RESEARCH ARTICLE

Resatorvid protects against hypoxic-ischemic brain damage in neonatal rats

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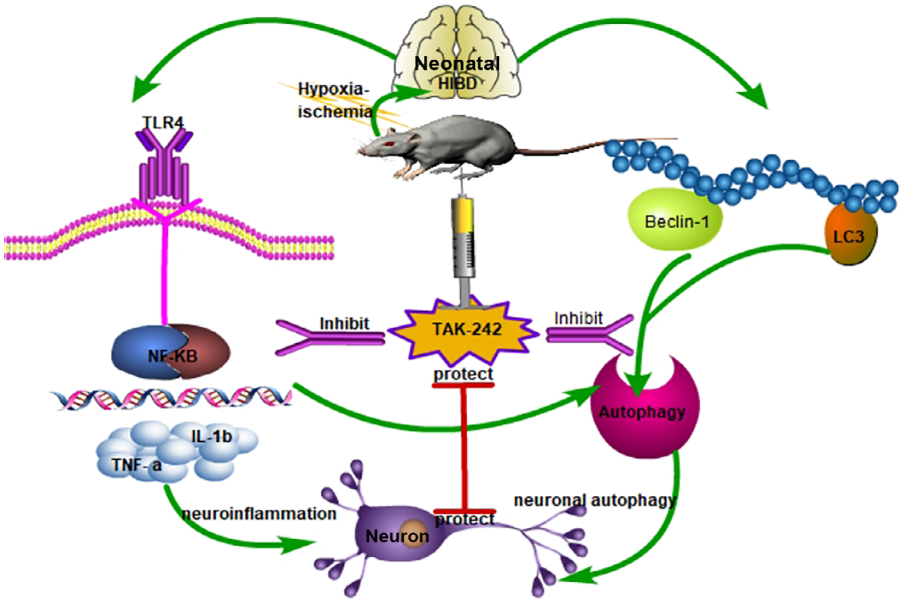
Graphical Abstract

Inhibiting neuronal autophagy and neuroinflammation via inhibition of Toll-like receptor 4 (TLR4)/ nuclear factor kappa-B (NF-κB) signaling pathway might play a neuroprotective role after hypoxic-ischemic brain damage (HIBD)



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Abstract

Secondary brain damage caused by hyperactivation of autophagy and inflammatory responses in neurons plays an important role in hypoxic-ischemic brain damage (HIBD). Although previous studies have implicated Toll-like receptor 4 (TLR4) and nuclear factor kappa-B (NF-κB) in the neuroinflammatory response elicited by brain injury, the role and mechanisms of the TLR4-mediated autophagy signaling pathway in neonatal HIBD are still unclear. We hypothesized that this pathway can regulate brain damage by modulating neuron autophagy and neuroinflammation in neonatal rats with HIBD. Hence, we established a neonatal HIBD rat model using the Rice-Vannucci method, and injected 0.75, 1.5, or 3 mg/kg of the TLR4 inhibitor resatorvid (TAK-242) 30 minutes after hypoxic ischemia. Our results indicate that administering TAK-242 to neonatal rats after HIBD could significantly reduce the infarct volume and the extent of cerebral edema, alleviate neuronal damage and neurobehavioral impairment, and decrease the expression levels of TLR4, phospho-NF-κB p65, Beclin-1, microtubule-associated protein l light chain 3, tumor necrosis factor-α, and interleukin-1β in the hippocampus. Thus, TAK-242 appears to exert a neuroprotective effect after HIBD by inhibiting activation of autophagy and the release of inflammatory cytokines via inhibition of the TLR4/NF-κB signaling pathway. This study was approved by the Laboratory Animal Ethics Committee of Affiliated Hospital of Yangzhou University, China (approval No. 20180114-15) on January 14, 2018.

Key Words: *autophagy; hypoxic-ischemic brain damage; neonatal hypoxic-ischemic brain damage; neuroinflammation; nuclear factor kappa-B; resatorvid; TAK-242; Toll-like receptor 4*

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Introduction

Neonatal hypoxic-ischemic brain damage (HIBD) occurs as a result of decreased or suspended cerebral blood flow, leading to partial or complete hypoxia of brain tissues. HIBD is a main cause of acute mortality and chronic neurological morbidity in neonates (Zhao et al., 2016), and affects approximately 5 of every 1000 neonates annually in developed countries (Gale et al., 2018). In addition, the mortality rate associated with HIBD in the neonatal period has been found to be as high as 40%, while another 30% of patients may suffer from neurological sequelae such as mental retardation and cerebral palsy, which can represent a substantial burden for children, their families, and society as a whole (Leigh et al., 2014; Smith et al., 2014; Li et al., 2017). The pathogenesis of neonatal HIBD is complex and involves a number of different factors. Neuronal apoptosis and autophagy caused by free radical production, ion imbalance, excitotoxicity, and inflammatory responses play a substantial role in the development of HIBD (Ginet et al., 2014; Zhao et al., 2016; Chang and Kong, 2019).

Autophagy is an important regulatory mechanism in cellular metabolism (Wang et al., 2018; He et al., 2019). When cells are threatened, multiple signaling pathways interact with activated autophagy-related proteins (Beclin-1 and microtubule-associated protein l light chain 3 (LC3)) to form autophages, which then selectively and non-selectively degrade cellular proteins, organelles, or pathogenic microorganisms (Kinsella et al., 2018; Eskelinen, 2019). Many studies have indicated that autophagic activation is a component of neonatal HIBD (Ginet et al., 2014; Chang and Kong, 2019; Huang et al., 2019). Inhibition of an autophagy gene was found to block hypoxia-ischemia-induced hippocampal neuron death (Kessel et al., 2012). Additionally, the prevention of autophagy via a gene knockout or drug blocking methods was observed to have neuroprotective effects on ischemic brain tissue (Tang et al., 2019; Zhang et al., 2019). Therefore, further investigation regarding the molecular protective mechanisms of inhibition of autophagy activation in the neuronal hypoxic-ischemic stress response is warranted.

