brain slice shows the position of immunofluorescence staining (small black box). n = 8 for each group. Scale bar = 100 μm.

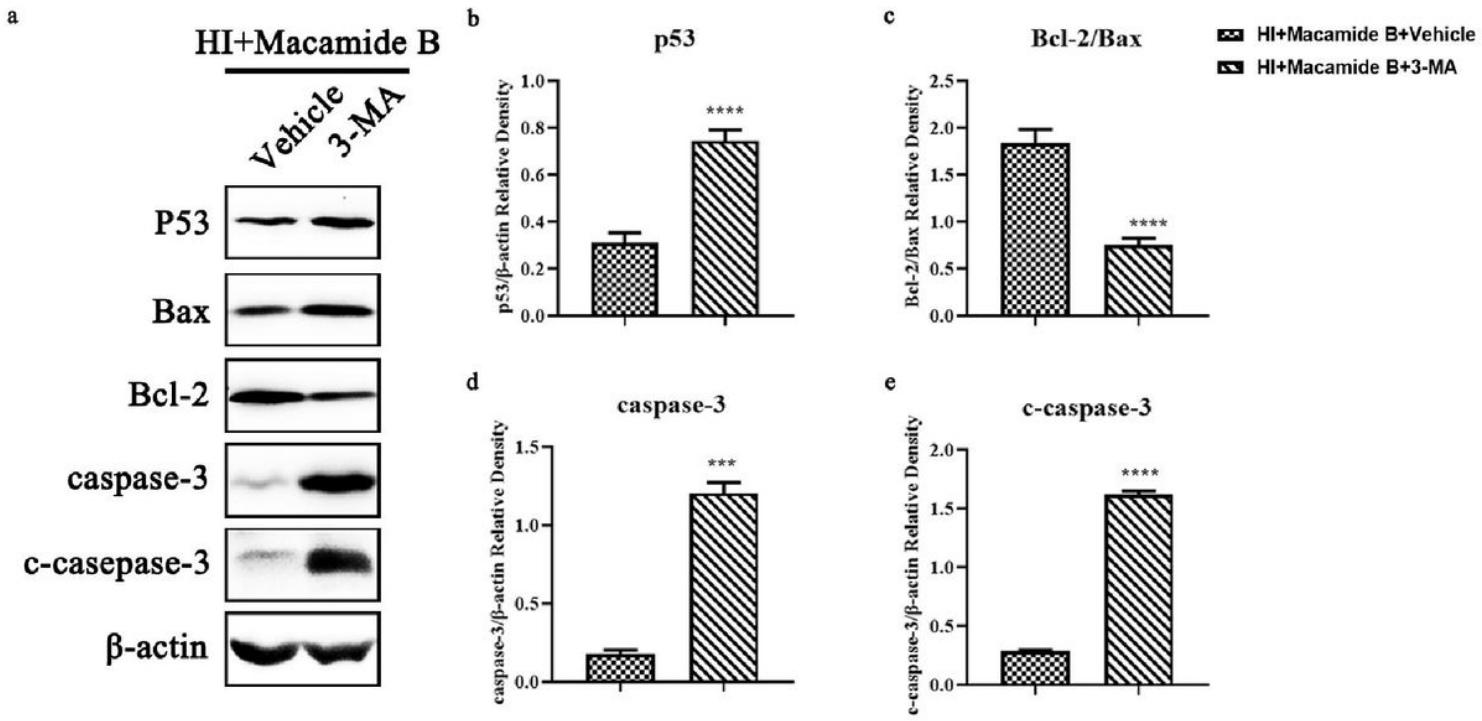

Figure 13

a Representative western blots and quantification data of b p53, c Bcl-2 / d Bax, e caspase-3, and f cleaved caspase-3 in the ipsilateral cerebral hemisphere were detected 24 h after HIBD. Compared with the macamide B + vehicle control group, the p53, caspase-3, and cleaved caspase-3 protein expression levels in the 3-MA intervention group were significantly increased, and the Bcl-2 / Bax ratio was significantly decreased. ****$p < 0.0001$ vs. sham group; ***$p < 0.0001$ vs. sham group. n = 6 for each group.