As secondary brain injury caused by neuroinflammatory responses plays an important role in HIBD, inhibition of inflammatory responses might protect hypoxic-ischemic brain tissues (Jenkins et al., 2012; Qin et al., 2019). Toll-like receptor (TLR) 4 has a significant effect on the triggering and modulation of neuroinflammatory responses (Tajal-li-Nezhad et al., 2019). To date, several studies have reported that brain damage is closely associated with TLR4 activity. The TLR4/nuclear factor kappa-B (NF-κB) signaling pathway plays an important role in the activation of neu-roinflammation and in the mechanisms of brain injury caused by cerebral ischemia-hypoxia, carbon monoxide poi-soning, brain trauma, among others. (Wang et al., 2014; Pang et al., 2016; Feng et al., 2017a, b; Liu and Fassbender, 2018; Zhao et al., 2018). Moreover, a TLR4 antagonist has been found to alleviate various types of brain damage, including ischemic stroke, subarachnoid hemorrhage, and brain trauma (Liu et al., 2018; Tang et al., 2018). Therefore, activation of the TLR4/NF-κB signaling pathway plays an important role in the neuroinflammatory response mechanism of brain injury. The TLR signaling pathway interacts with the autophagic signaling pathway. Myeloid differential protein 88 (MyD88), Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF), transforming growth factor β-activated kinase 1 (TAK1), and other junction proteins associated with TLRs can co-regulate autophagic signaling pathways via Bcl-2 and Beclin-1 (Shi and Kehrl, 2008; Kumar, 2019). However, the role and mechanisms of the TLR4-mediated autophagic signaling pathway in brain damage remain unclear.

To address this issue, we established a rat model of neonatal HIBD and intraperitoneally injected the TLR4 inhibitor resatorvid (TAK-242) into neonatal rats 30 minutes after hypoxic-ischemia. We then examined whether TAK-242 exerted a neuroprotective effect after HIBD by inhibiting activation of autophagy and neuroinflammation via inhibition of the TLR4/NF-κB signaling pathway. We hypothesized that the TLR4/NF-κB signaling pathway regulates brain damage by modulating neuron autophagy and neuroinflammation in neonatal rats with HIBD.

Materials and Methods

Animals

We purchased 7-day-old neonatal Sprague-Dawley (SD) rats from Yangzhou University Laboratory Animal Center, China (license No. SCXK [Su] 2017-0007). The neonatal rats were freely fed by female rats and were housed at 24 ± 2°C and 55 ± 5% humidity with an alternating 12-hour light/dark cycle. All animal experimental procedures were approved by the Laboratory Animal Ethics Committee of Affiliated Hospital of Yangzhou University, China (approval No. 20180114-15) on January 14, 2018 and were conducted in strict accordance with the Guidelines for the Nursing and Use of Laboratory Animals issued by the National Institutes of Health.

Animal grouping and HIBD model establishment

A total of 114 neonatal rats were randomly divided into sham-operated (n = 30), HIBD + vehicle (n = 42), HIBD + TAK-242 (0.75 mg/kg) (n = 6), HIBD + TAK-242 (1.5 mg/kg) (n = 6), and HIBD + TAK-242 (3 mg/kg) (n = 30) groups. We used the Rice-Vannucci method to establish the HIBD model (Vannucci et al., 1999). After anesthesia via inhalation of isoflurane (Merck Hoei Ltd., Osaka, Japan), the neonatal rats were maintained at a constant temperature, fixed in the supine position, and the skin on the middle of the neck was disinfected with 75% alcohol. We made a 0.5 cm long incision along the median line, and separated the left carotid artery. The blood vessel was ligated at both ends and disconnected in the middle. The incision was closed via sutures, and disinfectant was applied to the surgical site. After surgery, the neonatal rats were placed in an anoxic box with nitrogen-oxygen mixed gas (8% O_2 + 92% N_2), and the temperature was controlled at 36 ± 1°C for 2.5 hours. In the sham-operated group, the blood vessel was separated but no other action was performed, and no ligation or hypoxic treatments were carried out. Rats in the HIBD + TAK-242 (0.75, 1.5, 3 mg/kg) groups were intraperitoneally injected

30 minutes after hypoxic-ischemia with 0.75, 1.5, and 3 mg/kg of TLR4 inhibitor TAK-242 solution, respectively, while rats in the HIBD + vehicle group were given a volume of 7.5 mL/kg vehicle. The TAK-242 (Invitrogen, Carlsbad, CA, USA) solution was prepared with 1% dimethyl sulfoxide (Nacalai Tesque, Inc., Kyoto, Japan) and normal saline to final concentrations of 0.1, 0.2, and 0.4 mg/mL, and administered as previously described (Hua et al., 2015; Hwang et al., 2017).

Determination of infarct volume

We randomly selected 6 neonatal rats in each group for triphenyl tetrazolium chloride staining 48 hours after HIBD. This enabled us to determine the infarct volume using the following formula: infarct volume = (volume of the normal hemisphere − non-infarct volume of the infarct hemisphere) /volume of the hemisphere × 100% (Li et al., 2016).

Determination of brain water content

We randomly selected 6 neonatal rats in each group 48 hours after HIBD to assess the degree of cerebral edema. Neonatal rats were first deeply anesthetized. Their brains were then quickly extracted and the cerebellum removed. Water and blood were removed from the brain surface using filter paper. The brain tissues were then weighed, wrapped in aluminum foil, dried at 100°C in an oven, and taken out and weighed again at room temperature. The degree of cerebral edema was calculated using the dry-wet specific gravity method, where brain water content (%) = (brain wet weight-brain dry weight) / brain wet weight × 100 (Cui et al., 2014).

Assessment of neurobehavioral function

Short-term neurobehavioral tests

In this phase, we randomly selected 6 neonatal rats from each group to undergo short-term neurobehavioral tests 48 hours after HIBD. We performed the negative geotaxis test and the righting reflex test according to the methods used by Ye et al. (2018). In the negative geotaxis test, the rats were placed on a 45-degree incline with their heads down, and we recorded the amount of time it took for them to make a 180-degree turn. In the righting reflex test, we recorded the time it took for the rats to transition from a dorsal position to an upright position.

Long-term neurobehavioral tests

In this phase, we subjected rats to the rotarod and beam-walking tests at the 4th week after HIBD to investigate their motor integration and coordination abilities. The rotarod test was conducted as follows: the rats ran on a rotating rod, and we recorded the duration that passed before they lost their balance and fell from the rod (Feng et al., 2017b). The beam walking test was conducted as follows: A balance beam with a length of 100 cm and a width of 2.0 cm was placed at a height of about 50 cm, and we recorded the amount of time it took for the rats to cross the balance beam. We observed the rats as they crossed the balance beam and scored their motion according to Feeney's method (Feeney et al., 1981): 7 points: the rat quickly crossed the balance beam, the affected limbs were fully functional without obvious neurological impairment; 6 points: the rat quickly crossed the balance beam, the affected limbs were active during more than 50% of the overall motion; 5 points: the affected limbs were active during less than 50% of the overall motion; 4 points: the rat failed to cross the beam in a smooth motion, was close to falling during less than 50% of the overall motion; 3 points: the rat was close to falling for more than 50% of the overall motion; 2 points: the rat failed to cross, but was able to stand on the balance beam; 1 point: the rat was fully unable to cross or stand on the balance beam, and fell off the beam. The rats were trained for 3 days before undergoing the formal experiment. Each experiment had three identical trials, and the scores for the trials were averaged. The investigators performing and scoring the above-mentioned testing procedure were uninformed regarding group membership.

Hematoxylin-eosin staining

We randomly selected six neonatal rats from each group for hematoxylin-eosin (HE) staining. At 48 hours after HIBD, the rats were anesthetized and heart-perfused with phosphate-buffered saline and 4% paraformaldehyde. After removing the brains, the sections were immersed in 4% paraformaldehyde for re-fixation, and then subjected to gradient ethanol dehydration, transparency via xylene, paraffin embedding, and coronal sectioning with a thickness of 5 μm. Pathological lesions of the hippocampal CA1 region were observed via a light microscope (Nikon, Tokyo, Japan) after HE staining.

Immunohistochemistry

Paraffin-embedded brain sections containing the hippocampus were randomly selected from the HE-stained tissue samples and routinely dewaxed. We used 5% goat serum for mounting. We then added the following primary antibodies: rabbit anti-TLR4 polyclonal antibody (1:100, Proteintech, Wuhan, China), rabbit anti-phospho (p)-NF-κB p65 polyclonal antibody (1:100, ABclonal), rabbit anti-Beclin-1 polyclonal antibody (1:100, MBL, Nagoya, Japan), and rabbit anti-LC3 polyclonal antibody (1:100, MBL), and incubated the tissue overnight at 4°C. The next day, we exposed the tissue to a streptavidin-biotin complex-secondary anti-rabbit antibody (1:500, BosterBio, Wuhan, China) for 1 hour at room temperature. We used 3′-diaminobenzidine for color visualization, and performed counterstaining with hematoxylin. Analyses were performed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Western blot assay

In this stage, 6 neonatal rats in each group were randomly selected to undergo a heart perfusion with phosphate-buffered saline under anesthesia at 24, 48, and 72 hours after HIBD, and the left hippocampus was quickly removed. We conducted tissue homogenation, centrifugation, and protein extraction. Then, we took 50 μg of protein from each sample and separated the protein via 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis before transferring

the samples onto a polyvinylidene fluoride membrane. We used 5% skim milk powder to perform blocking for 2 hours, added the following primary antibodies: rabbit anti-TLR4 (1:500, Proteintech), rabbit anti-phospho-NF-κB p65 (1:1000, ABclonal), rabbit anti-Beclin-1 (1:1000, MBL), and rabbit anti-LC3 (1:750, MBL), and incubated the samples overnight at 4°C. The next day, the samples were incubated with secondary goat anti-rabbit antibody (1:5000, ABclonal) for 2 hours at room temperature, and then developed and fixed via enhanced chemiluminescence in a dark room. Protein expression levels were assessed in terms of the ratio of gray values between the target protein bands and the reference protein bands using Image J software.

Enzyme-linked immunosorbent assay

We used the supernatant of the left hippocampus after homogenizing and centrifuging the tissue. We determined the levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) at 24, 48, and 72 hours after HIBD using an enzyme-linked immunosorbent assay kit (ABclonal).

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (IBM, Armonk, NY, USA). The normally distributed data were expressed as the mean ± standard deviation (SD), and comparison among the groups was performed via one-way analysis of variance. We used the Student-Newman-Keuls test for pairwise comparisons. The non-normally distributed data were expressed as median values (interquartile range). We compared two independent samples using the Mann-Whitney *U* test, and compared multiple independent samples via the Kruskal-Wallis *H* test. $P < 0.05$ was considered statistically significant.

Results

Activation of the TLR4/NF-κB signaling pathway and autophagy, and release of inflammatory cytokines during brain damage after HIBD

We found that compared with the sham-operated group, rats that underwent HIBD exhibited significantly increased levels of TLR4, p-NF-κB p65, Beclin-1, LC3, TNF-α, and IL-1β in the left hippocampus after HIBD. These increases reached peak levels at 48 hours, and had gradually decreased by 72 hours after HIBD ($P < 0.01$, *vs*. sham-operated group; **Figure 1**).

TAK-242 reduces infarct volume and the extent of cerebral edema at 48 hours after HIBD

There was no significant evidence of cerebral infarct in the sham-operated group. While we observed a large-area infarct in the HIBD + vehicle group, the infarct volumes in the neonatal rats in the HIBD + TAK-242 (0.75, 1.5, 3 mg/kg) groups were significantly lower, with the lowest value in the 3 mg/kg TAK-242 group ($P < 0.01$; **Figure 2A** & **B**). Simultaneously, determination of brain water content showed that the extent of cerebral edema in the neonatal rats in the HIBD + vehicle group was notably elevated, while that in the HIBD + TAK-242 (3 mg/kg) group was significantly diminished ($P < 0.05$; **Figure 2C**).

TAK-242 improves neurobehavioral function after HIBD

The results of the short-term tests of neurobehavioral function indicated that neonatal rats had different degrees of neurobehavioral impairment after HIBD. In addition, TAK-242 (3 mg/kg) significantly alleviated neurological impairment and improved neurobehavioral function after HIBD ($P < 0.05$; **Figure 3A** & **B**).