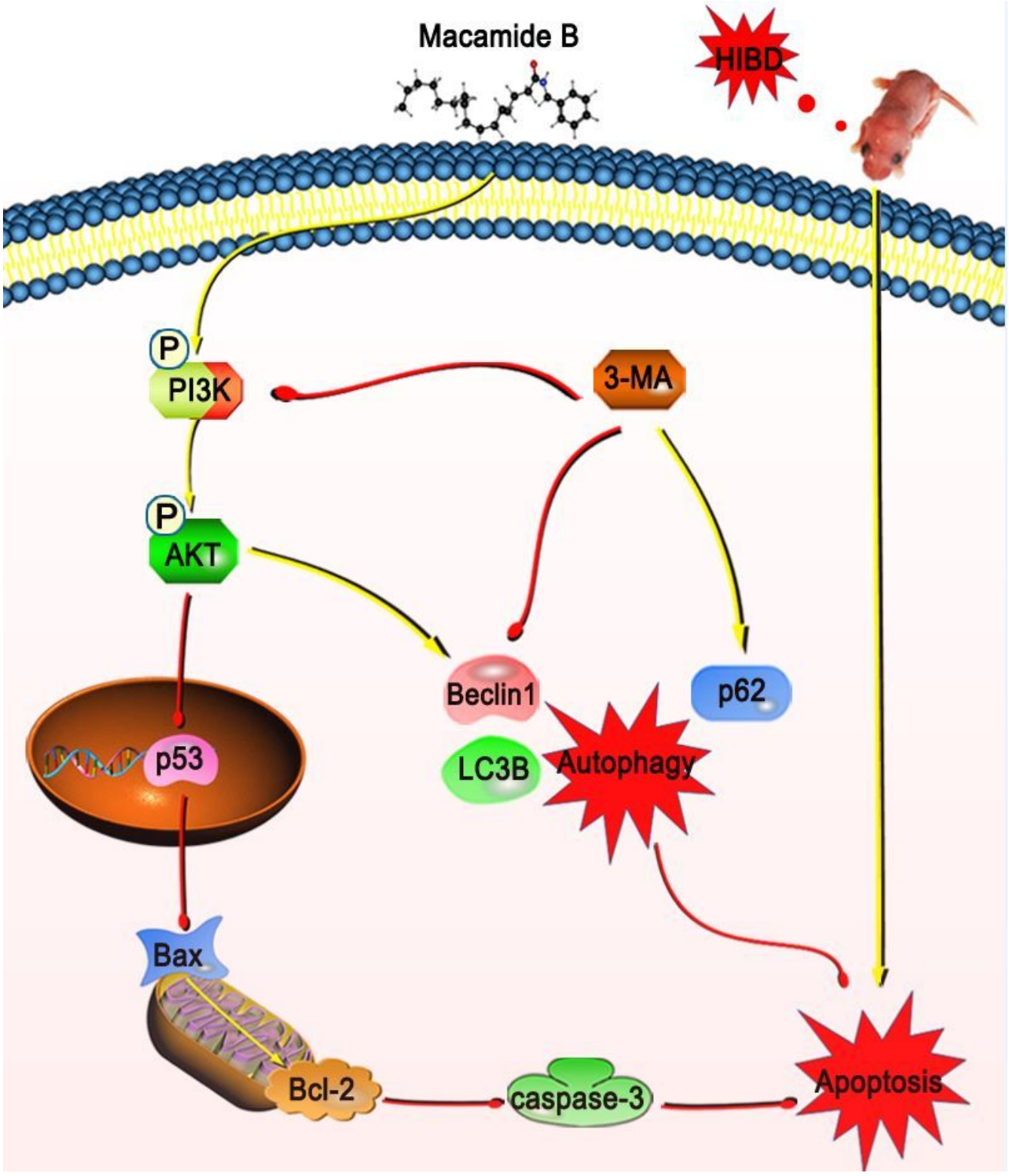

Figure 14

The neuroprotective mechanism of macamide B pretreatment on HIBD newborn mice.