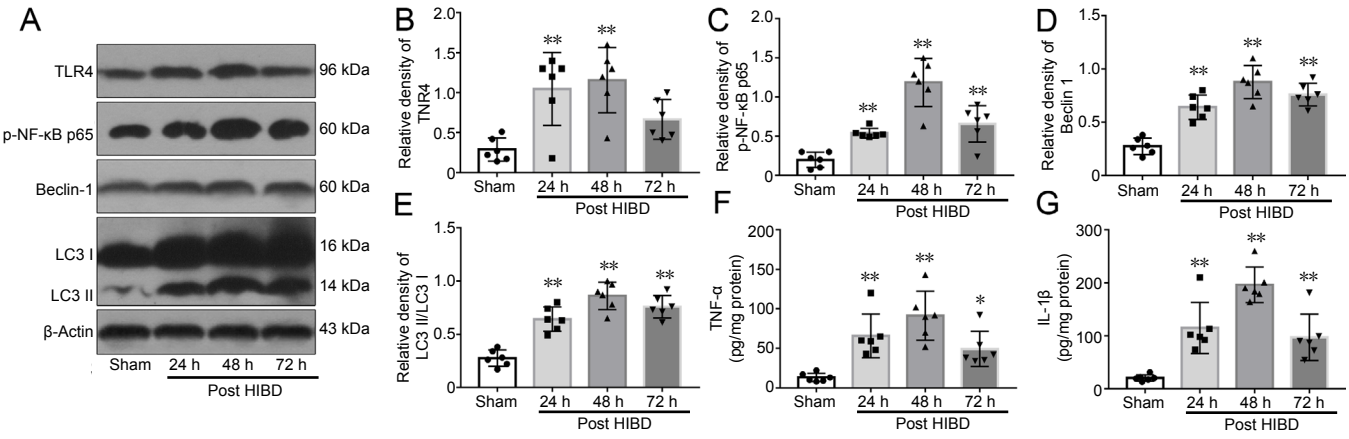

Figure 1 Activation of the TLR4/NF-κB signaling pathway and autophagy, and release of inflammatory cytokines in the hippocampus during brain damage after HIBD.

(A) Representative western blot bands of TLR4, p-NF-κB p65, Beclin-1, and LC3 at different time points after HIBD. (B–E) The protein expression levels of TLR4 (B), p-NF-κB p65 (C), Beclin-1 (D), and LC3 (E) were significantly increased and reached peak levels at 48 hours, and had gradually decreased by 72 hours. Protein expression levels of TLR4, p-NF-κB p65, and Beclin-1 were assessed according to the ratio of gray values between the target protein bands and β-actin bands. (F, G) An enzyme-linked immunosorbent assay showed that the expression levels of TNF-α (F) and IL-1β (G) were markedly elevated after HIBD such that they reached peak levels at 48 hours and had gradually decreased by 72 hours. Data are expressed as the mean ± SD ($n = 6$ per group) and were analyzed via one-way analysis of variance followed by the Student-Newman-Keuls test. $*P < 0.05$, $**P < 0.01$, *vs*. sham-operated group. HIBD: Hypoxic-ischemic brain damage; IL-1β: interleukin-1β; LC3: microtubule-associated protein l light chain 3; NF-κB: nuclear factor kappa-B; p-NF-κB: phos-pho-NF-κB; TLR4: Toll-like receptor 4; TNF-α: tumor necrosis factor-α.

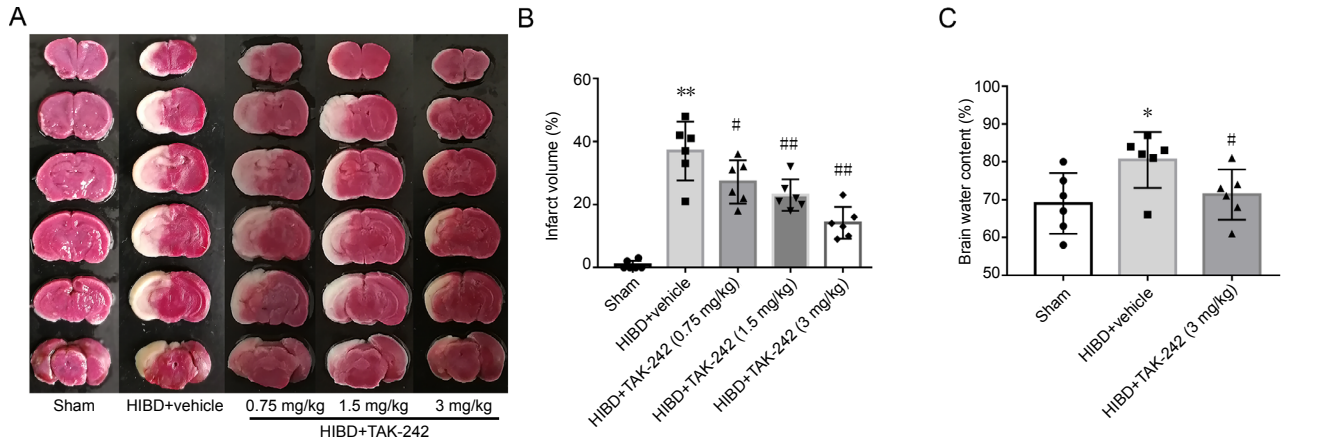

Figure 2 Resatorvid (TAK-242) reduces infarct volume and the extent of cerebral edema at 48 hours after HIBD.
(A) Triphenyl tetrazolium chloride staining of representative coronal brain sections. White and red indicate infarct and normal tissues, respectively. There was no significant evidence of cerebral infarct in the sham-operated group. We observed a large infarct in the HIBD + vehicle group, while the infarct volumes in the HIBD + TAK-242 (0.75, 1.5, 3 mg/kg) groups were significantly lower, with that in the 3 mg/kg TAK-242 being the lowest. (B) Quantitative infarct volume data (triphenyl tetrazolium chloride staining). (C) Quantitative brain water content data. Data are expressed as the mean ± SD (n = 6 per group) and were analyzed via a one-way analysis of variance followed by the Student-Newman-Keuls test. ∗P < 0.05, ∗∗P < 0.01, *vs.* sham-operated group; #P < 0.05, ##P < 0.01, *vs.* HIBD + vehicle group. HIBD: Hypoxic-ischemic brain damage.

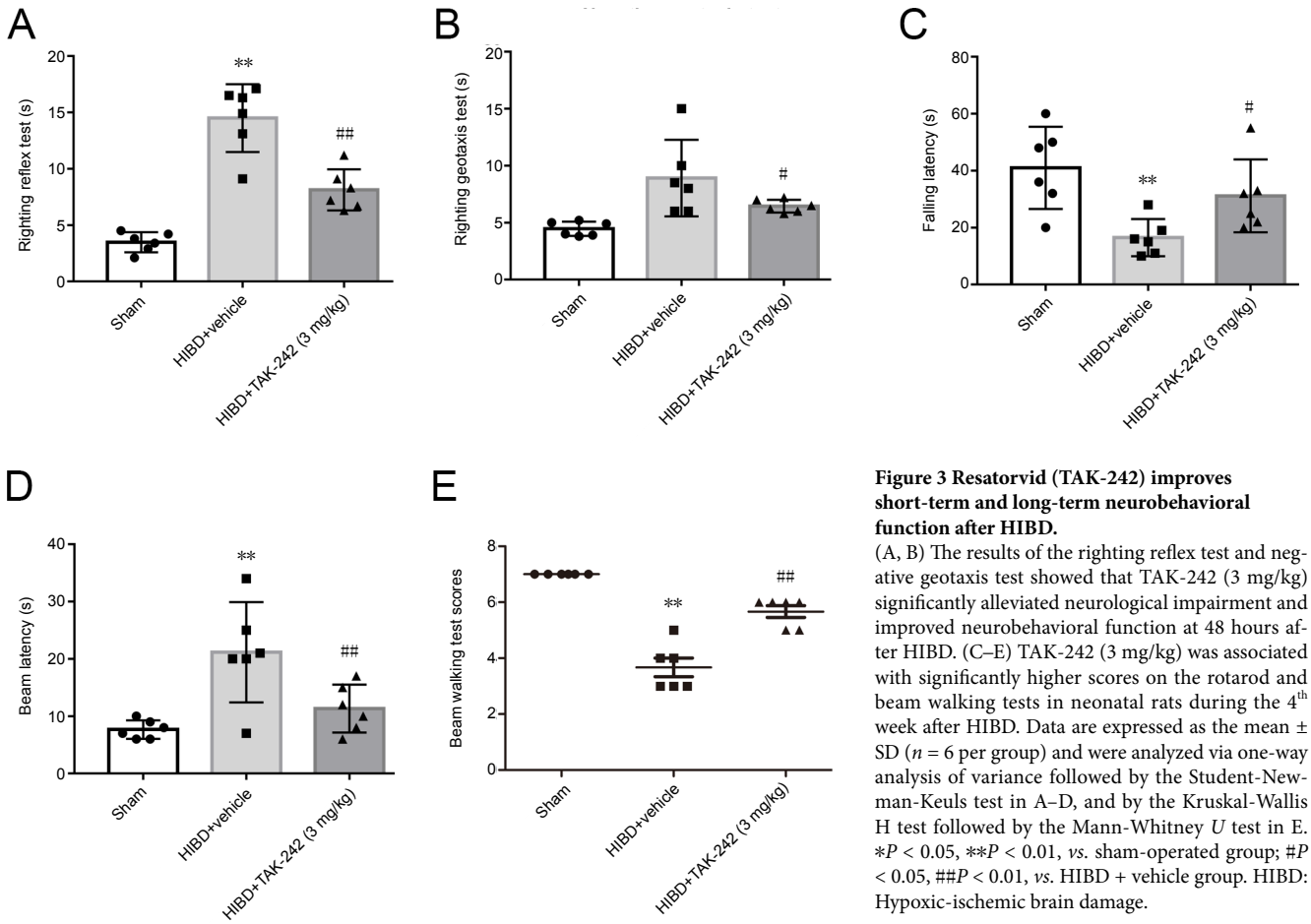

Figure 3 Resatorvid (TAK-242) improves short-term and long-term neurobehavioral function after HIBD.
(A, B) The results of the righting reflex test and negative geotaxis test showed that TAK-242 (3 mg/kg) significantly alleviated neurological impairment and improved neurobehavioral function at 48 hours after HIBD. (C–E) TAK-242 (3 mg/kg) was associated with significantly higher scores on the rotarod and beam walking tests in neonatal rats during the 4th week after HIBD. Data are expressed as the mean ± SD (n = 6 per group) and were analyzed via one-way analysis of variance followed by the Student-Newman-Keuls test in A–D, and by the Kruskal-Wallis H test followed by the Mann-Whitney U test in E. ∗P < 0.05, ∗∗P < 0.01, *vs.* sham-operated group; #P < 0.05, ##P < 0.01, *vs.* HIBD + vehicle group. HIBD: Hypoxic-ischemic brain damage.

We conducted long-term tests of neurobehavioral function to investigate motor integration and coordination abilities in rats during the 4th week after HIBD. We found that the rats in the sham-operated group had satisfactory motor inte-gration and coordination abilities of the left and right limbs, and that they were able to run quickly across the test beam without falling. The right-side limbs of rats in the HIBD + vehicle group were clearly weak, and these rats had poor motor integration and coordination. Scores on the rotarod test and beam walking test in the HIBD + vehicle group were remarkably lower than those in sham-operated group ($P < 0.01$). We found that TAK-242 (3 mg/kg) significantly increased Rotarod running time and improved beam walking performance, leading to significant improvements in motor integration and coordination abilities compared with the HIBD + vehicle group ($P < 0.05$, *vs*. HIBD + vehicle group; **Figure 3C–E**).

TAK-242 alleviates pathological injury in cerebral tissue after HIBD

In the sham-operated group, the structure of the left hippocampal CA1 region was clearly visible under a light microscope, and the neuronal cells were compact and properly aligned. Moreover, the cell morphology was unremarkable. In the HIBD + vehicle group, the structure of the hippocampus was disordered, and the neuronal cells were loosely arranged and irregular. Furthermore, we observed cellular edema, karyopyknosis, karyorrhexis, and necrosis of nerve cells. In the HIBD + TAK-242 (3 mg/kg) group, the damage to the hippocampus was mild, such that only a few nerve cells had edema and necrosis (**Figure 4**).

TAK-242 inhibits activation of autophagy and the TLR4/NF-κB signaling pathway, as well as the release of inflammatory cytokines in the hippocampus after HIBD

The expression levels of TLR4, p-NF-κB p65, Beclin-1, LC3, TNF-α, and IL-1β in the left hippocampus were low in the sham-operated group, while the corresponding levels in the HIBD + vehicle group reached a peak at 48 hours after HIBD. Administration of TAK-242 (3 mg/kg) significantly decreased the levels of TLR4, p-NF-κB p65, Beclin-1, LC3, TNF-α, and IL-1β in the hippocampus at 48 hours after HIBD ($P < 0.01$, *vs.* HIBD + vehicle group; **Figures 5E–I** and **6E–G**). Immunohistochemical data further demonstrated that the expression levels of TLR4, p-NF-κB p65, Beclin-1, and LC3 positive cells in the hippocampus in the HIBD + vehicle group were significantly higher than those in the sham-operated group at 48 hours after HIBD ($P < 0.01$), while the expression levels in the HIBD+TAK-242 group were markedly lower than those in the HIBD + vehicle group ($P < 0.01$; **Figures 5A–D** and **6A–D**).

Discussion

In this study, we demonstrated that the TLR4 inhibitor TAK-242 could significantly reduce the infarct volume and the extent of cerebral edema in neonatal rats after HIBD. Further, TAK-242 alleviated neuronal damage and neurobehav-ioral dysfunction, and decreased the expression levels of TLR4, p-NF-κB p65, Beclin-1, LC3, TNF-α, and IL-1β in the hippocampus. Therefore, TAK-242 might exert a neuroprotective effect after HIBD by inhibiting neuronal autophagy and neuroinflammation associated with the TLR4/NF-κB signaling pathway.

The pathogenesis of neonatal HIBD involves free radical production, ion imbalance, excitotoxicity, oxidative stress, and inflammatory response, among other factors. The secondary brain injury caused by inflammation and immune responses plays a substantial role in cerebral ischemic injury (Ystgaard et al., 2019; Zhou et al., 2019). TLR4 can play the role of a pro-inflammatory cytokine, thereby inducing and regulating immune inflammatory response (Brown et al., 2011). To date, several studies have revealed that the TLR4/NF-κB signaling pathway is closely associated with brain damage. Activation of the TLR4/NF-κB pathway has been observed as a consequence of cerebral ischemia-reperfusion injury in rats, and inhibition of this pathway has been found to alleviate brain injury after hypoxic-ischemia (Kong and Le, 2011; Wang et al., 2014; Zhao et al., 2018). Furthermore, activation of the TLR4/NF-κB pathway is part of the neu-roinflammatory process associated with brain damage caused by trauma, carbon monoxide poisoning, and cerebral hemorrhage, and inhibition of this pathway has been found to reduce the degree of brain damage (Wang et al., 2013; Pang et al., 2016; Feng et al., 2017b). In the present study, HIBD induced significant impairments in neurobehavioral function and damage to brain tissue in neonatal rats. We also observed HIBD-induced massive cerebral infarction in the left side of the brain, as well as cerebral edema, and levels of TLR4 and p-NF-κB p65 in the left hippocampus sig-nificantly increased. Western blot and enzyme-linked immunosorbent assays demonstrated that levels of TLR4, p-NF-κB p65, TNF-α, and IL-1β in the left hippocampus were notably increased. These results were similar to the findings of Yao et al. (Yao et al., 2013). Therefore, we speculate that activation of the TLR4/NF-κB pathway and neuroinflammation further aggravated brain injury after HIBD, which is an important pathogenesis of neonatal HIBD.

Additionally, we observed a significant increase in levels of Beclin-1- and LC3-positive cells in the left hippocampus of neonatal rats after HIBD. Beclin-1 and LC3 are key regulators of the activation of neuronal autophagy. After cerebral hypoxic-ischemia, Beclin-1 and LC3 induce formation of autophagosomes, and their expression increases gradually with the process of neuronal autophagy (Koike et al., 2008; Gao et al., 2012). Our western blot data further demonstrated that levels of Beclin-1 and LC3 were notably increased after HIBD, implicating the activation of au-tophagy during brain damage after HIBD. Although autophagy regulation is involved in brain injury, whether autophagy after brain injury is protective or harmful remains controversial. At present, it is believed that neuronal autophagy can be divided into neuronal basal autophagy and neuronal inducible autophagy. Neuronal basal autophagy protects neurons, while neuronal inducible autophagy damages neurons because its intensity is extremely strong (Hou et al., 2019). Many experiments have

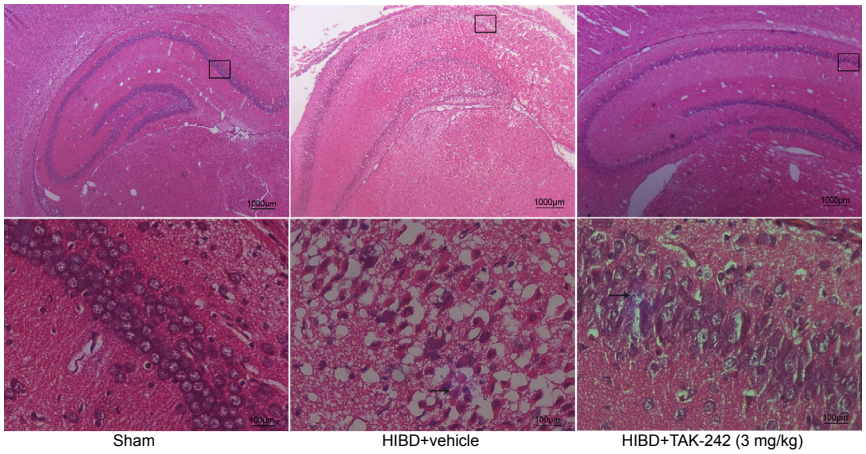

Figure 4 Resatorvid (TAK-242) alleviates pathological damage to brain tissue after HIBD (light microscope, original magnification 400×, scale bars: 100 μm (upper)/1000 μm (lower)).
The representative hematoxylin-eosin stained cells in the left hippocampal CA1 region in each group demonstrate that TAK-242 (3 mg/kg) alleviated pathological damage to brain tissue in neonatal rats at 48 hours after HIBD. The arrowheads indicate cellular edema, karyopyknosis, karyorrhexis, and necrosis of nerve cells. The black rectangles correspond to the magnified sections of the left hippocampal CA1 region. HIBD: Hypoxic-ischemic brain damage.

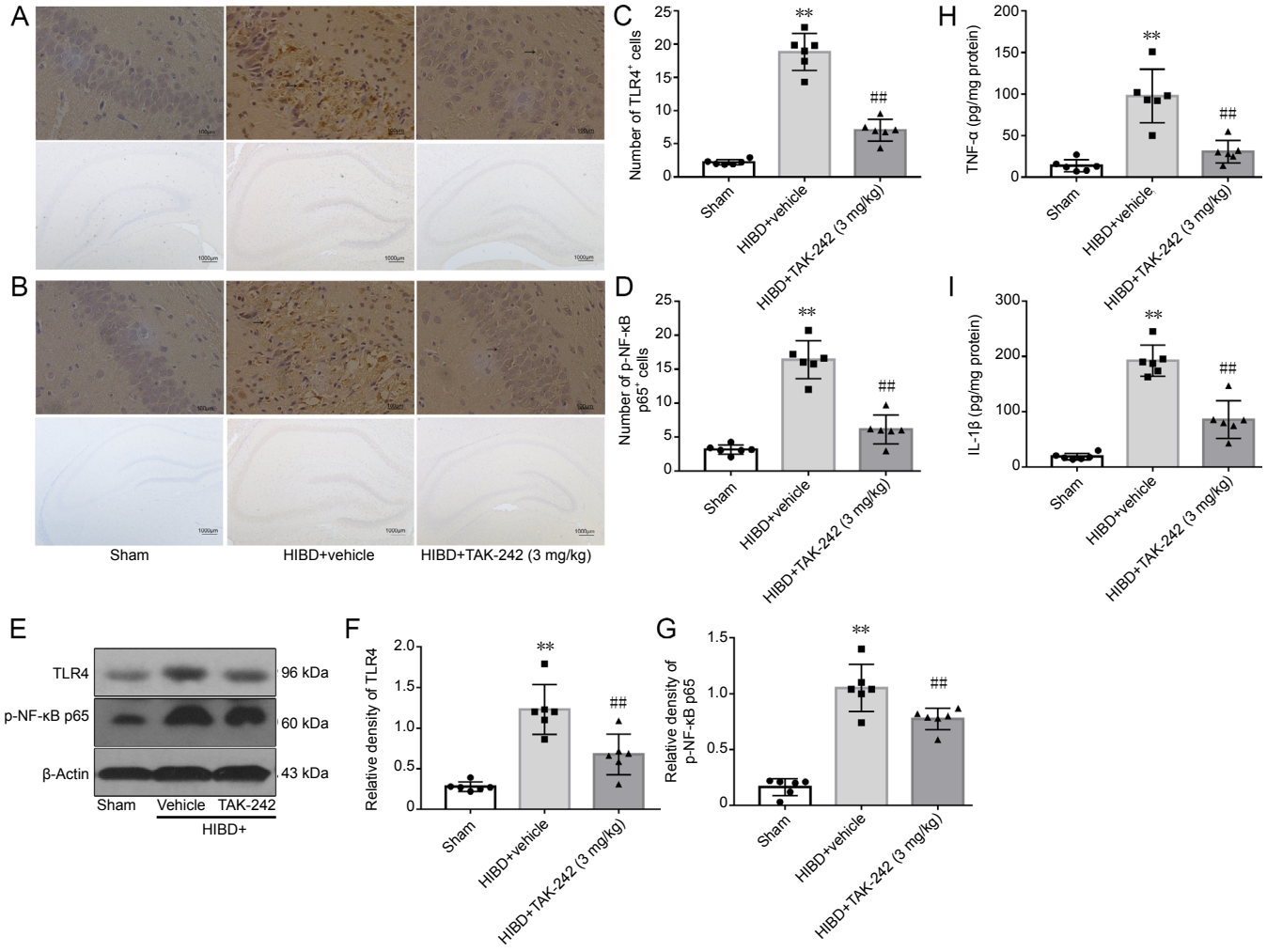

Figure 5 Resatorvid (TAK-242) inhibits expression levels in the TLR4/NF-κB signaling pathway and expression levels of downstream inflammatory cytokines in the hippocampus.
(A, B) TLR4 (A) and p-NF-κB p65 (B) immunopositivity in the left hippocampal CA1 region (light microscope, original magnification 400×, scale bars: 1000 μm for lower resolution (lower panel) and 100 μm for higher magnifications (upper panel)). The arrowheads indicate reactive neurons with robust TLR4 and p-NF-κB p65 immunoreactivity after HIBD. (C, D) Immunohistochemical analysis showed that the number of TLR4- (C) and p-NF-κB p65-positive cells (D) in the hippocampus were significantly increased at 48 hours after HIBD, and that administration of TAK-242 (3 mg/kg) significantly decreased their expression. (E–G) Representative western blot bands and western blot analysis showing that TAK-242 (3 mg/kg) significantly inhibited protein expression levels of TLR4 and p-NF-κB p65 in the hippocampus at 48 hours after HIBD. (H, I) Enzyme-linked immunosorbent assay showed that TAK-242 (3 mg/kg) significantly decreased the expression levels of TNF-α (H) and IL-1β (I) in the hippocampus at 48 hours after HIBD. Data are expressed as the mean ± SD (n = 6 per group) and were analyzed via one-way analysis of variance followed by the Student-Newman-Keuls test. $*P < 0.05$, $**P < 0.01$, *vs.* sham-operated group; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* HIBD + vehicle group. HIBD: Hypoxic-ischemic brain damage; IL-1β: interleukin-1β; p-NF-κB: phospho-nuclear factor kappa-B; TLR4: Toll-like receptor 4; TNF-α: tumor necrosis factor-α.

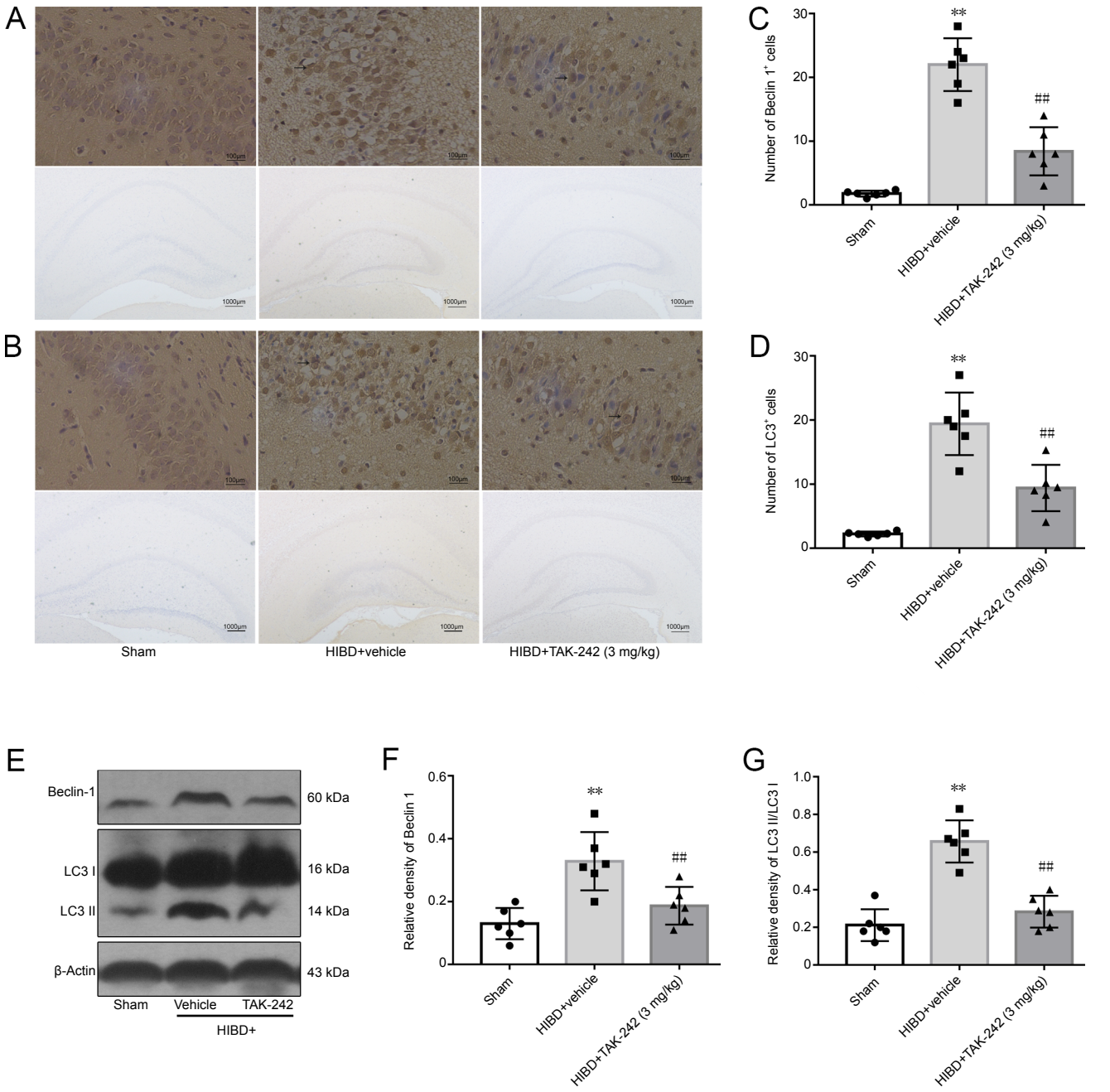

Figure 6 Resatorvid (TAK-242) inhibits protein expression associated with autophagy in the hippocampus.
(A, B) Beclin-1 (A) and LC3 (B) immunopositivity in the left hippocampal CA1 region (light microscope, original magnification 400×, scale bars: 1000 μm for lower resolution (lower panel) and 100 μm for higher magnifications (upper panel)). Arrowheads indicate reactive neurons with robust Beclin-1 and LC3 immunoreactivity after HIBD. (C, D) Immunohistochemical analysis showed that the number of Beclin-1- (C) and LC3-positive cells (D) in the hippocampus were significantly increased at 48 hours after HIBD, and that TAK-242 (3 mg/kg) markedly decreased the expression levels of Beclin-1 (C) and LC3 (D) positive cells. (E–G) Representative western blot bands and western blot analysis showing that TAK-242 (3 mg/kg) notably inhibited protein expression levels of Beclin-1 and LC3 in the hippocampus at 48 hours after HIBD. Data are expressed as the mean ± SD (n = 6 per group) and were analyzed via one-way analysis of variance followed by the Student-Newman-Keuls test. $*P < 0.05$, $**P < 0.01$, *vs.* sham-operated group; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* HIBD + vehicle group. HIBD: Hypoxic-ischemic brain damage; LC3: microtubule-associated protein l light chain 3; TLR4: Toll-like receptor 4.

shown that autophagy can directly participate in the mediation of neuronal cell death or apoptosis (Puyal et al., 2013; Xie et al., 2016; Wang et al., 2017). The previous finding that over-autophagy is an important cause of neuronal apoptosis and brain damage after cerebral hypoxic-ischemia (Shi et al., 2012) is con-sistent with the results of this study. The TLR signaling pathway interacts with the autophagy signaling pathway, and TLR4, which serves as an autophagy environment sensor, can regulate autophagy (Xu et al., 2007). TLR4 might activate autophagy through MyD88- and TRIF-dependent signaling pathways, and promote the release of inflammatory cytokines, further aggravating the degree of

traumatic brain injury (Feng et al., 2017a). Taken together, these data indicate that the TLR4/NF-κB signaling pathway plays a major role in the activation of autophagy and release of in-flammatory cytokines in brain injury. Thus, inhibition of neuronal autophagy and neuroinflammation via modulation of signaling in the TLR4/NF-κB pathway may be a novel target for neonatal HIBD treatment.

TAK-242 is a specific TLR4 inhibitor with high liposolubility and low molecular weight, and has been found to bind to Cys747 in the intracellular domain of TLR4 and inhibit its gene expression (Li et al., 2014; Lei et al., 2016). TAK-242 is rapidly distributed and can penetrate the blood-brain barrier, leading to a substantial blood concentration in the plasma as well as brain tissues. Thus, it exerts a neuroprotective effect on brain ischemia-reperfusion and cerebral hemorrhage (Wang et al., 2013; Hua et al., 2015). However, no studies have examined the effects of TAK-242 on the TLR4/NF-κB pathway and autophagy after neonatal HIBD. In the present study, intraperitoneal injection of TAK-242 revealed a notable reduction in neurobehavioral functional damage in neonatal HIBD rats, and also improved the pathological morphology of brain tissue. Moreover, we found significant decreases in the extent of cerebral edema and infarct volume, as well as significantly reduced levels of TLR4, p-NF-κB p65, Beclin-1, LC3, TNF-α, and IL-1β in the hippocampus. These results indicate that TAK-242 might alleviate brain damage after HIBD by inhibiting activation of autophagy and the release of inflammatory cytokines via inhibition of the TLR4/NF-κB signaling pathway.

In conclusion, this study represents the first use of a neonatal HIBD rat model to support the neuroprotective effect of TAK-242 after HIBD via prevention of neuronal autophagy and neuroinflammation via inhibition of the TLR4/NF-κB signaling pathway. This study therefore provides a theoretical basis for clinical research regarding new HIBD treatment methods. Comprehensive studies with larger samples and different HIBD models are needed to address the potential use of TAK-242 for treating HIBD.

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